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JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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JOURNAL OF CHROMATOGRAPHY

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(*Continued overleaf*)

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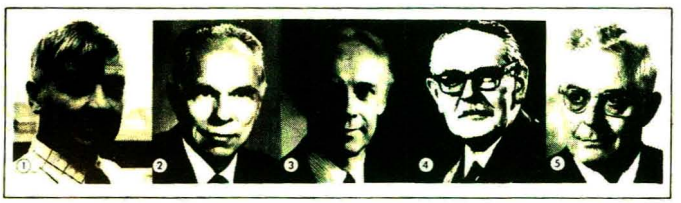
75 Years of Chromatography

A Historical Dialogue

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

Journal of Chromatography Library - Volume 17

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



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

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(*Nobel Prize laureates)

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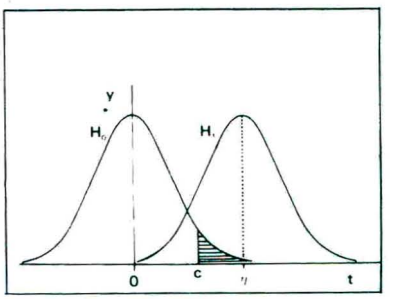
Statistical Treatment of Experimental Data

by J. R. GREEN, *Lecturer in Computational and Statistical Science, University of Liverpool, and* D. MARGERISON, *Senior Lecturer in Inorganic, Physical and Industrial Chemistry, University of Liverpool.*

Physical Sciences Data, Vol. 2



First published in 1977 and now reprinted with some minor revisions, this book is intended for researchers wishing to analyse experimental data using statistical methods. Statistical concepts and methods which may be employed, are explained, and the ideas and reasoning behind statistical methodology clarified. Formal results are illustrated by many numerical worked examples mainly taken from the laboratory. Concepts, practical methodology, and worked examples are integrated in the text.

Consideration is given in this work to a large number of practical topics which are often omitted from standard texts. These include: obtaining an approximate confidence interval for a function of some unknown parameters; testing for outliers, stabilization of heterogeneous variances, and significant differences between means; estimation of parameters after performing tests; deciding what numbers of significant figures to quote for sample means and variances; straight-line and polynomial regression, through the origin or not, using weighted points, and testing the homogeneity of a set of such lines or curves.

The many examples provided throughout the text will serve as models for the various problems encountered by the readers when employing statistical methods to treat experimental data.

In addition to research workers in universities and industry, the book will be of use for first-year students of statistics, and will be especially suitable as the basis of a graduate course in experimental sciences.

CONTENTS: Chapters: 1. Introduction. 2. Probability. 3. Random Variables and Sampling Distributions. 4. Some Important Probability Distributions. 5. Estimation. 6. Confidence Intervals. 7. Hypothesis Testing. 8. Tests on Means. 9. Tests on Variances. 10. Goodness of Fit Tests. 11. Correlation. 12. The Straight Line Through the Origin or Through Some Other Fixed Point. 13. The Polynomial Through the Origin or Through Some Other Fixed Point. 14. The General Straight Line. 15. The General Polynomial. 16. A Brief Look at Multiple Regression. Appendices: 1. Drawing a Random Sample Using a Table of Random Numbers. 2. Orthogonal Polynomials in x. References. Index.

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CHROMBIO. 568

ÜBER DAS EINSCHLEPPEN VON VERUNREINIGUNGEN BEI VERWENDUNG VON EXTRELUT®-FERTIGSÄULEN

MANFRED ENDE*, PETER PFEIFER und GERHARD SPITELLER

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(Eingegangen am 22. Januar 1980)

SUMMARY

Contamination by use of Extrelut® columns

Extrelut® columns commonly used to isolate metabolites from biological fluids often contain fatty acids, phthalates, alcohols and especially resin acids which occassionally cause great analytical problems. Therefore purification before use of Extrelut® columns is strongly recommended.

EINLEITUNG

Zur Isolierung von Stoffwechselprodukten aus Körperflüssigkeiten werden vor allem in der Arzneimittelanalytik in zunehmendem Masse Extrelutsäulen benutzt, weil so wesentlich rascher als bei der flüssig—flüssig-Extraktion und ohne Störungen durch Emulsion ein Extrakt erhalten wird, der direkt für die nachfolgende chromatographische Trennung und Identifizierung verwendet werden kann [1—5].

In unserem Labor wird dieses Extraktionsverfahren zur Isolierung von Säuren und Steroiden aus Urin bzw. Plasma eingesetzt, die wir nachfolgend mit der Kombination Glaskapillargaschromatographie—Massenspektrometrie untersuchen. Wir fanden nun, dass Extrelutsäulen organische Verbindungen enthalten, die insbesondere die Identifizierung von Spurenbestandteilen stören. Im folgenden wollen wir über die Art der Verunreinigungen und ihre Beseitigung berichten.

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EXPERIMENTELLER TEIL

Verwendete Geräte

Glaskapillargaschromatographie—Massenspektrometrie (GC—MS): Die Trennung der Substanzgemische und die Aufnahme der Massenspektren erfolgte mit einem LKB 2091 Kombinationsgerät von Gaschromatograph mit dem Massenspektrometer. Zur Registrierung der Spektren diente das LKB 2130-Datensystem (PDP 11/05-Rechner).

Messbedingungen: 25-m Glaskapillarsäule (Innendurchmesser 0.3 mm, SE-30-Dünnfilm), Helium 2 ml/min, Injektortemperatur 275°C, Säulentemperatur von 150°C bis 300°C mit 2°C/min programmiert. Ionisierungsenergie 70 eV. Der Totalionenstrom wurde bei 20 eV registriert.

Die Gaschromatogramme zur Ermittlung der Retentionsindices wurden an einem Siemens L 402 Gaschromatograph aufgenommen: 25-m Glaskapillarsäule (Innendurchmesser 0.3 mm, SE-30-Dünnfilm), Injektortemperatur 275°C, Detektortemperatur (Flammenionisationsdetektor) 280°C, Wasserstoff 2 ml/min, Säulentemperatur von 150°C bis 300°C mit 2°C/min programmiert.

Blindaufarbeitung zur Identifizierung der Verunreinigungen

Dest. Wasser (20 ml) wurde mit verdünnter Salzsäure auf pH 2—3 eingestellt und auf einer Extrelut®-Fertigsäule (E. Merck, Darmstadt, B.R.D.) verteilt. Nach 30 min wurde die Säule mit 50 ml Essigsäureethylester eluiert. Das Eluat wurde zur Trockne eingeengt, derivatisiert (siehe unten) und der GC—MS bzw. der GC-Analyse unterworfen.

Isolierung der Steroidfraktion aus Urin

Urin (10 ml) wurde mit 10 ml 0.5 *M* Acetatpuffer (pH 4.5) und 100 µl β-Glucuronidase/Arylsulfatase (Boehringer, Mannheim, B.R.D.) versetzt und 72 Std. bei 37°C inkubiert. Diese Lösung wurde anschliessend auf einer Extrelutsäule verteilt und die Säule nach 30 min mit 50 ml Essigsäureethylester eluiert.

Das Eluat wurde dreimal mit je 10 ml einer Lösung aus 5% Natriumhydrogencarbonat und 10% Natriumchlorid und nachfolgend zweimal mit je 5 ml einer gesättigten Natriumchloridlösung gewaschen [6]. Nach dem Trocknen über Natriumsulfat und einengen der Lösung wurden 1/5 des Extraktes mit 6 µl N-Methyl-N-trimethylsilyltrifluoracetamid (MSTFA, Macherey-Nagel, Düren, B.R.D.) derivatisiert (siehe unten).

Derivatisierung

Darstellung der Methylester. Die Proben wurden in wenig Methanol gelöst und mit einem Überschuss einer etherischen Diazomethanlösung versetzt. Nach 10 min wurde die Lösung im Stickstoffstrom zur Trockne eingeengt, in einigen Mikroliter Methanol gelöst und diese Lösung zur GC-, bzw. GC—MS-Analyse verwendet.

Silylierung. Die gut getrocknete Probe wurde in einem zugeschmolzenen Schmelzpunktröhrchen mit 5—10 µl MSTFA 24 Std. bei Raumtemperatur oder 45 min bei 60—80°C umgesetzt.

Reinigung des Extrelutsäulenmaterials

In einer Soxhlett-Apparatur wird das Säulenmaterial jeweils 4 Std. mit Aceton, Toluol und Ethanol oder Methanol extrahiert.

Die "Inhaltsstoffe" von Extrelutsäulen

Fig. 1 zeigt das Glaskapillargaschromatogramm des Extraktes aus Extrelutsäulen nach einer Blindaufarbeitung (Experimenteller Teil). Die Peaks sind nach steigenden Retentionsindices numeriert. Die zur Ermittlung der Retentionsindices beigefügten geradkettigen Kohlenwasserstoffe wurden mit C-10, C-12 usw. gekennzeichnet.

In der Tabelle I sind die in Extrelut gefundenen Verbindungen entsprechend der Kennzeichnung in den Chromatogrammen aufgelistet. Zusätzlich sind die bei Temperaturprogrammierung (150—300°C, 2°C/min, siehe Experimenteller Teil) gemessenen Retentionsindices sowie Literaturhinweise bzw. die charakteristischen Fragmentionen der Massenspektren angegeben.

Besonders störend sind die relativ grossen Mengen an Harzsäuren. Da die Hauptverbindungen [8(14),15-Pimaradien-18-säure, 7,15-Isopimaradien-18-säure und Dehydroabietinsäure] für Extrelutverunreinigungen geradezu typisch sind, wurden die Massenspektren ihrer Trimethylsilylester in Fig. 2 reproduziert.

Der Effekt, den eine derartige Verunreinigung verursacht, ist in dem in Fig. 3 dargestellten Gaschromatogramm veranschaulicht.

Harn (10 ml) wurde in der angegebenen Weise (Experimenteller Teil) auf Steroide aufgearbeitet. Das nach Extrelutextraktion erhaltene Gaschromatogramm der Steroidfraktion zeight, dass die durch Extrelut eingeschleppten Verunreinigungen (Peak 9′—14′ in Fig. 3) in gleicher Konzentration wie die Hauptsteroidmetabolite (b—f in Fig. 3) vorliegen.

Die Identifizierung von Metaboliten, die im Bereich zwischen C-22 und C-24 angezeigt werden (Androsten-3-ol-isomere) wird dadurch erschwert, bzw. unmöglich gemacht.

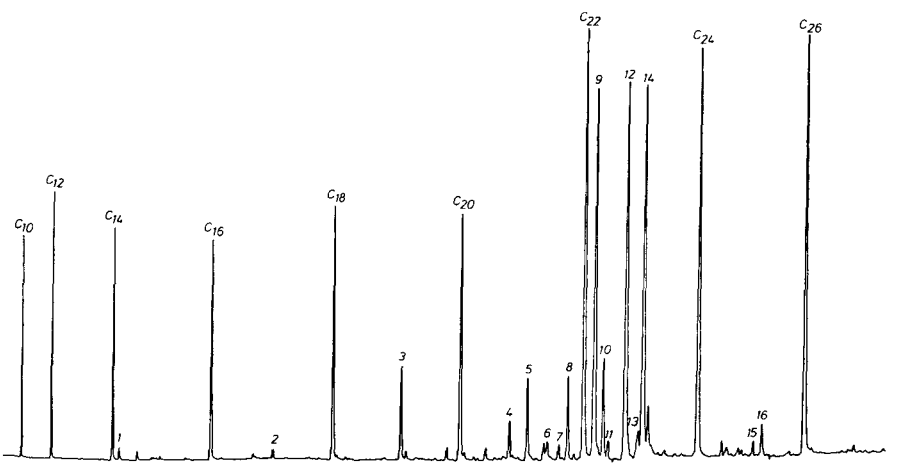

Fig. 1. Glaskapillargaschromatogramm eines Blindextraktes von Extrelutsäulen. In Tabelle I sind die den GC-Peaks entsprechenden Verbindungen angegeben.

TABELLE I

ZUSAMMENSTELLUNG IN EXTRELUT® ENTHALTENEN VERUNREINIGUNGEN

GC-Peak-Nr.	Name der Verbindung	Retentionsindex*	Molekulargewicht	Massenspektrum, charakteristische Ionen [*m/e* (%)] bzw. Literaturhinweise
1	Phthalsäuredimethylester**	1415	194	50 (10), 76 (12), 77 (24),92 (9), 104 (6), 105 (4), 120 (3), 132 (5), 135 (7), 163 (100), 194 (8, M^+)
	Myristinsäure-			
2	-methylester	1707	242	Lit. 7
	-trimethylsilylester	1842	300	Lit. 8
	Palmitinsäure-			
3	-methylester	1907	270	Lit. 7
	-trimethylsilylester	2041	328	Lit. 8
	Ölsäure-			
4	-methylester	2080	296	Lit. 7
	-trimethylsilylester	2235	354	Lit. 8
	Stearinsäure-			
5	-methylester	2108	298	Lit. 7
	-trimethylsilylester	2242	356	Lit. 8
6	1α-Carbomethoxy-2α-[2′(*m*-isopropylphenyl)ethyl]-1β,3α-dimethylcyclohexan	2143	316	Lit. 7
7	isomere Verbindung zu der im GC-Peak 6 enthaltenden Verbindung 8, 15-Isopimaradien-18-säure-	2160	316	133 (45), 146 (100), 192 (12), 284 (16), 316 (17, M^+)
8	-methylester	2173	316	Lit. 7
8	-trimethylsilylester	2229	374	73 (100), 241 (98), 256 (26), 257 (27), 359 (18), 374 (13, M^+)
	8(14), 15-Pimaradien-18-säure (Pimarsäure)			
9	-methylester	2218	316	Lit. 7, 9
9	-trimethylsilylester	2285	374	Fig. 2a
	8(14), 15-Isopimaradien-18-säure-			
10	-methylester	2235	316	Lit. 7
10′	-trimethylsilylester	2298	374	73 (100), 121 (63), 241 (11), 256 (10), 257 (25), 359 (18), 374 (12, M^+)
	Harzsäure nicht bekannter Struktur			
11	-methylester	2242	316	105 (89), 121 (100), 133 (49), 148 (68), 180 (25), 185 (28), 241 (58), 256 (42), 257 (58), 301 (34), 316 (25, M^+)
	7, 15-Isopimaradien-18-säure-(Isopimarsäure)			
12	-methylester	2272	316	Lit. 7, 9
12′	-trimethylsilylester	2311	374	Fig. 2b

TABELLE I (Fortsetzung)

GC-Peak-Nr.	Name der Verbindung	Retentionsindex*	Molekulargewicht	Massenspektrum, charakteristische Ionen [*m/e* (%)] bzw. Literaturhinweise
	Harnsäure nicht bekannter Struktur -methylester			
13		2295	318	107 (80), 121 (100), 136 (30), 181 (44), 215 (39), 243 (78), 259 (32), 271 (12), 275 (28), 303 (30), 318 (22, M^+)
	Dehydroabietinsäure-methylester			
14		2304	314	Lit. 7, 10, 11
14'	-trimethylsilylester	2352	372	Fig. 2c
In anderen Lieferchargen von Extrelut wurden folgende Phthalester gefunden:				
	Phthalsäuremethylester**	1482	208	50 (15), 65 (8), 76 (18), 77 (23), 92 (14), 104 (11), 105 (11), 121 (5), 133 (5), 135 (9), 136 (10), 149 (54), 163 (100), 176 (9), 177 (8), 208 (7, M^+)
	Phthalsäurediethylester	1583	222	Lit. 12—14
	Phthalsäuredi-isobutylester	1825	278	Lit. 15, 16
	Phthalsäure-*n*-butylisobutylester	1868	278	Lit. 15
	Phthalsäuredi-*n*-butylester	1916	278	Lit. 14—17
Als Trimethylsilylether (TMS) wurden folgende primäre Alkohole [11] identifiziert				
	Tetradecanol-TMS	1764	286	73 (56), 75 (78), 83 (28), 103 (24), 271 (100)
	Hexadecanol-TMS	1964	314	73 (60), 75 (75), 83 (29), 103 (29), 299 (100)
	Octadecanol-TMS	2162	342	73 (38), 75 (100), 103 (42), 327 (90)

*Die Retentionsindices wurden bei Temperaturprogrammierung gemessen: 150—300°C, 2°C/min (siehe Experimenteller Teil).
**Nach Derivatisierung mit Diazomethan.

Der Gehalt an Verunreinigungen ist schwankend. Fig. 1 zeigt das Gaschromatogramm einer Durchschnittsprobe. In einer Liefercharge waren erheblich geringere Mengen an Beimengungen, in einer anderen Lieferung war die Konzentration an Harzsäuren gleich, an Phthalestern sehr viel höher.

Selbstverständlich schlagen bei Aufarbeitung grosser Probenmengen die Verunreinigungen nicht so sehr zu Buche. Dies gilt insbesondere für die Untersuchung von basischen Arzneistoffmetaboliten, da bei der Aufarbeitung auf

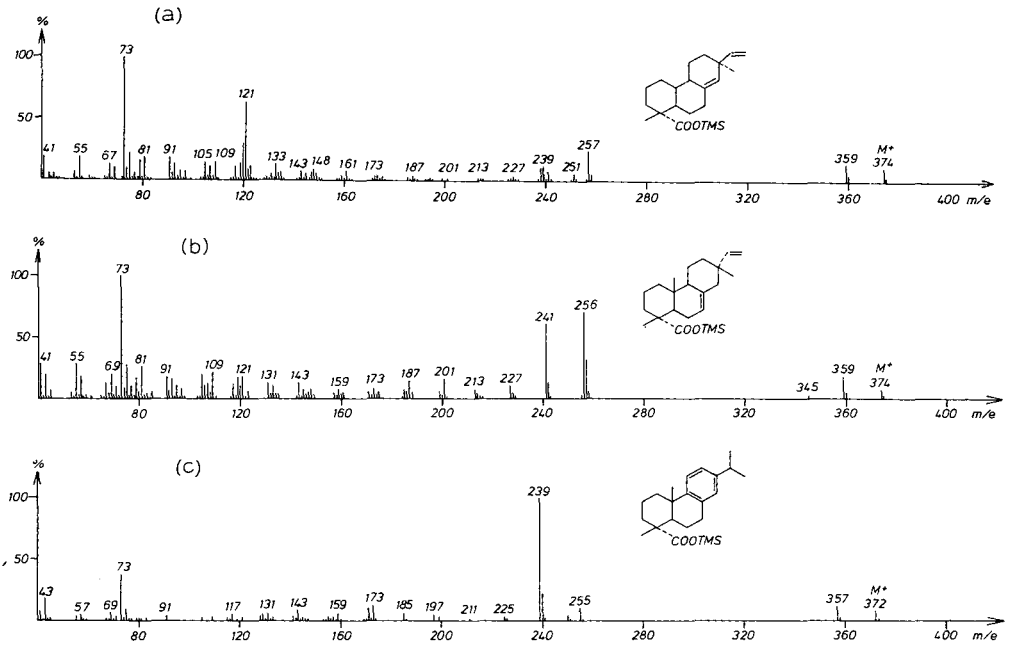

Fig. 2. Massenspektren der Trimethylsilylester von (a) 8(14),15-Pimaradien-18-säure (Pimarsäure), (b) 7,15-Isopimaradien-18-säure (Isopimarsäure), (c) Dehydroabietinsäure.

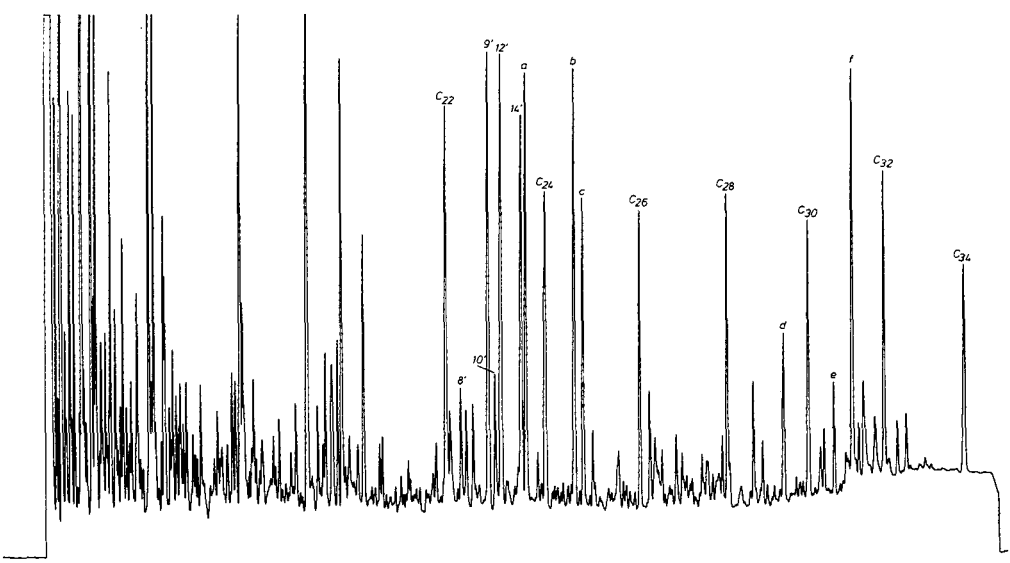

Fig. 3. Chromatogramm einer Steroidfraktion (analysiert in Form der Trimethylsilylderivate), die mit Hilfe von Extrelut®-Fertigsäulen isoliert wurde. Messbedingungen sind im Experimenteller Teil angegeben. Verunreinigungen aus Extrelut: (9') 8(14),15-Pimaradien-18-säure-trimethylsilylester (Pimarsäure-TMS-ester); (10') 8(14),15-Isopimaradien-18-säure-trimethylsilylester; (12') 7,15-Isopimaradien-18-säure-trimethylsilylester (Isopimarsäure-TMS-ester); (14') Dehydroabietinsäuretrimethylsilylester. a = Interner Standard. Hauptsteroidmetabolite als Trimethylsilylderivate: (b) Androsteron; (c) Etiocholanolon; (d) 3α,17α,20α-Trihydroxy-5β-pregnan-3α,20α-di-TMS; (e) 3α,17α,20α,21-Tetrahydroxy-5β-pregnan-11-on (α-Cortolon); (f) Tetrahydrocortison.

Basen die als Verunreinigung enthaltenen Säuren nicht stören. Sind jedoch nur kleine Mengen an Säuren und Neutralstoffen als Metabolite zu erwarten, so ist eine massenspektrometrische Kontrolle der Gaschromatogramme zur Identifizierung der aus Extrelut stammenden Verunreinigungen unerlässlich.

Entfernung der Verunreinigungen

Ein einfacher Weg zur Entfernung der Verunreinigungen besteht in einer der Verwendung vorausgehenden 4-stündigen Extraktion des Säulenmaterials jeweils mit Aceton, Toluol und Ethanol bzw. Methanol in einer Soxhlett-Apparatur (siehe Experimenteller Teil).

ZUSAMMENFASSUNG

Extrelutsäulen® die zur Isolierung von Stoffwechselprodukten aus Körperflüssigkeiten verwendet werden, enthalten oft Fettsäuren, Phthalester, Alkohole und insbesondere Harzsäuren, die die Analyse von Metaboliten stören. Daher ist eine Reinigung des Säulenmaterials vor der Verwendung dringend erforderlich.

DANK

Wir danken der Deutschen Forschungsgemeinschaft, dem Fonds der Chemischen Industrie und der Dr. Robert Pfleger-Stiftung für Sachbeihilfen. Herrn Dr. J. Reiner danken wir für die Herstellung der Glaskapillarsäulen.

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CHROMBIO. 583

SEPARATION OF PEPTIDES AND AMINO ACIDS BY ION-EXCHANGE CHROMATOGRAPHY OF THEIR COPPER COMPLEXES

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SUMMARY

The method of separating peptides and amino acids using their copper (II) complexes has been reexamined by studying model compounds. A marked improvement in the separation was achieved on DEAE Sephadex columns by variation of the ionic strength of the eluting buffers. The use of the method is illustrated by an examination of a protein hydrolysate used in intravenous feeding (Aminosol).

INTRODUCTION

The separation of peptides from amino acids by ion-exchange chromatography of their copper(II) complexes was first described by Tommel et al. [1, 2]. The method has since been applied to a number of biological fluids including urine [3, 4], serum [5], cheese [6] and wort [7]. We have previously used this method to separate radioactive metabolites of peptides in mouse urine [8].

The method is based on the charge differential, at alkaline pH, between Cu(II) complexes of amino acids (no net charge) and peptides (negative net charge). All of the above quoted applications employ the technique of eluting the amino acid complexes from a high pH anion-exchange column and then acidifying to elute the peptide complexes. This has the disadvantage that, because of the instability of the complexes below pH 6—7, elution of the complex cannot be directly monitored by its spectral properties.

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A modification by Giliberti and Niederwieser [9], using two columns, allowed resolution of neutral dipeptides from tripeptides, but did not allow a full resolution. We describe here a method allowing a considerable resolution of a mixture of peptides, with continuous monitoring of the column eluate.

MATERIALS AND METHODS

Peptides were obtained from Sigma (St. Louis, MO, U.S.A.), BDH (Poole, Great Britain) or Cyclo Chemicals (Los Angeles, CA, U.S.A.) (tri-L-leucine), DEAE Sephadex A-25 from Pharmacia (Uppsala, Sweden). Aminosol powder (Vitrum, Sweden) was obtained from Paynes and Byrne (Greenford, Great Britain).

Copper complexes were formed by adding an excess of basic copper carbonate to the aqueous solution of peptide. Sodium hydroxide was added to maintain the pH at 9—9.5 and the tube shaken for 10—15 min. The tube was then centrifuged and the copper complex solution aspirated into a clean tube.

Copper complex (5—50 μmol) contained in 0.5—1.0 ml solution was applied to a column of DEAE Sephadex A-25 equilibrated in 5 mmol/l sodium borate buffer pH 8.5. Column dimensions were 15 × 1.5 cm, on which the sample was chromatographed. The column was eluted with sodium borate buffers pH 8.5 with sodium chloride added to increase ionic strength. The absorbance of the eluate was recorded at 570 nm by passage through a flow-cell in a Vitatron spectrophotometer. Fractions of 1—3 ml were collected.

Peptide-enriched samples were prepared by ligand-exchange chromatography using copper-loaded Chelex 100 [10, 11]. The column was equilibrated with 0.1 mol/l ammonia and the sample, adjusted to pH 10 with ammonia, applied to it. The peptides and acidic amino acids were not retained and were eluted with 0.1 mol/l ammonia. The neutral and basic amino acids were then eluted with 4 mol/l ammonia. The fractions were concentrated in vacuo to remove ammonia, copper complexes were then formed as above.

Spectra were measured in a Pye-Unicam SP800 recording spectrophotometer in the range 400—700 nm using a 1-cm glass cell against water. Concentrations are given as concentration of Cu(II) complex, assuming a 2:1 acid:copper stoichiometry for amino acid complexes, and 1:1 for peptide complexes.

RESULTS

The effect of variation in pH and ionic strength of the eluate on the retention characteristics of different amino acids and peptides was investigated using columns equilibrated with the buffer under test. The relative retention was expressed as R_g values, where $R_g = V_e/V_g$, V_e being the elution volume of the substance under test and V_g the elution volume of glycine in the same buffer system. For all except the very high ionic strength V_g is 18—20 ml. Variations in V_g elution volume of neutral [Cu(II)—amino acid] complexes can be at least partially ascribed to gel shrinkage in high salt concentrations. The results are tabulated in Tables I and II. The effect of ionic strength on the elution of a series of oligo-glycines is shown in Fig. 1. The retention data all have coefficients of variation of $< \pm 5\%$.

TABLE I

RETENTION DATA FOR COPPER COMPLEXES ON DEAE-SEPHADEX A-25

Variation with ionic strength of eluting buffer. Elution at pH 8.5. Each result is the average of at least three determinations. Results are expressed as R_g values (see text).

Ligand	Ionic strength						
	0.0075	0.03	0.06	0.1	0.16	0.26	0.5
Gly	1	1	1	1	1	1	1
Arg	0.49	0.57	0.58	0.6	0.65	0.75	0.82
Lys	0.52	0.59	0.55	0.56	0.65	0.66	0.75
Pro	0.97	0.99	0.97	1	1.03		
Ser	1.34	1.17	1.07	1.05	1.05		
Thr	1.42	1.13	1.08	1.03	1.02		
Glu			10.8	5.65	3.16	1.86	1.29
Asp			10.9	5.65	2.99	1.85	1.22
GlyGly	5.38	2.94	2.18	1.89	1.57	1.35	1.13
LeuLeu	4.32	2.51	1.82	1.64	1.43	1.29	
GlyGlyGly	19	5.72	3.45	2.77	2.14	1.17	1.39
AlaGlyGly	16.5		3.92	2.68	2.15	1.58	
LeuLeuLeu		5.67	3.38	2.75	2.1	1.73	
tetra-Ala			6.25	3.25	2.2	1.45	1.22
tetra-Gly			5.7	3.7	2.38	1.73	1.3
penta-Gly			15	8.1	4.75	2.52	1.45
hexa-Gly				7	3.63	2.34	1.34
Tyr	7.3	3.05		2.5			

TABLE II

RETENTION DATA OF COPPER COMPLEXES ON DEAE-SEPHADEX A-25

Variation with pH and ionic strength of eluting buffer. Results are expressed as R_g values (see text). Each result is the average of at least three determinations.

Ligand	Ionic strength				
	0.0075	0.03	0.06	0.10	0.16
pH 9.0					
Gly	1	1	1	1	1
Lys	0.49	0.51	0.525	0.57	0.62
Ser	1.52	1.23	1.1	1.05	1.01
GlyGly	7	4	2.66	1.99	1.63
GlyGlyGly			3.7	2.7	2.3
pH 9.5					
Gly	1		1		1
Lys	0.63		0.56		0.65
Ser	2.92		1.2		1.08
GlyGly			2.27		1.66
GlyGlyGly			3.5		2.34
pH 8.0					
Gly	1				
Lys	0.48				
Ser	1.10				
GlyGly	3.05				

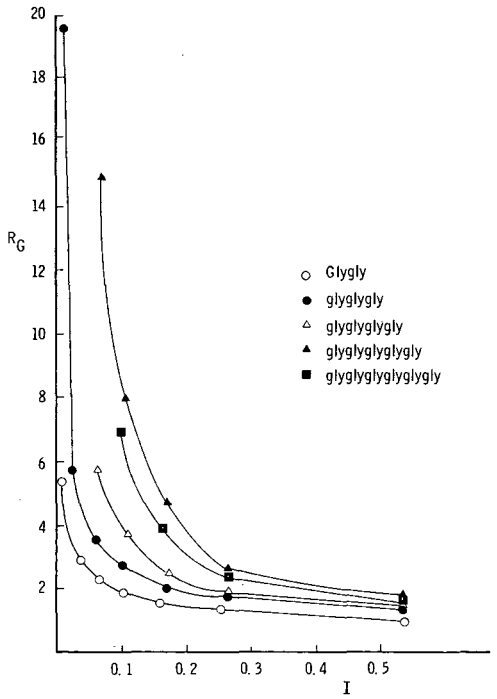

Fig. 1. Retention of Cu(II)—oligopeptide complexes on DEAE-Sephadex A-25 at pH 8.5. Effect of ionic strength.

The order of elution of amino acids is basic > neutral > acidic and aromatic. Weakly acidic amino acids (i.e. Ser, Thr) are relatively retarded, but not resolvable from neutral amino acids. Aromatic amino acids are presumably retarded by aromatic interaction with the column material [12]. The effect of acidic, aromatic and basic groupings on peptide elution is illustrated in Table III.

The application of a stepwise increase in ionic strength of the eluting buffer to the separation of a mixture of oligo-glycines is shown in Fig. 2. Total resolution of a five-component mixture is reproducibly accomplished. The anomalous behaviour of hexaglycine precludes any resolution of the peptide. At this stage it is uncertain whether this is due to the presence of a lower charge on the hexaglycine—Cu(II) complex, but it is possible that hydrolysis of the hexapeptide to pentapeptide plus amino acid is occurring [13].

TABLE III

EFFECT OF FUNCTIONAL GROUPS ON RETENTION OF DIPEPTIDE—Cu(II) COMPLEXES

Ligand	Ionic strength of buffer				
	0.0075	0.03	0.06	0.1	0.16
GlyPhe	6.5	3.88	3.01	2.05	2.26
PheTyr	>40	10			
GlyAsp			4.75	3.0	2.28
ProGly	3.9	2.4		1.65	
GlyGly	5.38	2.94	2.18	1.89	1.57

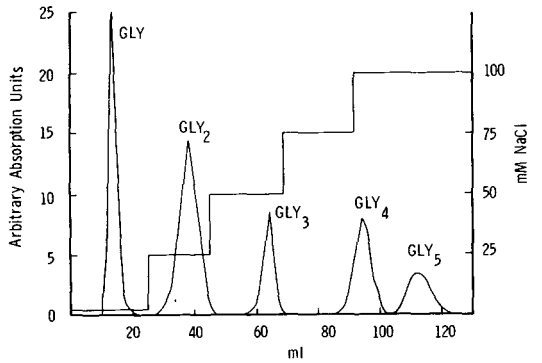

Fig. 2. Separation of Cu(II) complexes of oligo-glycines on DEAE-Sephadex A-25.

The separation scheme has been further tested using a protein hydrolysate, Aminosol. This is an enzymatic hydrolysate of casein from which the larger peptides have been removed by dialysis [14]. The separation illustrated in Fig. 3 shows that ten distinct peaks can be identified. Many of these are retained more strongly than any of the model peptides tested, and may be due to the high acidic amino acid content of the peptide fraction of Aminosol (information supplied by Vitrum).

Quantitation of the results was demonstrated by estimation of peak areas as recorded. The calibration lines obtained for glycine, glycylglycine and triglycine were linear between 5 and 20 μmol although the glycine calibration line showed a pronounced curvature below 5 μmol.

Recovery of the same three oligo-glycines was determined by collecting the total eluate and calculating the amount of Cu(II) complex from the measured molar extinction coefficients. The data in Table IV indicate a recovery in excess of 94%.

The spectra of several complexes were determined under varying pH conditions. The wavelength, 570 nm, used to estimate the complexes in the eluate, is a compromise, being optimum only for tri- and tetrapeptides. This allows only

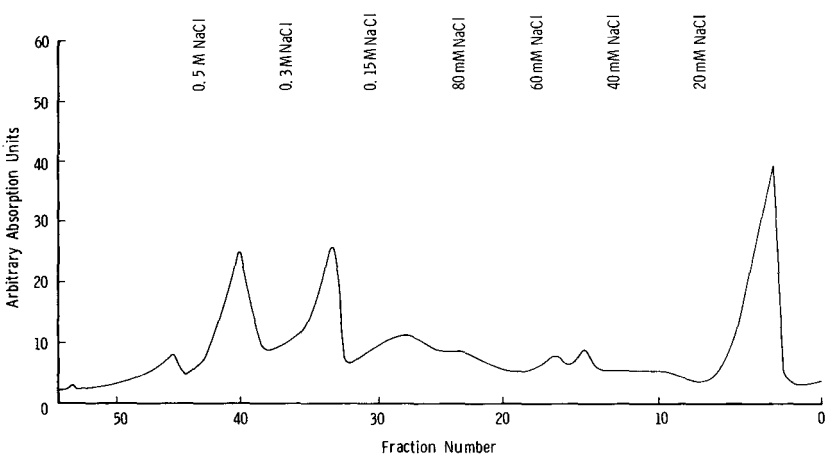

Fig. 3. Fractionation of Aminosol—copper(II) complexes on DEAE-Sephadex A-25.

TABLE IV

RECOVERY OF COMPLEXES FROM THE DEAE-SEPHADEX A-25 COLUMN

Sample	Recovery (%) (mean ± S.D.)	*n*
Gly	94.4 ± 7.4	11
GlyGly	108 ± 11.4	8
GlyGlyGly	95.1 ± 4.3	3

60% of maximum sensitivity for pentapeptides, but these are nevertheless well detected at 570 nm. The spectra are not significantly affected by changes in ionic strength.

DISCUSSION

The methods described here allow the resolution into peptide-chain length related fractions of a mixture of neutral peptides, up to pentapeptides. The stability in the conditions used of larger peptides is open to question. The separation is based largely on the increasing net negative charge on the Cu(II) complexes in alkaline solution [15—18]. As the pH of the solution of the copper complex was increased, there was a tendency for the wavelength of maximum absorption to move to shorter wavelengths and at the same time, the molar extinction coefficient tended to increase [19].

In analysis of biological samples (i.e. plasma or urine) prior removal of the amino acids by ligand-exchange chromatography [10, 11] will help decrease the interference of amino acids in the resolution of the dipeptide fraction. This stage may also be used to concentrate the peptides by allowing removal of many other interfering substances.

Subsequent analysis of the separated peptide fraction may be performed after extraction of copper and desalting. Application of such techniques (i.e. gel filtration and thin-layer chromatography of dansyl derivatives) have been described elsewhere [19], including analysis of protein hydrolysates and of urine.

With the method as described here, an optical density change of about 0.001 (i.e. 1% of full scale deflection) was obtained with 2 μmol tripeptide. Further increases in sensitivity may be achieved in several ways. Use of small columns would limit diffusion-caused band spreading of strongly retained peptides, as would more rigorously controlled ionic strength gradients. The use of the higher absorbance at 280 nm of the Cu(II)—peptide complex has been described by Giliberti and Niederwieser [9]. Atomic absorption estimation of copper in the eluate has been used by Jones and Manahan [20] in the sensitive estimation of chelating agents. Such techniques would considerably enhance the application of these methods to the study of peptides in clinical samples.

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CHROMBIO. 572

ANALYSIS OF SALSOLINOL AND SALSOLINE IN BIOLOGICAL SAMPLES USING DEUTERIUM-LABELLED INTERNAL STANDARDS AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Salsolinol and salsoline were labelled with deuterium using an acidic exchange reaction in $^2HCl—^2H_2O$. Two deuterium atoms were incorporated in both compounds. The deuterium-labelled salsolinol and salsoline were used as internal standards to determine picomol amounts of the corresponding unlabelled compound in the urine, cerebrospinal fluid, brain and liver. The salsolinol was purified on alumina and salsoline collected in the effluent. The compounds were analysed as their pentafluoropropionyl derivatives by gas chromatography on a 1% OV-17 column and were selectively detected with electron-impact mass spectrometry at the molecular ions M^+ and M^+-15.

With human urine the precision of the methods were ± 4.9% (coefficient of variation, $n = 10$) for salsolinol and ± 2.2% for salsoline at a level of 0.100 nmol/ml.

Administration of salsolinol to rats intraperitoneally (0.4 mmol/kg) resulted in levels of 1—2 nmol/g in striatum and limbic forebrain after 2 h, whereas the corresponding liver values were about 550 nmol/g. Control animals showed salsolinol values in liver of about 2 nmol/g and in striatum and limbic forebrain 1 nmol/g tissue.

INTRODUCTION

Tetrahydroisoquinoline alkaloids have been suggested to be involved in the biochemical mechanism causing alcohol addiction [1, 2].

In vivo and in vitro experiments have shown that biogenic amines such as dopamine can condense with acetaldehyde derived from ethanol metabolism to form 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol). This compound has been shown to be a substrate for COMT (catechol methyltransferase, EC 2.1.1.6) (see Fig. 1), thus forming 1-methyl-6-hydroxy-7-

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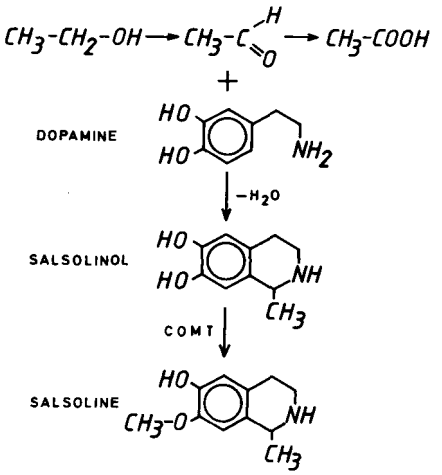

Fig. 1. The condensation of acetaldehyde and dopamine to give salsolinol, and its methylation to salsoline.

methoxy-1,2,3,4-tetrahydroisoquinoline (salsoline) [3]. It is still controversial whether these compounds are formed in vivo as a consequence of alcohol consumption. One reason for this might be that the amounts formed are below the detection limits of the analytical methods previously used [4, 5]. Another reason might be that the compound is conjugated and/or methylated to an extensive degree and is thus not detected.

The purpose of this study was to develop a highly selective and sensitive method to measure salsolinol and salsoline* in biological samples. To achieve optimal selectivity and reproducibility we decided to use gas chromatography—mass spectrometry with deuterium-labelled internal standards.

EXPERIMENTAL

Materials

Salsolinol was synthesized according to the description of Schöpf and Bayerle [6]. The purity was checked by melting point, thin-layer chromatography and gas chromatography. Salsoline was purchased from Aldrich Nova Kemi, Stockholm, Sweden.

The alumina oxide (Aktivitätstufe II—III, 0.063—0.200 mm) was obtained from Merck, Darmstadt, G.F.R. It was purified according to the method of Anton and Sayre [7]. All solvents used were of analytical grade. The acetaldehyde was freshly distilled before use. Solvents were extracted with water containing semicarbazide hydrochloride in order to trap any acetaldehyde present in the solvents.

Pentafluoropropionic anhydride was obtained from Massanalys, Bromma, Sweden. ^2H_2O was obtained from Norsk Hydro, Rjukan, Norway and ^2HCl in ^2H_2O 20% from Merck. Sulfatase was obtained from Sigma Chemical Company, St. Louis, MO, U.S.A., and semicarbazide hydrochloride from Fluka, Buchs, Switzerland.

*If salsolinol is methylated in the 6-position yielding isosalsoline, this isomer would be determined as salsoline by this method.

Preparation of 1-methyl-5,8-dideutero-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline ([$^2H_2$] salsolinol)

Salsolinol (100 mg) was heated in 10% ^{2}HCl in ^2H_2O at 120—130°C for 40 h. After evaporation the compound was crystallized in methanol—diethyl ether (1:100). The isotopic background at *m/z* M^+ and M^+-15, $H/^2H_2$ was 3.6%. The deuterium-labelled salsolinol was stored as the hydrochloride and dissolved in water before mixing with the samples as internal standard.

Preparation of 1-methyl-5,8-dideutero-6-hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline ([$^2H_2$] salsoline)

Salsoline (100 mg) was dissolved in 5% ^{2}HCl in ^2H_2O (1.5 ml) and heated at 120—130°C for 40 h. After evaporation and crystallization in the diethyl ether the compound was purified from traces of [$^2H_2$] salsolinol (formed through demethylation) on a column of alumina. The purified [$^2H_2$] salsoline contained an isotopic background at M^+ and M^+-15, $H/^2H_2$ = 1.8%. The deuterium-labelled salsoline was dissolved in a small amount of ethanol before being added to the samples.

Treatment of urine, cerebrospinal fluid and brain tissue

To the fresh biological samples 50 mg of semicarbazide hydrochloride were added. To 10 ml of urine was added 0.1 ml of 2% $NaHSO_3$, 0.2 ml of 10% ethylenediaminetetraacetate (EDTA), 1 nmol of [$^2H_2$] salsolinol and 1 nmol of [$^2H_2$] salsoline. The urine samples were titrated to pH 6.0—6.5 with 0.1 *M* sodium hydroxide before 10 mg of sulfatase were added. The samples were then incubated for 16 h at 37°C.

Following incubation, samples were buffered to pH 8.4 with 3 ml of 1 *M* trishydroxymethylaminomethane (Tris) buffer and put on Al_2O_3 columns (600 mg) prewashed with 0.1 *M* Tris (5 ml). The columns were washed through with 20 ml of water. The effluent was first extracted at pH 1—2 with ethyl acetate (2 × 10 ml). When it was of interest this organic phase was further processed for analysis of acidic and neutral catecholamine metabolites. The pH of the water phase was then adjusted to 10—11 and extracted with ethyl acetate (2 × 10 ml); the organic phase was evaporated and derivatized. In this fraction salsoline was recovered. The Al_2O_3 columns were eluted with 6 ml of 0.2 *M* formic acid. The eluate was freeze-dried overnight and the samples were then derivatized prior to analysis for salsolinol.

Samples (5 ml) of cerebrospinal fluid (CSF) were run through the same procedure but without incubation with sulfatase.

Tissue samples were weighed and homogenized with a loose-fitting PTFE pestle in 0.1 *M* HCl (2+1 ml) and centrifuged at 30,000 *g*. The supernatant was run through the above procedure. Liver samples were hydrolyzed with sulfatase but brain tissues were processed without hydrolysis.

Derivatization

The samples were dissolved in ethyl acetate—pentafluoropropionic anhydride (50 µl : 50 µl) and left for 0.5 h at 60°C. Solvent and reagent were evaporated under a stream of nitrogen. The samples were redissolved in 50 µl of ethyl acetate and 1 µl was injected into the gas chromatograph—mass spectrometer.

The work-up procedure is outlined in Fig. 2.

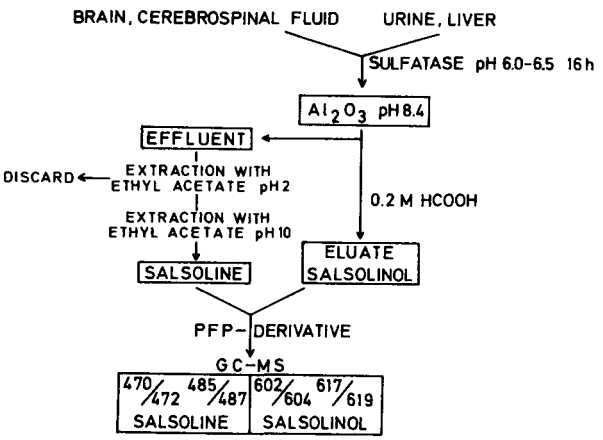

Fig. 2. Flow-diagram of the work-up procedure.

Standard curves

Standard curves were prepared for each analysis using varying amounts of salsolinol and salsoline and a constant amount of the corresponding deuterium-labelled compound. All standard samples were run through the whole analytical procedure.

Instrumental conditions

An LKB 2091 combined gas chromatograph—mass spectrometer was used. The instrument was equipped with a multiple ion detector and a 1% OV-17 column, length 2.5 m. The flow-rate of the carrier gas (helium) was 20—25 ml/min. Temperatures were: column 190°C, flash heater 230°C, ion source 270°C, the trap current was 50 μA and the energy of the electrons 22.5 eV.

The ions selected for salsolinol and [$^2H_2$]salsolinol were the base peaks *m/z* 602 and 604, respectively, and the molecular ion M^+ 617 and 619. For salsoline and [$^2H_2$]salsoline the corresponding ions were *m/z* 470 and 472 and M^+ 485 and 487.

Precision and recovery

To study the precision and recovery 1 nmol of salsolinol was added to 10 ml of urine. Ten equivalent samples were processed according to the procedure described above.

For salsoline the recovery and precision were also measured after addition of 1 nmol of the compound to 10 ml of urine (n = 9). The samples were acidified to pH 2 and extracted twice with ethyl acetate. The organic phases were discarded. The water phase was adjusted to pH 10—11 and extracted again with ethyl acetate twice.

Animal experiments

Male Sprague—Dawley rats (200 g, n = 6) were injected intraperitoneally (i.p.) with salsolinol · HBr (0.4 mmol/kg) dissolved in 200 μl of dilute HCl (pH 4). Control animals (n = 6) were injected with 200 μl of the dilute HCl. Two hours after the animals had received salsolinol they were anaesthetized with

chloroform and decapitated. The brains were removed and the striatum and limbic forebrain (containing tuberculum olfactorium, medial part of nucleus accumbens and amygdala) were dissected out. The liver was also removed and a 100 mg specimen was processed from each animal. The tissues were weighed and deuterium-labelled standards were added. The tissues were homogenized in 2+1 ml of 0.1 *M* HCl and centrifuged for 45 min at 30,000 *g*. The supernatant was stored at −20°C until analysis, which was carried out according to the method described above for urine and CSF.

Conjugation test

Male Sprague—Dawley rats (200 g, $n = 5$) were injected with salsolinol · HBr (0.4 mmol/kg). Two hours after the animals received salsolinol they were anaesthetized (chloroform) and decapitated. The brains and livers were taken out, weighed and homogenized. [$^2H_2$] Salsolinol and [$^2H_2$] salsoline were added and the homogenate was centrifuged and divided into two equal parts. One part was analysed as described above without incubation and the other was analysed after incubation with sulfatase overnight.

RESULTS

A mass spectrum of the pentafluoropropionyl derivative of deuterium-labelled salsolinol is presented in Fig. 3. The base peak in the spectrum was *m/z* 604 ($M^+ - 15$) and the intensity of the molecular ion *m/z* 619 (M^+) was about 30% of the base peak. The isotope background was 3.8 for *m/z* 602/604 and 617/619. The recovery for salsolinol, calculated at *m/z* 602/604 and 617/619, was found to be 102% and 95%, respectively. The precision at the 0.1 nmol/ml level expressed as coefficient of variation was ± 4.5% and ± 9.5%, respectively. For salsoline the corresponding derivative gave a mass spectrum with the base peak at *m/z* 472 and the M^+ at *m/z* 487 (see Fig. 3). The M^+ ion

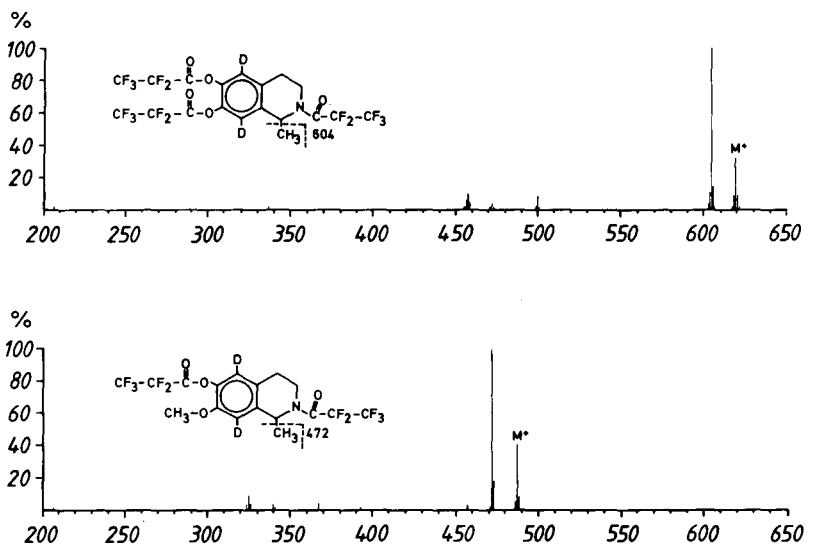

Fig. 3. The mass spectra of the pentafluoropropionyl derivatives of deuterium-labelled salsolinol (top) and salsoline (bottom).

was about 40% of the base peak and the isotopic background 1.8% for *m/z* 470/472 and 485/487. The recovery of salsoline was found to be 92% and 101% at *m/z* 470/472 and *m/z* 485/487 and the precision ± 2.2% and ± 5.0%, respectively.

The high sensitivity of the present method enables quantitative analysis of salsolinol down to 10 pmol/ml of urine and 10 pmol per 100 mg of tissue, and for salsoline down to 4 pmol/ml urine and 4 pmol per 100 mg of tissue. The minimum detectable amount of pure standards was 0.4 pmol of salsolinol and 0.2 pmol of salsoline.

The conjugation test showed that salsolinol was conjugated to 99% in the liver and to 25% in the brain, and the metabolite salsoline was completely conjugated in the liver but only conjugated to 7% in the brain.

Fig. 4 shows the monitoring of the selected ions for salsolinol and salsoline in a sample of rat limbic forebrain. Table I gives the results obtained in the rat experiment. Levels of 550 nmol/g of salsolinol (free + conjugated) could be recovered in the liver. Salsolinol was metabolized to an extensive degree to salsoline in the rat. Small but measurable amounts of salsolinol were recovered in the dopamine-rich regions of the brain. The amounts of salsoline found in the same regions were seven times higher than the levels found for salsolinol, while the metabolite in the liver amounted to about 34% of the salsolinol concentration. In the control rats small amounts of both salsolinol and salsoline were obtained.

DISCUSSION

During deuterium labelling of salsoline it was found to be important not to have too acid conditions otherwise salsoline was demethylated to salsolinol. Even under the conditions used trace amounts of salsolinol were formed. For this reason the [$^2H_2$]salsoline was purified on an alumina column before use.

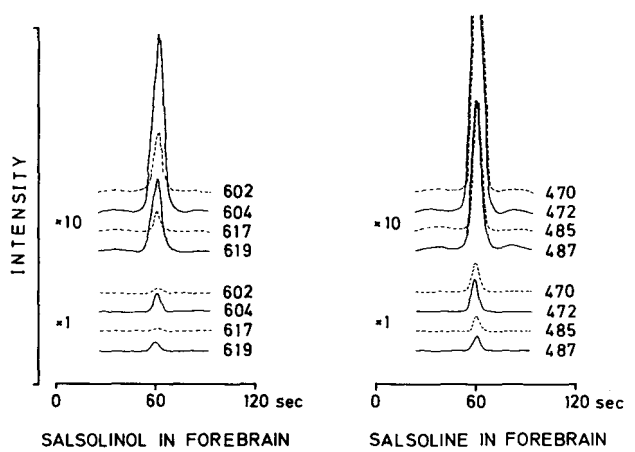

Fig. 4. Monitoring of *m/z* 602/604 and *m/z* 617/619 for the pentafluoropropionyl derivative of salsolinol, and *m/z* 470/472 and 485/487 for the corresponding derivative of salsoline, in a sample from limbic forebrain of a rat. Broken lines represent the unlabelled compounds and the solid lines the deuterium-labelled compounds.

TABLE I

LEVELS OF SALSOLINOL AND SALSOLINE IN RATS

Determinations were made 2 h after the i.p. administration of salsolinol · HBr 0.4 mmol/kg. The measurements were made at *m/z* 602/604 and 617/619 for salsolinol and *m/z* 470/472 for salsoline. Values are given as the mean ± S.D. (n = 6).

Sample	Salsolinol (nmol/g)		Salsoline (nmol/g)	
	m/z 602/604	*m/z* 617/619	*m/z* 470/472	*m/z* 485/487
Control rats				
Liver*	1.96 ± 0.92	2.27 ± 0.81	0.70 ± 0.15	0.65 ± 0.12
Striatum	0.85 ± 0.46	0.90 ± 0.38	0.13 ± 0.03	—
Forebrain	1.03 ± 0.41	1.02 ± 0.43	0.19 ± 0.12	—
Salsolinol-treated rats				
Liver*	550 ± 194	533 ± 180	185 ± 50	184 ± 49
Striatum	1.60 ± 0.46	1.39 ± 0.52	9.29 ± 0.72	9.11 ± 0.71
Forebrain	1.91 ± 0.48	1.58 ± 0.41	9.49 ± 0.91	9.31 ± 0.92

*Liver values were obtained after enzymatic hydrolysis with sulfatase.

The greatest advantage of these gas chromatographic—mass spectrometric methods compared to the earlier published gas chromatographic method with electron-capture detection [4] and the high-performance liquid chromatographic method with electrochemical detection [5], is the selectivity of the detection, particularly since the molecular ion and $M^+ - 15$ ions could be used.

The recovery data are relative to the deuterium-labelled standards and should thus ideally be 100% even if the absolute recovery is lower. Since the internal standards and the corresponding isoquinolines are similar chemically, most of the deviation from 100% is probably due to inaccuracy in pipetting.

Since both salsolinol and salsoline were found to be almost completely conjugated in the liver but only conjugated in the brain tissue to a minor degree, we decided to incubate liver and urine samples with sulfatase under the same conditions used in previous work on catecholamine metabolites [8] and to analyse brain and CSF samples without enzymatic hydrolysis.

The determinations were made at molecular ion M^+ and $M^+ - 15$ for both salsolinol and salsoline. The agreement between these determinations was good except for salsolinol in brain tissue of salsolinol-treated rats. The determinations at *m/z* 602/604 would seem to be most reliable since the peak intensity was three times those at *m/z* 617/619.

The discrepancy between the ratios between salsolinol and salsoline in brain and liver could either depend on a more extensive methylation of salsolinol in the central nervous system than in the periphery or on a difference in the penetration of salsolinol and salsoline through the blood—brain barrier. Since salsoline is more lipophilic than salsolinol, it would pass the blood—brain barrier more easily.

The origin of the low but measurable amounts of salsolinol and salsoline found in the control rats is obscure. These compounds could be formed as an artifact in the work-up procedure if acetaldehyde is present in the solvents used and dopamine in the biological samples. To avoid this we pretreated samples

and solvents with semicarbazide to trap acetaldehyde as the semicarbazone. We also made additions of acetaldehyde and dopamine to the samples and could not detect formation of salsolinol when semicarbazide had been used. A contribution of salsolinol from the food has not been excluded; post-climacteric bananas, for instance, have been reported to contain high amounts of salsolinol [9]. Another possibility is that small amounts of acetaldehyde are normally present in the organism and react with dopamine in the body. Since reliable methods to measure acetaldehyde at levels of less than 1 μM are lacking, this last possibility cannot be ruled out.

To summarize, this study describes the deuterium labelling of salsolinol and salsoline, the usefulness of these compounds as internal standards in quantitating the corresponding protium compounds in biological samples by a highly selective gas chromatographic—mass spectrometric method and the fate of i.p. administered salsolinol in the rat.

ACKNOWLEDGEMENTS

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CHROMBIO. 569

DETERMINATION OF TRANYLCYPROMINE IN HUMAN PLASMA AND URINE USING HIGH-RESOLUTION GAS—LIQUID CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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SUMMARY

A precise and sensitive method is described for the determination of the antidepressant drug tranylcypromine in human plasma and urine using high-resolution gas chromatography. The drug, together with an added internal standard, is extracted from the plasma or urine sample, derivatized with heptafluorobutyric anhydride and analysed on an OV-225 support-coated open-tubular glass capillary column with nitrogen-sensitive detection. The method has been applied to the measurement of tranylcypromine levels in plasma and urine from both healthy volunteers and from psychiatric patients receiving a therapeutic dosage of the drug.

INTRODUCTION

Although the monoamine oxidase inhibitor tranylcypromine (Parnate) has been used as an antidepressant for well over a decade our knowledge of its pharmacokinetic properties is very limited. We are particularly interested in this psychotropic drug because of its reported enhanced antidepressant effect when administered in combination with the antimanic agent lithium [1, 2]. In order to study the kinetics of this drug in man and to investigate what effect lithium may have on therapeutic plasma and urinary levels it was necessary to develop a sensitive and selective method for its determination. Previously described methods which have relied on fluorimetry [3] or gas chromatography

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with flame ionisation detection [4] are not suitable for quantifying the drug at the therapeutic level due to their lack of selectivity and sensitivity. More recently a method has been described utilizing gas chromatography with a packed column and electron-capture detection which has been used to measure the drug at these low concentration levels [5]. This same technique has also been used to measure plasma concentrations of tranylcypromine in an overdose patient [6].

In the present paper we describe an alternative sensitive gas chromatographic assay for tranylcypromine in human plasma and urine based on the use of a high-resolution glass capillary column with a nitrogen-sensitive detection system.

MATERIALS AND METHODS

Reagents

Tranylcypromine (*trans*-2-phenylcyclopropylamine) hydrochloride was obtained from Sigma (London) Chemical Co., Great Britain. The internal standard, 3-phenylpropylamine was obtained from Fluorochem (Glossop, Great Britain). Diethyl ether, hexane, and ethyl acetate (BDH, Poole, Great Britain) of AnalaR grade were each purified as described previously [7].

Stock standard solutions of tranylcypromine hydrochloride and 3-phenylpropylamine were prepared by dissolving these compounds in methanol to give concentrations of 1 mg/ml calculated as base. These solutions were stable for several weeks when stored at 0°C. On each experimental day dilutions of the standards were made to give concentrations of 1 μg/ml.

Sample collection

Blood samples in lithium-heparin tubes were centrifuged immediately after collection and the plasma stored in silanized glass tubes at −20°C until analysed. The volume and pH of urines were recorded and 20-ml aliquots stored at −20°C until analysed.

Gas—liquid chromatography

A Pye Unicam Series 104 Model 64 gas chromatograph modified to incorporate a Perkin-Elmer alkali-bead nitrogen-sensitive detector was used. The instrument was fitted with a falling needle solid injection device similar to that described by Van den Berg and Cox [8]. Analyses were made on a 20 m × 0.30 mm I.D. Pyrex capillary column coated with OV-225. Prior to coating with the stationary phase a layer of microparticles of sodium chloride were deposited on the walls of the column using a procedure similar to that described by Franken et al. [9]. The modified wall surface was then deactivated by coating a thin layer of Carbowax 20M followed by a 1-h flow conditioning at 280°C [10]. The capillary was coated using a 20% solution of OV-225 in methylene chloride according to the mercury plug dynamic procedure developed by Schomburg et al. [11]. The chromatographic conditions used in the analyses were as follows: helium carrier gas flow-rate 2 ml/min measured at 180°C. Injection and detector temperature 220°C. Hydrogen, air and make-up gas flow-rates to the nitrogen-sensitive detector were 5, 150 and 20 ml/min respectively. Samples

were introduced into the capillary immediately after the solvent had evaporated from the glass injector needle. The column was maintained initially at a temperature of 70°C for 3 min followed by a 30°/min programme rise to 160°C. The analyses were then carried out isothermally at this upper temperature. The amplifier setting was 1×10^{-11} A and results were recorded simultaneously on two Servoscribe pen recorders, one set at 10 mV and the other at 20 mV.

Gas chromatography—mass spectrometry

Mass spectra were recorded with a VG Micromass 70-70 F mass spectrometer equipment with a PDP 8a VG data system. The OV-225 capillary column installed in a Pye Unicam Series 204 gas chromatograph was linked directly to the ion source of the mass spectrometer using 0.4 mm I.D. glass-lined stainless-steel tubing. The mass spectrometer was operated with an ionization potential of 70 eV, an accelerating voltage of 4 kV and a trap current of 200 μA. The ion source and gas chromatograph interface temperatures were 200°C and 250°C respectively.

Extraction and derivatization

All glassware was cleaned and silanized as described previously [7]. To a 4-ml plasma sample or a diluted urine sample (1:10 with distilled water) contained in a 30-ml glass stoppered centrifuge tube were added 20 μl of the appropriate internal standard solution of 3-phenylpropylamine. Following the addition of 0.5 ml 5 *N* sodium hydroxide and 8 ml diethyl ether the mixture was shaken on a mechanical shaker for 10 min and then centrifuged. The organic layer was transferred using a pasteur pipette into a similar glass tube containing 2.4 ml 1 *N* hydrochloric acid and the mixture shaken for 10 min and centrifuged. The organic layer was aspirated and discarded and the remaining aqueous phase washed with 3 ml hexane followed by 3 ml diethyl ether. Hexane (3 ml) and 0.8 ml 5 *N* sodium hydroxide were then added and the tube shaken for 10 min and centrifuged. The hexane layer was then transferred with a pasteur pipette into a glass tube with a constricted tip and fitted with a PTFE-lined cap. The hexane phase was then taken to dryness under dried nitrogen while the tube was immersed in ice-water. Derivatization was performed by adding 100 μl ethyl acetate and 5 μl heptafluorobutyric anhydride to the residue and reacting at 60°C for 10 min. The reaction mixture was taken to dryness under dried nitrogen while again immersing the tube in ice-water and finally reconstituted to 100 μl with hexane containing 1% heptafluorobutyric anhydride. Aliquots of this solution were subjected to analysis on the gas chromatograph.

Quantitation

Quantitation was achieved by reference to a standard calibration curve prepared with each batch of samples analysed. This standard curve was obtained by analysing 4-ml aliquots of pooled blank plasma or diluted urine containing 20 ng internal standard and from 2.5 to 125 ng/ml tranylcypromine. The ratio of the peak height of tranylcypromine to the peak height of the added internal standard plotted against the concentration of the drug gave a linear curve which passed through the origin.

RESULTS AND DISCUSSION

In a previous publication [7] we demonstrated the potential of high-resolution gas chromatography with nitrogen-sensitive detection for the analysis of psychotropic drugs applied to the determination of the antidepressant Nomifensine in plasma. This technique has now been successfully employed to the described method for tranylcypromine determination in human plasma and urine. In order to avoid a derivatization step in the assay procedure an attempt was initially made to quantitate the drug as its free base. The underivatized drug exhibited good gas chromatographic properties on deactivated polar glass capillaries but irreversible absorption effects were apparent at concentration levels below 5 ng. The heptafluorobutyrate (HFB) derivatives used to overcome this problem are readily prepared in quantitative yield if the described precautions are taken to prevent physical losses during the removal by evaporation of the derivatizing reagents. The structure of tranylcypromine HFB and its mass spectrum are shown in Fig. 1. The derivatized drug and internal standard have methylene unit values [12] on the OV-225 support coated glass capillary of 21.2 and 20.6 respectively. The limit of detection of the nitrogen-sensitive detector for tranylcypromine HFB was 500 pg with a signal-to-noise ratio of 10:1. The extraction procedure gave extracts both for plasma and urine which contained few detectable impurities none of which interfered with the analysis of the drug even down to the 2 ng/ml level. Fig. 2 shows a chromatogram from the analysis of a plasma sample from a patient receiving a therapeutic dosage of the drug.

The accuracy of the method was determined from recovery experiments of

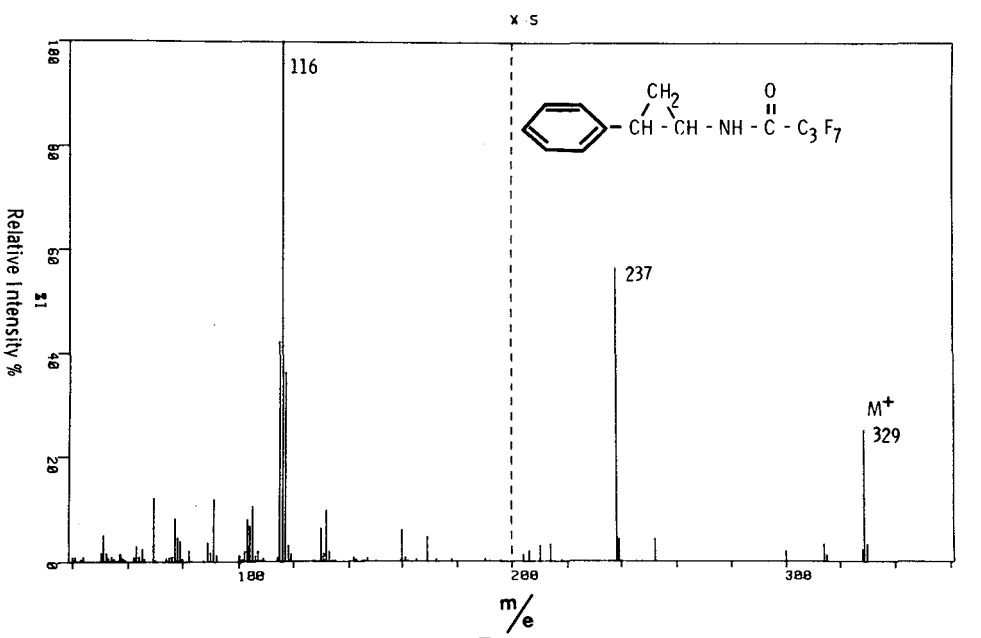

Fig. 1. Electron-impact mass spectrum and structure of the heptafluorobutyrate derivative of tranylcypromine.

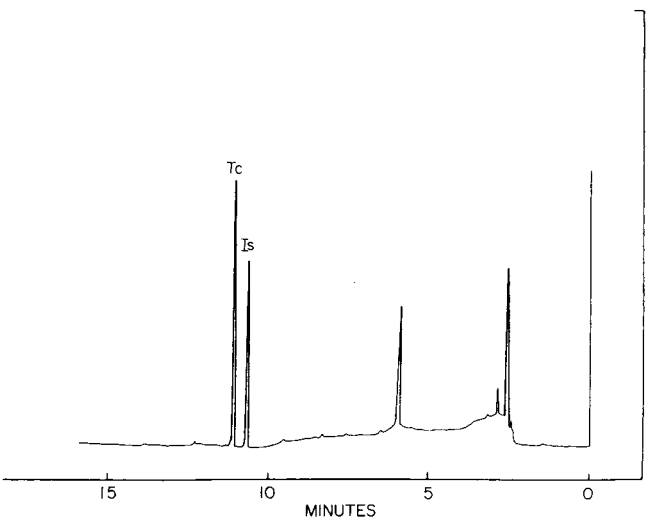

Fig. 2. Gas chromatogram from the analysis of a plasma sample from a patient receiving a therapeutic dosage of tranylcypromine. Peaks: Tc, tranylcypromine and I.S., internal standard, 3-phenylpropylamine.

authentic tranylcypromine added to blank plasma and urine samples at different concentration levels. The calculated recoveries from eight plasma samples analysed over the range 5—50 ng/ml varied from 83.5 to 100.2% with a mean of 92.1% (S.D. ± 5.92) and from eleven plasma samples analysed over the range 100—400 ng/ml the values varied from 88.7 to 99.5% with a mean of 92.2% (S.D. ± 3.89). From eight urine samples the calculated recoveries over the range 50—500 ng/ml varied from 88.1 to 99.2% with a mean of 93.3% (S.D. ± 3.62). The absolute recovery of the drug from plasma using phenylpropylamine as an external standard gave, in ten analyses, a mean value of 85.3% (S.D. ± 4.76).

The precision of the method was determined from duplicate estimations in plasma using the method of Snedecor [13]. The results are given in Table I.

Combined gas chromatography—mass spectrometry was carried out on a number of plasma and urine extracts. The separation was made on an OV-225 coated capillary column similar to that used in the assay procedure. The mass spectra of the peaks at the retention time of tranylcypromine HFB and phenylpropylamine HFB were identical with the spectra from the derivatized authentic standards.

TABLE I

PRECISION OF THE METHOD FROM ESTIMATES OF DUPLICATE DETERMINATIONS

	Range (ng/ml)	
	2—20	50—200
Number of duplicates	6	6
Mean (ng/ml)	10.22	111.27
Standard deviation	0.99	4.1
Coefficient of variation (%)	9.69	3.68

A study of the specificity of the method towards other drugs or metabolites has not yet been made. However the use of the nitrogen detector together with a high-resolution capillary column has the advantage of reducing possible interference from contaminating compounds.

Application

The method has been applied to a study of the plasma and urinary concentration profiles of the drug in three healthy adult volunteers. Each subject was administered 50 mg Parnate after overnight fasting and plasma and urine samples were collected over the following 24 h. The plasma concentration—time curves for the three subjects are given in Fig. 3. After a rapid rise in plasma concentration peak levels of 75.5, 79.5 and 89.4 ng/ml were reached 1.5, 1.75 and 2.25 h respectively after administration of the drug. These findings are comparable to those obtained by Baselt et al. [5] who carried out a similar study on two normal subjects following an oral dosage of 30 mg of the drug.

The excretion rate of urinary tranylcypromine in Subject 1 over the 24-h collection period is shown in Fig. 4. Only 2.16% of the administered dose in this subject was excreted as unchanged drug which is in concurrence with previous results [5, 14] suggesting that tranylcypromine undergoes extensive metabolism before excretion.

A preliminary study has been made using the described assay procedure to elucidate a possible relationship in depressed patients between plasma concentration of the drug and clinical effect. An investigation has also been carried out on the effect of lithium therapy on therapeutic plasma and urinary levels of tranylcypromine. The results of these investigations will be reported elsewhere.

In conclusion the method described here was found to be accurate and reliable for measuring tranylcypromine in human plasma and urine at the

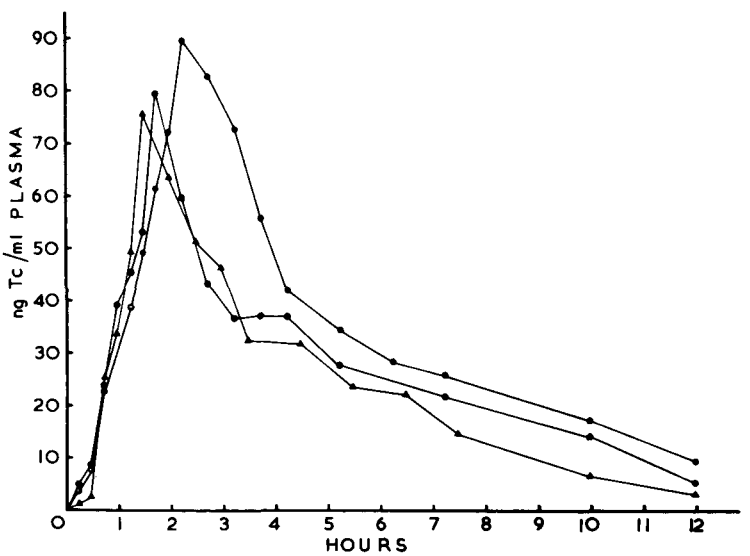

Fig. 3. Plasma concentration—time profiles in three healthy adult volunteers following oral administration of 50 mg Parnate.

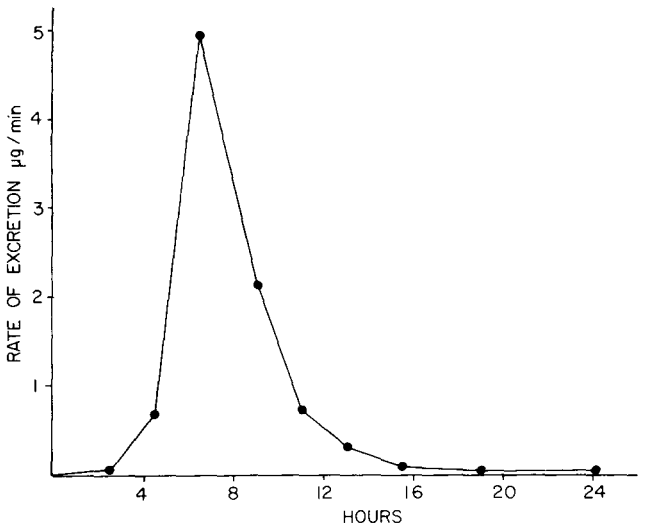

Fig. 4. Excretion rate of tranylcypromine in urine of a healthy adult volunteer following oral administration of 50 mg Parnate.

therapeutic level. The use of high-resolution capillary gas chromatography enhances both the sensitivity and specificity of the method. The alkali-bead nitrogen detector used in the method has an advantage over the electron-capture detector in having a greater stability and reliability.

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CHROMBIO. 575

GAS CHROMATOGRAPHIC DETERMINATION OF AMANTADINE HYDROCHLORIDE (SYMMETREL) IN HUMAN PLASMA AND URINE

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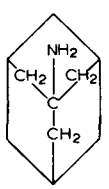
SUMMARY

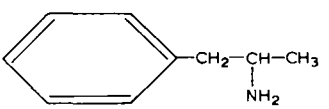
A method for the determination of amantadine hydrochloride at concentrations down to 10 ng/ml in human plasma and urine is described. After addition of a known amount of amphetamine sulphate as internal standard to 1 ml of plasma or urine, amantadine is extracted at basic pH in toluene. Both compounds are derivatized with trichloroacetyl chloride. The derivatives are determined by gas chromatography using a ^{63}Ni electron-capture detector. The technique was applied in a study of the elimination of amantadine after oral administration to man; plasma concentrations are reported.

INTRODUCTION

Amantadine* hydrochloride is an antiviral agent, active against various strains of influenza virus.



Amantadine



Amphetamine

Several methods for the assay of amantadine by gas chromatography have been described. Bleidner et al. [1] studied the absorption, the distribution and the excretion of amantadine hydrochloride using gas chromatography with flame-ionization detection, but this method lacks sensitivity. Montanari et al. [2] studied the urinary excretion of amantadine in the elderly using a gas chro-

*Symmetrel®, Ciba-Geigy.

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matograph with a flame-ionization detector. Biandrate et al. [3] described a laborious gas chromatographic method with electron-capture detection for the determination of amantadine in human plasma down to 25 ng/ml, using amphetamine sulphate as internal standard.

This paper reports on a rapid and sensitive procedure which permits the determination of amantadine down to 10 ng/ml of plasma or urine.

EXPERIMENTAL

Chemicals and reagents

Amantadine hydrochloride was supplied by Ciba-Geigy (Basle, Switzerland) and amphetamine sulphate by Specia, Rhône-Poulenc (Paris, France).

All reagents and solvents are of analytical grade: sodium hydroxide (Titrisol, Merck 9956; E. Merck, Darmstadt, G.F.R.); toluene (Mallinckrodt, St. Louis, MO, U.S.A.); trichloroacetyl chloride purum (Fluka, Buchs, Switzerland).

The aqueous solution of internal standard contains 200 ng of amphetamine sulphate per ml.

Equipment

The glass tubes are pretreated to prevent adsorption. They are immersed in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1% v/v each) for 15 min and rinsed with methanol. The treatment is repeated every two months. Between such treatments, the tubes are cleaned as usual.

A Hewlett-Packard Model 5713A gas chromatograph equipped with a Hewlett-Packard electron-capture detector (Model 18713A) is used. The peak areas are given by a Hewlett-Packard electronic integrator (Model 3380A).

The column is operated at 200°C, the injector at 250°C and the detector at 300°C, with an argon—methane (90:10) flow-rate of 75 ml/min. The glass column is washed with 1 *M* hydrochloric acid, distilled water, acetone and benzene and then silanized with a 1% (v/v) solution of hexamethyldisilazane in benzene. After this treatment the column is washed again with benzene and dried at 100°C.

The column packing is 5% SE-30 on Chromosorb W HP, 80—100 mesh (Applied Science Labs., State College, PA, U.S.A.). The filled column (2 m × 3 mm I.D.) is flushed with the carrier gas at a flow-rate of 40 ml/min and heated to 300°C at a rate of 1°C min. The column temperature is held overnight at 300°C and throughout the next day at 270°C. The temperature is then repeatedly increased from 150 to 250°C over a period of 24 h. During these programmed cycles, the column is further conditioned by injection 100 μl of Silyl 8 (Pierce, Rockford, IL, U.S.A.) by fractions between 150°C and 220°C. After this treatment, the column is ready for use.

Extraction

One millilitre of the internal standard solution is measured into a stoppered glass tube. Then, 1 ml of the sample (plasma or urine), 1 ml of 1.0 *M* sodium hydroxide and 2 ml of toluene are added. The tube is shaken mechanically (Infors shaker) for 15 min at a speed gradually increasing up to 300 rpm to prevent emulsification, and centrifuged at 4800 *g* for 10 min.

Derivatization and chromatography

An aliquot of the toluene phase is transferred to another tube, 10 μl of 2% trichloroacetyl chloride solution in toluene (prepared immediately before use) are added, and the stoppered tube is heated at 70°C for 30 min.

After cooling to room temperature, excess trichloroacetyl chloride is removed with 1 ml of 1.0 *M* sodium hydroxide and the tube is shaken mechanically for 5 min and centrifuged. A 3-μl portion of the organic layer is injected into the gas chromatograph by the solvent-flush technique using 1 μl of toluene as the guard.

The amantadine hydrochloride content is calculated from the peak area ratio by reference to a calibration curve. This curve is obtained by extraction of plasma or urine spiked with increasing amounts of amantadine hydrochloride (from 10 to 1000 ng/ml) and a constant amount of internal standard (200 ng/ml plasma or urine).

Study in man

Eight healthy subjects, who had been advised to take no drugs during the week preceding the experiment and none besides amantadine throughout the duration of the study, received 100 mg of amantadine hydrochloride as one soft-gelatin capsule of Symmetrel. Blood samples were collected before and 0.5, 1, 2, 4, 6, 8, 12, 24 h after the administration of the drug and centrifuged. Plasma was removed and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Precision and recovery

Tables I and II give the results obtained when the described procedure was applied to spiked plasma and spiked urine samples, respectively. These tables demonstrate the good reproducibility of the assay down to concentrations of 10 ng amantadine hydrochloride per ml of plasma or urine. This concentration (10 ng/ml) may be taken as the sensitivity limit of the method, although lower concentrations could still be detected.

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF AMANTADINE HYDROCHLORIDE APPLIED TO SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Amount found (ng/ml) (mean, n = 6)	Precision/ reproducibility (CV, %)	Recovery/accuracy (mean, %)
10	9.2	6.8	92.3
20	20.3	1.6	101.6
50	52	3.7	104.3
100	105	1.5	104.8
200	213	1.2	106.6
1000	927	0.5	92.7
			100.4 ± 6.5 (± S.D.)

TABLE II

PRECISION AND RECOVERY OF THE DETERMINATION OF AMANTADINE HYDROCHLORIDE APPLIED TO SPIKED HUMAN URINE SAMPLES

Amount added (ng/ml)	Amount found (ng/ml) (mean, n = 6)	Precision/ reproducibility (CV, %)	Recovery/accuracy (mean, %)
10	10.8	5.2	108.2
25	24.1	7.6	96.5
100	105	1.9	104.6
			103.1 ± 7.1 (± S.D.)

Plasma and urine interference

Fig. 1 shows the chromatograms of an extract of human plasma and of the same extract spiked with 200 ng of amphetamine sulphate and 200 ng of amantadine hydrochloride. There is no interference from the normal components of the plasma extract.

Fig. 2 shows the chromatograms of an extract of human urine and of the same extract spiked with 200 ng of amphetamine sulphate and 200 ng of amantadine hydrochloride.

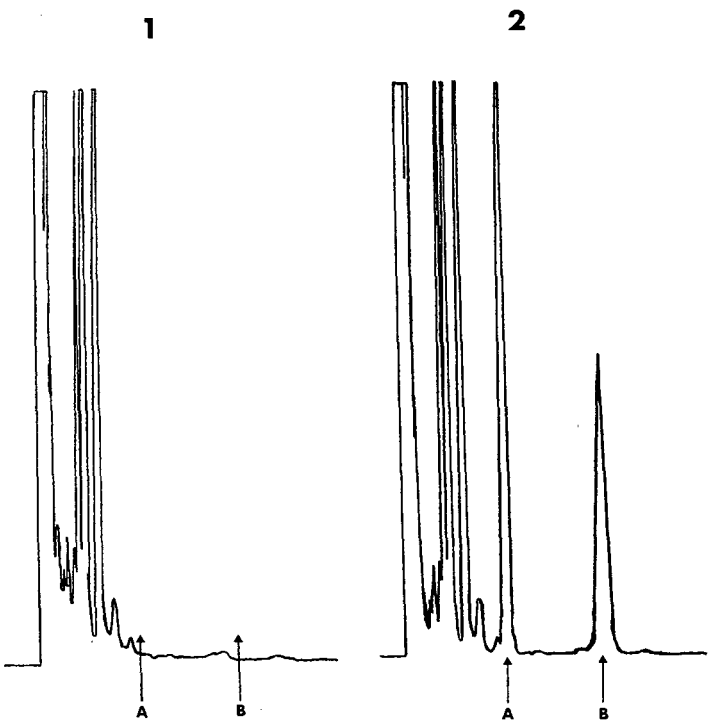

Fig. 1. Examples of chromatograms: (1) human plasma blank (1 ml of plasma); (2) the same plasma spiked with 200 ng/ml internal standard (A), and 200 ng/ml amantadine hydrochloride (B).

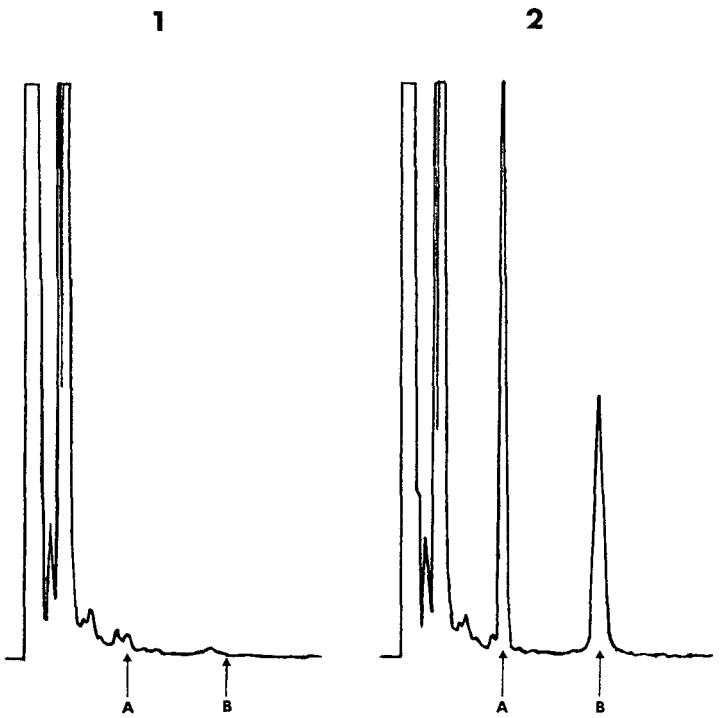

Fig. 2. Examples of chromatograms: (1) human urine blank (1 ml of urine); (2) human urine spiked with 200 ng/ml internal standard (A), and 200 ng/ml amantadine hydrochloride (B).

Specificity

Bleidner et al. [1] showed that the cumulative amount of amantadine recovered unchanged from human urine approached an asymptotic value of 93% of the administered dose. There was no evidence of acetylated or methylated forms of amantadine in any of the urine samples examined, and no extraneous peaks were observed that could be attributed to metabolites of the drug.

Storage stability of amantadine hydrochloride in human plasma

Table III shows that no decrease in the amantadine hydrochloride content (20 ng/ml and 100 ng/ml) was observed in plasma samples when stored frozen for six months at −20°C.

Application

The technique was applied in a study of the elimination of amantadine after oral administration to eight healthy subjects. Fig. 3 shows an average curve obtained from the plasma samples of the eight subjects given 100 mg of amantadine as one Symmetrel capsule. The sensitivity of the method thus appears sufficient to determine amantadine in bioavailability or pharmacokinetic assays.

TABLE III

STORAGE STABILITY OF AMANTADINE HYDROCHLORIDE IN HUMAN PLASMA FOR SIX MONTHS AT −20°C

Duration of storage at −20°C	Amount of amantadine hydrochloride added to plasma (ng/ml)	
	20	100
	Amount of amantadine hydrochloride found (average of two assays) (ng/ml)	
1 day	19	105
8 days	18	103
15 days	18	104
1 month	20	98
3 months	19	96
6 months	19	106

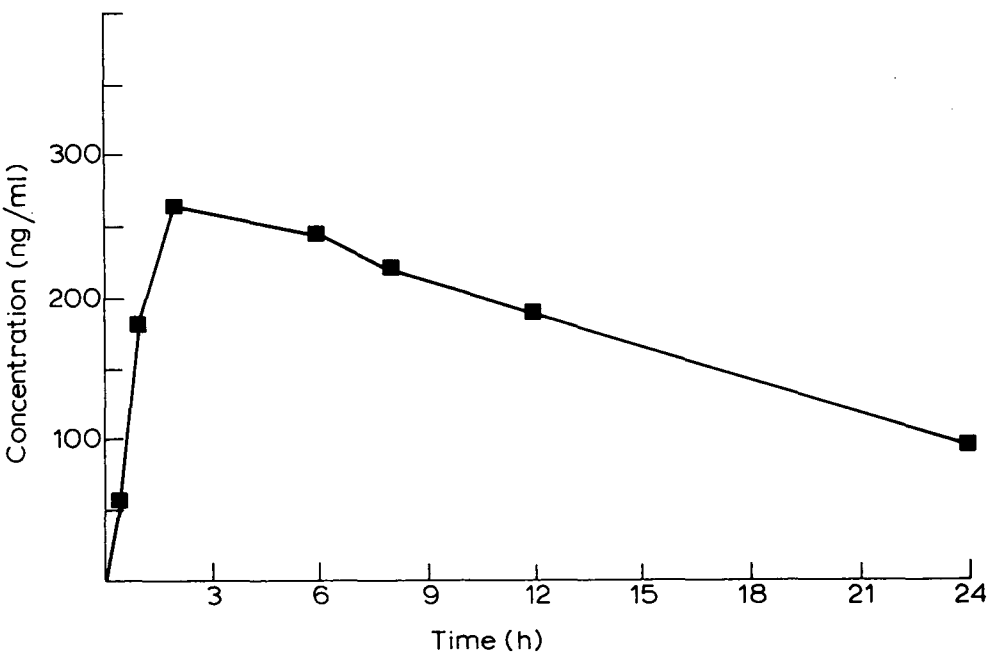

Fig. 3. Average amantadine hydrochloride plasma concentrations obtained in eight healthy subjects after administration of 100 mg of Symmetrel.

CONCLUSION

The proposed technique permits the quantitative assay of amantadine hydrochloride in human plasma and urine at concentrations down to 10 ng/ml. It is fast, reproducible and sufficiently sensitive for determinations of the absorption of amantadine in man after administration of a single dose.

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CHROMBIO. 578

IMPROVED GAS CHROMATOGRAPHIC METHOD OF DETERMINING DICLOFENAC IN PLASMA

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SUMMARY

Diclofenac was converted into either its methyl or ethyl ester in methanol or ethanol containing 0.1% or 0.5% sulfuric acid, respectively. The ester was extracted and subjected to gas—liquid chromatography with electron-capture detection. The esterification resulted in an increased sensitivity of the gas chromatographic detection, three times better than that previously reported for the formation of indolone ring in trifluoroethanol containing 0.5% sulfuric acid.

INTRODUCTION

Diclofenac [sodium *o*-(2,6-dichlorophenyl)-aminophenylacetate; the active constituent of Voltaren] is pharmacologically active as an anti-inflammatory and antirheumatic agent. Two methods for assaying diclofenac in biological materials have been reported [1, 2]. Both methods are based on indolone ring formation: one in trifluoroethanol containing 0.5% sulfuric acid [1], the other in methanol saturated with HCl gas [2]. We applied both methods to the determination of diclofenac in rabbit plasma after drug administration. The second method did not give good reproducibility because of interference in the reaction by concomitant moisture. In the first method, however, the presence of methanol in the trifluoroethanol gave a sensitivity about three times higher than that previously reported. For convenience, we routinely used the methanol—trifluoroethanol method for the quantitation of diclofenac. Meanwhile, using thin-layer chromatography and gas chromatography—mass spectrometry

(GC—MS), we identified a GC peak with a retention time of 16 min as the indolone derivative and the methyl ester of diclofenac. On the basis of these observations and the fact that trifluoroethanol is a relatively expensive material, we sought an improved and inexpensive method for the determination of diclofenac. In the present paper, the search for more available reagents for derivative formation and the assay procedure using those reagents are described.

EXPERIMENTAL

Reagents

All chemicals were of analytical grade, and the organic solvents for extraction were of especially fine grade; these were purchased from Wako Pure Chemicals, Osaka, Japan.

Extraction of diclofenac from plasma

Extraction was carried out with slight modifications of the method previously reported by Geiger et al. [1]. A 0.5-ml volume of plasma was acidified with 1 ml of 2.7 *M* phosphoric acid, and diclofenac in the solution was extracted with 5 ml of benzene under continuous shaking for 30 min. The mixture was centrifuged at 1000 *g* for 10 min to separate the phases. A 4-ml aliquot of the benzene extract thus obtained was added to 2 ml of 0.1 *N* sodium hydroxide solution, and the mixture was stirred with a Vortex mixer. After centrifugation at 1000 *g* for 10 min, as much of the benzene phase as possible was removed by aspiration and the residual benzene was evaporated under a stream of nitrogen gas. The aqueous phase was acidified with 0.2 ml of 43% phosphoric acid, and diclofenac in the solution was re-extracted with 3 ml of benzene using a Vortex mixer. A 2.5-ml aliquot of the benzene layer in a test-tube was evaporated to dryness at 40°C under reduced pressure. The dried residue was dissolved in diethyl ether, and transferred to a 5-ml ready-made ampoule. The test-tube was washed twice with ether. The ether washes were combined in the ampoule and evaporated to dryness in a gentle stream of nitrogen gas. Diclofenac in the residue was subjected to cyclization or esterification as described below.

Cyclization of diclofenac

The procedure described by Geiger et al. [1] was modified as follows. The dried residue in the ampoule was dissolved in 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid, and the ampoule was sealed. After reaction in a water-bath at 75°C for 75 min, the ampoule was opened, and 0.4 ml of 25% potassium hydrogen carbonate solution and 2 ml of *n*-hexane were added. The mixture was shaken on a Vortex mixer, and 1.5 ml of the hexane phase were transferred to a 2.5-ml test-tube. Then hexane was evaporated to dryness in a gentle stream of nitrogen gas below 40°C. The residue was dissolved in an aliquot of benzene containing aldrin (25 ppb), of which a 1-μl aliquot was injected into the gas chromatograph.

Esterification of diclofenac

The dried residue in the ampoule was dissolved in 0.15 ml of methanol or

ethanol containing 0.1% (v/v) or 0.5% (v/v) sulfuric acid, respectively, and the ampoule was sealed. After reaction in a water-bath at 60°C for 1 h, the ampoule was opened and treated as described above.

Gas—liquid chromatography

The gas—liquid chromatographic (GLC) analysis was carried out on a Shimadzu Model GC-4CMPFE gas chromatograph, which was operated at a pulse-rate of 10 kHz and a temperature of 290°C. The glass column, 2 m × 3 mm I.D., was packed with 1.5% Silicone OV-17 on Shimalite W AW DMCS, 80—100 mesh. The carrier gas was nitrogen at a flow-rate of 50 ml/min. The electrometer setting was continuously kept at range 10^2, attenuation 8. The injection port temperature was 290°C, column oven 260°C. The peak height ratio was used for quantitation. Typical gas chromatograms obtained using the present procedures are shown in Fig. 1.

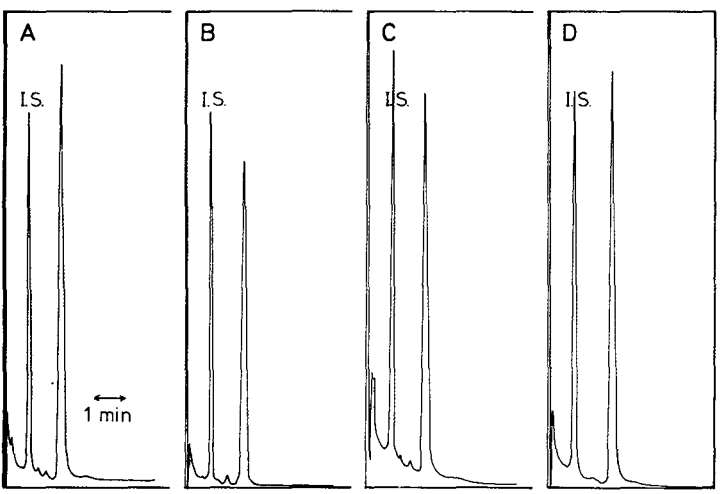

Fig. 1. Typical gas chromatograms, with electron-capture detection, of the reaction products obtained using four different reaction systems: trifluoroethanol—H_2SO_4 (A), trifluoroethanol—methanol—H_2SO_4 (B), methanol—H_2SO_4 (C) and ethanol—H_2SO_4 (D). Analytical conditions were as described in Experimental. I.S., internal standard (aldrin).

RESULTS AND DISCUSSION

Derivative formation from diclofenac

Since we had noticed that the presence of methanol in trifluoroethanol caused about a threefold increase in sensitivity compared to trifluoroethanol alone, the determination of diclofenac in plasma was carried out using the mixture of methanol and trifluoroethanol. Thereafter, we attempted to react diclofenac in methanol or ethanol alone, instead of in trifluoroethanol, in the presence of sulfuric acid under the same reaction conditions of Geiger et al. [1]. Reaction in methanol or trifluoroethanol yielded a single peak with a retention time of 16 min when analyzed by GC—MS under the conditions described above except that the column oven temperature was maintained at 200°C and

the flow-rate of carrier gas (helium) was 40 ml/min; reaction in ethanol yielded a peak at the retention time of 19 min. At this point, it was thought that the indolone derivative and the methyl ester of diclofenac might overlap each other on the gas chromatogram. To analyze the reaction products in five independent reactions, thin-layer chromatography was carried out (Fig. 2). It was shown

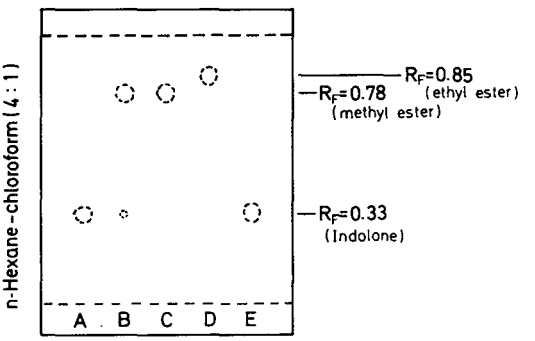

Fig. 2. Thin-layer chromatogram of reaction products obtained in five different reaction systems. Chromatography was performed on silica gel (Wakogel B5FM for UV detection), and each spot was visualized by UV absorption. Diclofenac (1 mg) was reacted with each reagent [trifluoroethanol—H_2SO_4 (A), trifluoroethanol—methanol—H_2SO_4 (B), methanol—H_2SO_4 (C), ethanol—H_2SO_4 (D) or methanol—HCl (E)] and each reaction product extracted with *n*-hexane was subjected to thin-layer chromatography.

that the chief reaction product in a mixture of trifluoroethanol (0.15 ml) and methanol (0.05 ml) is the methyl ester of diclofenac. The reaction products produced in those systems were subjected to GC—MS, and were confirmed to contain the indolone or the ester (see Fig. 3). Geiger et al. tried using diazomethane for the esterification of diclofenac and found the simultaneous formation of the ester and the indolone. We examined the methylation reaction using diazomethane; we detected no indolone, but dimethylated diclofenac in addition to the monoester, which were detected by GC—MS analysis.

Reaction conditions for derivative formation

To establish optimum reaction conditions, the reaction temperature and sulfuric acid concentration were changed in three reaction systems. From the results (Fig. 4), it was concluded that the most preferable conditions were the methanol system with 0.1% (v/v) sulfuric acid, and with reaction temperature and time of 60°C and 1 h, respectively.

Standard curves and calibration curves

Fig. 5 shows standard curves (without extraction procedure) of diclofenac in four systems. The curves are linear up to at least 10 μg. Fig. 6 shows calibration curves for each analytical procedure with the extraction procedure. A good linearity is seen up to at least 10 μg/ml. As little as 0.1 μg of diclofenac can be measured by the present method.

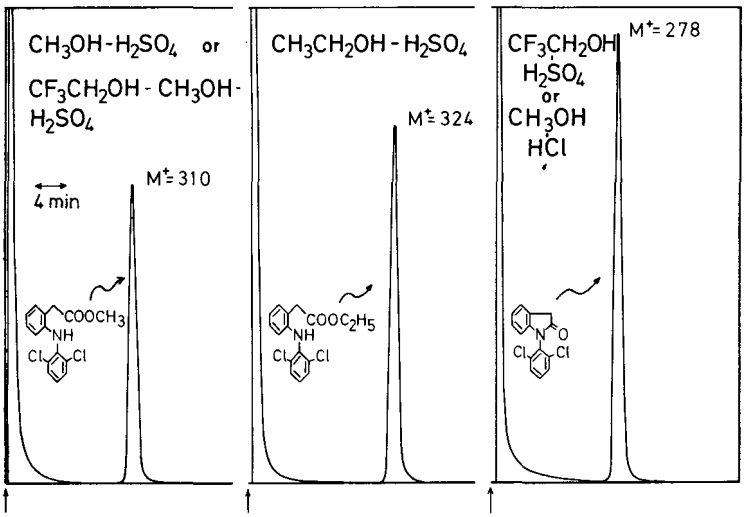

Fig. 3. Gas chromatograms of the reaction products obtained in five different reaction systems by GC—MS. The reaction products obtained as described in Fig. 2 were also subjected to GC—MS, which was performed on a Shimadzu LKB 9000 gas chromatograph—mass spectrometer. The column oven temperature was maintained at 200°C, and the flow-rate of carrier gas (helium) was 40 ml/min. Column packings and size were as described in Experimental. Reaction of diclofenac with methanol—H_2SO_4 or trifluoroethanol—methanol—H_2SO_4 gave the peak at 310 as M^+ (the methyl ester), while reaction with trifluoroethanol—H_2SO_4 or methanol—HCl gave the peak at 278 as M^+ (indolone). Reaction with ethanol—H_2SO_4 gave the peak at 324 as M^+ (the ethyl ester).

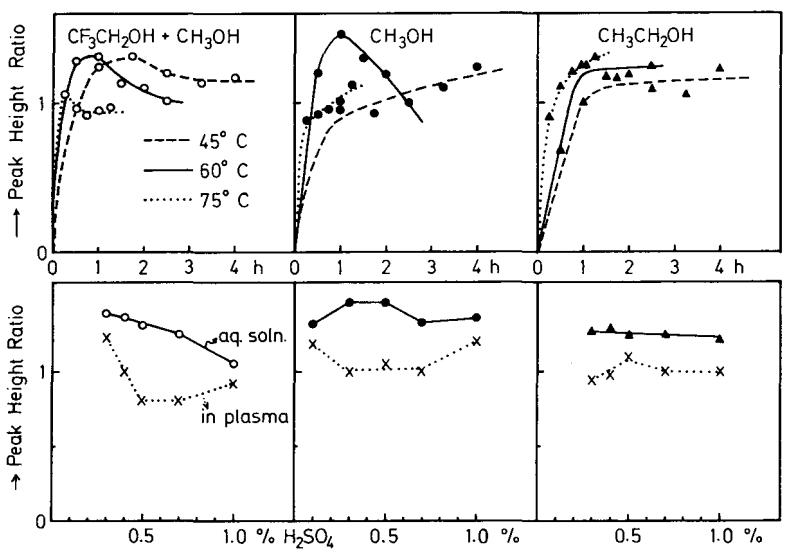

Fig. 4. The effect of temperature and time and sulfuric acid concentration on the esterification of diclofenac. Diclofenac was reacted with 0.15 ml of alcohol containing 0.5% sulfuric acid at three different temperatures (upper panels). To test the optimum concentration of sulfuric acid, diclofenac was reacted with 0.15 ml of alcohol containing various concentrations of sulfuric acid at 60°C for 1 h (lower panels). Extraction and determination were carried out as described in Experimental.

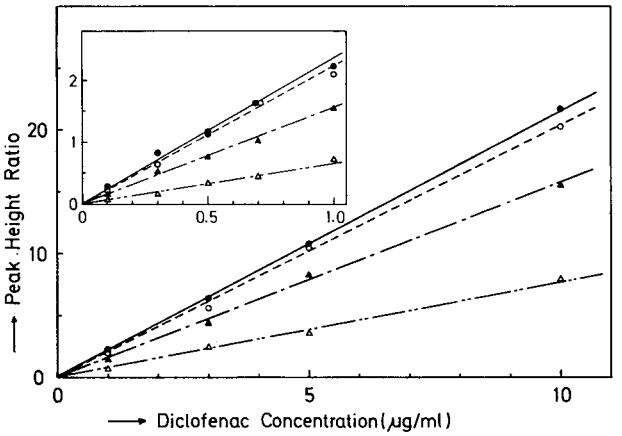

Fig. 5. Standard curves of diclofenac. Varying amounts of diclofenac methanol solution in an ampoule were evaporated to dryness, reacted in four different systems, and measured by GC with electron-capture detection after being dissolved in an aliquot of benzene containing 25 ppb of aldrin. •——• represents the standard curve obtained by reaction of diclofenac with 0.15 ml of methanol containing 0.1% (v/v) sulfuric acid at 60°C for 1 h, ○— — —○ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid and 0.05 ml of methanol at 60°C for 1 h, ▲— · —▲ by reaction with 0.15 ml of ethanol containing 0.5% (v/v) sulfuric acid at 60°C for 1 h, and △—· · —△ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid at 75°C for 75 min.

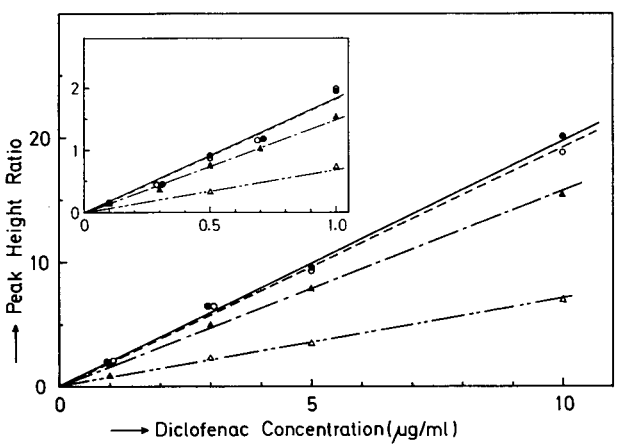

Fig. 6. Calibration curves of diclofenac. Varying amounts of aqueous diclofenac solution were treated as described in Extraction of diclofenac from plasma, then reacted in four different systems and measured by GC with electron-capture detection after bing dissolved in an aliquot of benzene containing 25 ppb of aldrin. •——• represents the calibration curve obtained by reaction with 0.15 ml of methanol containing 0.1% (v/v) sulfuric acid at 60°C for 1 h, ○— — —○ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid and 0.05 ml of methanol at 60°C for 1 h, ▲— · —▲ by reaction with 0.15 ml of ethanol containing 0.5% (v/v) sulfuric acid at 60°C for 1 h, and △— · · —△ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid at 75°C for 75 min.

Recovery tests

In order to apply the esterification methods to the assay of diclofenac in plasma, recovery tests were carried out. Various amounts of diclofenac were added to each sample of plasma and the amount of diclofenac was measured by the procedure given above. The results are summarized in Table I. Recovery data obtained from the esterification method are comparable with those from the method of Geiger et al. [1].

TABLE I

RECOVERY OF DICLOFENAC ADDED TO PLASMA

Diclofenac extracted from plasma was reacted in three different systems, trifluoroethanol—methanol—H_2SO_4 (B), methanol—H_2SO_4 (C) or ethanol—H_2SO_4 (D). Each value is the mean of duplicate determinations.

Diclofenac added (μg/ml)	Diclofenac determined (μg/ml)		
	B	C	D
1.0	0.97	0.96	1.00
2.0	1.87	1.84	1.96
4.0	3.64	3.58	3.26
6.0	5.72	6.23	5.79
Recovery (%)	94.1 ± 2.6	95.2 ± 6.3	94.0 ± 8.5

ACKNOWLEDGEMENT

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CHROMBIO. 579

SOME ASPECTS OF THE GAS—LIQUID CHROMATOGRAPHIC ANALYSIS OF CYCLOPHOSPHAMIDE IN PLASMA

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SUMMARY

The gas—liquid chromatography of cyclophosphamide has been extensively investigated. Several methods for the assay of cyclophosphamide in plasma are reported, those with the nitrogen—phosphorus-specific detection being most sensitive and selective.

INTRODUCTION

The widely applied drug cyclophosphamide (CP) with antitumor and immunosuppressive properties is known to act through its activated forms 4-hydroxycyclophosphamide, aldophosphamide and phosphoramide mustard [1, 2]. Our research program aims at developing analytical methods for these metabolites and the parent compound by gas—liquid chromatography (GLC) with nitrogen—phosphorus (N—P) specific detection. Our starting point was to establish methods for the quantification of CP in biological fluids. Known analyses include: radioactivity measurements of ^{14}C-labelled CP [3], mass spectrometry [4, 5], gas chromatography—mass spectrometry [2, 6] and high-performance liquid chromatography [7]. CP was determined by GLC in pharmaceutical formulations [8], in sheep tissue [9] and in plasma of mice [10]. Recently methods to determine CP in human plasma have been published [11—13]. In this study we present a general insight into the GLC behaviour of CP and we discuss several GLC methods. The structural formulae of CP and two of its congeners are given in Fig. 1.

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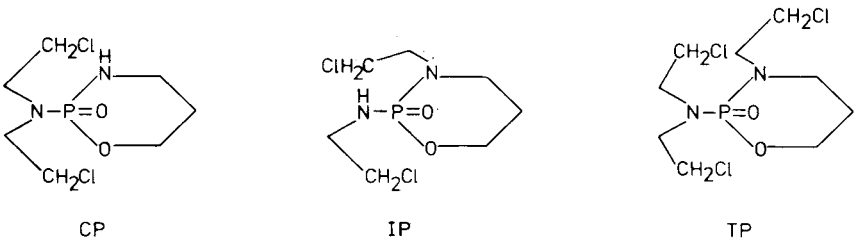

Fig. 1. Structural formulae of cyclophosphamide (CP), isophosphamide (IP) and trophosphamide (TP).

MATERIALS AND METHODS

Apparatus

A Packard-Becker Model 417 gas chromatograph with flame ionisation detector, and a Packard-Becker Model 420 gas chromatograph equipped with a Hewlett-Packard dual nitrogen—phosphorus flame ionisation detector Model 18789A were used. The radiochemical experiments were performed on a Packard Model 2450 Tri-Carb liquid scintillation spectrometer.

Solvent, standards and reagents

The solvents, analytical grade, obtained from Baker (Deventer, The Netherlands) and Merck (Darmstadt, G.F.R.) were distilled over a 50-cm Vigreux column before use. Versamid 900, SE-30, Carbowax 20M and Gas-Chrom Q were obtained from Chrompack (Middelburg, The Netherlands). Tullanox was from Cabot Corporation (Boston, MA, U.S.A.). Cyclophosphamide as the monohydrate, isophosphamide (IP) and trophosphamide (TP) were kindly supplied by Asta (Bielefeld, G.F.R.). Cyclophosphamide monohydrate (ring-4-^{14}C) (11.25 Ci/*M*) was purchased from New England Nuclear (Boston, MA, U.S.A.). Instafluor was from Packard (Downers Grove, IL, U.S.A.) and Triton X-100 from Koch-Light Labs. (Colnbrook, Great Britain). Trifluoroacetic anhydride was obtained from Fluka (Buchs, Switzerland) or Merck.

Extraction procedure

To 0.2 ml of plasma in a glass vial was added 0.1 ml of 0.6 *N* aqueous sodium hydroxide, the internal standard (200 ng of TP or 250 ng of IP) and 1 ml of ethyl acetate. After shaking on a Vortex and centrifuging for 5 min, the upper layer was transferred to a clean vial. The aqueous layer was extracted two more times with 1 ml of ethyl acetate.

The combined ethyl acetate extracts were evaporated under nitrogen and dissolved in 0.5 ml of methanol—water (90:10, v/v); then 1 ml of hexane was added. After shaking on a Vortex and centrifuging the hexane layer was discarded. The purification procedure with hexane was repeated twice, the methanol layer was evaporated under nitrogen and the residue was reconstituted in 100 µl of ethyl acetate for GLC assay.

Derivatization procedure

The residue from the extraction procedure was dissolved in 50 μl of trifluoroacetic anhydride in order to prepare the trifluoroacetyl derivative of CP. After standing for 0.5 h at room temperature the solution was evaporated in a stream of nitrogen. The residue was reconstituted in 100 μl of ethyl acetate for the GLC procedure.

GLC procedure

A 10-μl aliquot of the solution in 100 μl of ethyl acetate resulting from the extraction or derivatization procedure was applied to the upper part of a stainless-steel rod. After evaporation of the solvent the rod was inserted in a solid-state injector which had been assembled from a Hoke ball valve [14].

The 3% Versamid 900 and 3% SE-30 columns were packed on Gas-Chrom Q (140—160 and 200—220 μm). Tullanox was used as a support material for the capillary Carbowax 20M column. The experimental conditions are summarized in Table I. Further GLC details were as reported by Driessen et al. [14].

Radiochemical recovery experiments

A stock solution of [ring-4-^{14}C]cyclophosphamide monohydrate in ethyl acetate was prepared (500 pCi/μl). For the recovery experiments 40 μl of this solution, 1 μg of CP and 1 μg of IP were dissolved in 0.2 ml of human plasma. Subsequently the extraction procedure was performed using ethyl acetate or chloroform. However, the organic extracts were now evaporated in a counting vial and reconstituted in the scintillation liquid (Instafluor—Triton X-100, 2:1). The aqueous residues were directly dissolved in the scintillation liquid. The experiments were carried out in triplicate.

RESULTS AND DISCUSSION

The presence of an amide group in CP previously prevented a suitable GLC analysis of this compound in its underivatized form. The chromatograms ob-

TABLE I

EXPERIMENTAL GLC CONDITIONS AND DATA

	3% SE-30	3% Versamid 900	SCOT Carbowax 20M
Column length (cm)	75	130	2000
Column I.D. (mm)	1.2	0.8	0.45
Temperature (°C)			
column	195	215	220
injector	250	300	300
detector	300	300	300
Flow-rate (ml/min)			
carrier gas	6 (N_2)	10 (He)	12 (He)
auxiliary gas		20 (He)	18 (He)
hydrogen	30	3	3
air	300	100	100
Retention times (min)	trifluoroacetyl CP 1.6	CP 2.4	CP 2.3
			IP 3.2
	CP 2.5		
	TP 4.4		

tained invariably showed considerable peak broadening and decomposition of CP. Pantarotto et al. [10] have protected the amide function by derivatization with trifluoroacetic anhydride. In this way the trifluoroacetyl derivative of CP, extracted from plasma of mice, could be determined on a 3% SE-30 column using flame ionisation or electron-capture detection. Whiting et al. [12] were unsuccessful in reproducing this method with human plasma, probably because of the co-elution of breakdown products. In a careful reinvestigation of the method of Pantarotto et al. [10] we have established that it can be reproduced for human plasma using flame ionisation detection. TP was used as an internal standard. Representative chromatograms are given in Fig. 2. Calibration yielded a straight line in the range 10—200 ng of CP per injection (correlation coefficient 0.9990). Incidentally co-extracted impurities of unknown origin may interfere with the CP or TP peak.

Whiting et al. [12] and Juma et al. [13] have measured the trifluoroacetyl derivatives of CP and IP on a 10% Apiezon L column with electron-capture detection and on a 5% OV-17 column using N—P detection. The application of N—P detection results in considerably better sensitivity and selectivity. During our investigations of trifluoroacetyl CP on the 3% SE-30 column we found that under the same experimental conditions CP itself could be determined without derivatization; TP was again applied as an internal standard. Representative chromatograms are shown in Fig. 3.

In a recent report [11] an analysis of underivatized CP on a 3% OV-17 column using N—P detection and imipramine as an internal standard has been described. The application of N—P detection for the analysis of CP results here also in the elimination of a number of undesired peaks and a remarkable increase in sensitivity (see below).

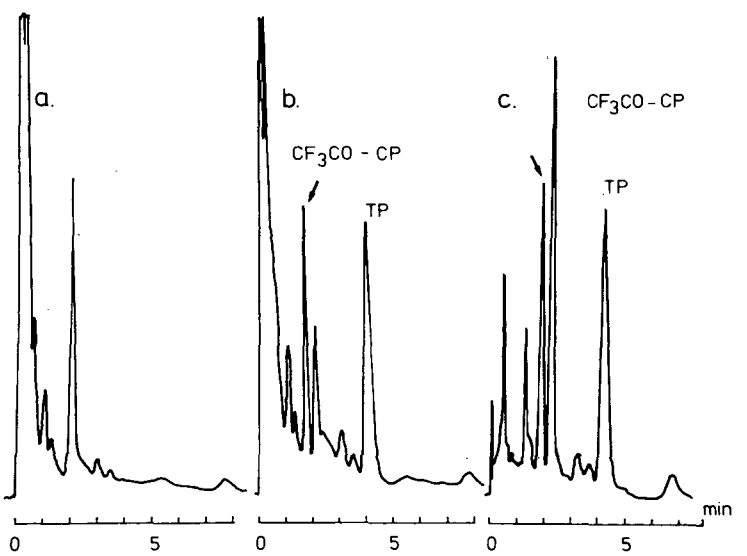

Fig. 2. Chromatograms of the assay of CP as trifluoroacetyl CP on the 3% SE-30 column. (a) Derivatized blank plasma; (b) 100 ng of CP converted into trifluoroacetyl CP, and 200 ng of TP from plasma; (c) reference sample of 100 ng of CP converted into trifluoroacetyl CP, and 200 ng of TP.

During the GLC analysis of underivatized CP the appearance of an additional peak in the chromatogram due to decomposition of CP is often observed. We studied the decomposition of CP on a 3% Versamid 900 column applying various experimental conditions of which the injection temperature and injected amount of CP were found to be very important. The decomposition reaction, represented in Fig. 4, is the intramolecular alkylation of the NH group by one of the chloroethyl substituents in the nor-nitrogen mustard part of the CP molecule. We confirmed this by mass spectrometry (see also ref. 6).

Rather than designing precautions against the intramolecular alkylation reaction we have searched for experimental conditions where the conversion of CP into the bicyclic compound intramolecular alkylated CP (CP*) was quantitative. The decomposition reaction of CP appeared to depend upon the injection and column temperatures and upon the amount of CP injected. At higher temperatures CP reacted faster under formation of CP*. In Fig. 5 the amounts of unchanged CP and CP* leaving the column are represented as a function of the amount of CP injected on the column.

If at the proper injection and column temperatures of 300°C and 215°C respectively, less than 200 ng of CP was injected, it was safely assumed that quantitative conversion into CP* occurred. In Fig. 6 three examples of chro-

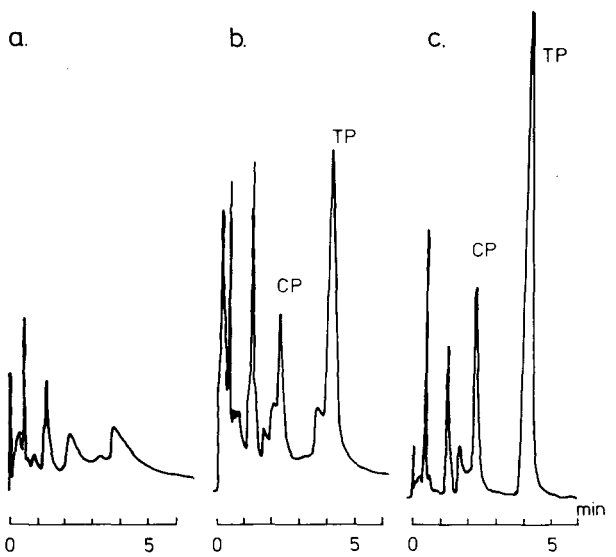

Fig. 3. Chromatograms of the assay of underivatized CP on a 3% SE-30 column. (a) Blank plasma; (b) 100 ng of CP and 200 ng of TP (internal standard) extracted from plasma; (c) reference sample of 100 ng of CP and 200 ng of TP.

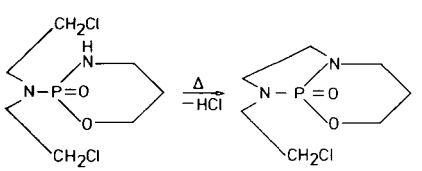

Fig. 4. The intramolecular alkylation of CP.

matograms are presented, which were recorded in the N—P detection mode. Since no acceptable internal standard was found, peak heights were used for the construction of a reference line; uracil may be applied as an external standard. Interference of IP which on decomposition also yields the same product CP*, does not occur because the conversion of IP into CP* proceeds very slowly under the experimental conditions used.

Finally we have initiated research on CP in the area of capillary GLC and we have developed another novel method to assay underivatized CP in human plasma. In the N—P detection mode CP was analyzed on a capillary Carbowax 20M SCOT column; IP was used as an internal standard. Examples of chromatograms are presented in Fig. 7.

For the extraction of CP from biological fluids ethyl acetate and chloroform were found to be very suitable. After an extraction with ethyl acetate the results showed a recovery of CP of 97% (n=3, S.D.=1%), while 1% (n=3, S.D.= 0.1%) was present in the aqueous residue. After extraction with chloroform the organic layer accounted for 97% (n=3, S.D.=0.5%) of CP, and the aqueous layer for 0.7% (n=3, S.D.=0.04%) of CP. After an extraction procedure using ethyl acetate followed by the purification method with hexane the recovery was 90% (n=3, S.D.=1%), the aqueous residue contained 1% (n=3, S.D.=0.2%) of CP and the combined hexane layers 3% (n=3, S.D.=0.3%) of CP.

The availability of various methods for the analysis of CP in human plasma offers the possibility of adequate assays to be carried out. Thus in the case of undesired compounds due to co-medication or caused by CP-derived com-

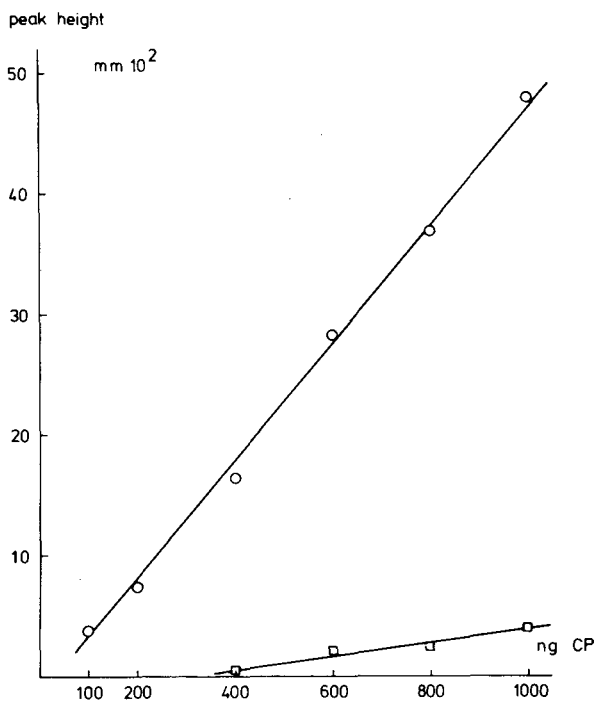

Fig. 5. The peak height of CP* formed from CP (○), and CP not converted into CP* (□) plotted versus the amount of CP injected. Results from three duplicate experiments.

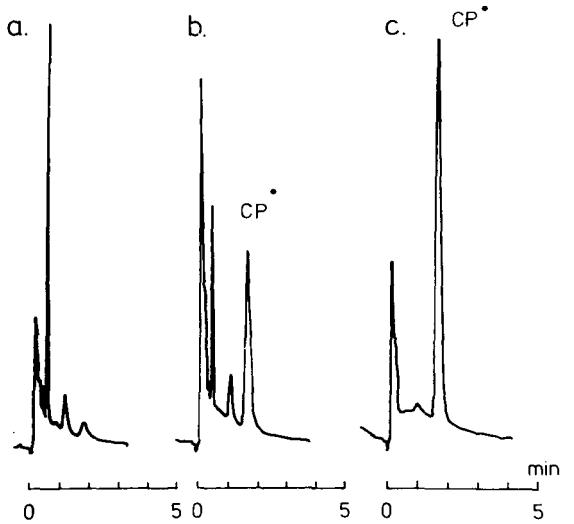

Fig. 6. Chromatograms of the assay of CP as intramolecular alkylated compound CP* on a 3% Versamid 900 column. (a) Blank plasma; (b) CP* from 100 ng of CP extracted from plasma; (c) CP* from 100 ng of CP of a reference solution.

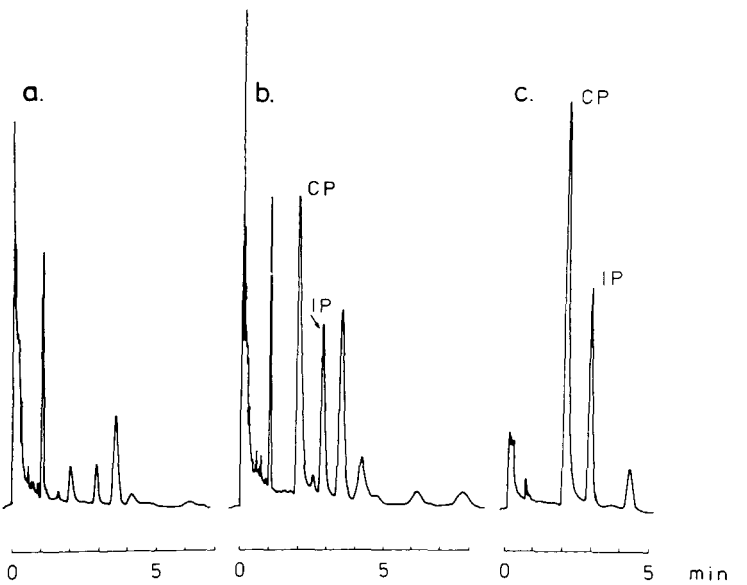

Fig. 7. Chromatograms of the assay of CP on the capillary Carbowax 20M SCOT column. (a) Blank plasma; (b) 30 ng of CP and 25 ng of IP (internal standard) extracted from plasma; (c) 30 ng of CP and 25 ng of IP from a reference solution.

pounds or CP-induced breakdown products, another GLC method may be applied. Because of their sensitivity and selectivity, methods with N—P detection are preferred to those with flame ionisation detection. For the latter methods the limit of sensitivity is 1 μg/ml, for those with N—P detection 10 ng/ml. The accuracy of the methods is usually approximately 7%.

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CHROMBIO. 576

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DIPYRIDAMOLE IN PLASMA AND WHOLE BLOOD

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic method is described for the quantitative analysis of dipyridamole in plasma and whole blood. The method involves a single extraction of an alkalinized sample with diethyl ether followed by evaporation of the organic solvent and ion-pair chromatography using fluorescence detection. The lower limit of sensitivity for dipyridamole is 1 ng/ml. Concentrations of dipyridamole between 1 and 500 ng per sample are measured with an average coefficient of variation of 4.5% in plasma and 7.4% in whole blood.

INTRODUCTION

Several methods have been reported for the analysis of the vasodilating and antiplatelet agent dipyridamole in biological samples (Table I). The spectrofluorometric methods involve solvent extraction of the biological sample with diethyl ether, followed by back-extraction into an aqueous phase, pH adjustment, and fluorescence determination [1, 2], or extraction of the biological sample with an acidic solution, followed by filtration, pH adjustment, and fluorescence determination [3]. In addition to being time-consuming, these methods suffer from the lack of specificity inherent in non-chromatographic analytical methods of drugs in biological samples.

Two high-performance liquid chromatographic (HPLC) methods have been described for the quantitative analysis of dipyridamole in serum or plasma, both of which are based on reversed-phase column chromatography. One HPLC method [4] uses two different procedures for sample preparation, i.e., ethanol protein precipitation for samples with expected concentrations higher than 0.1 μg/ml, and solvent extraction of a buffered sample with diethyl ether followed by partial evaporation and back-extraction into acid for samples with expected

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TABLE I

COMPARISON OF METHODS FOR ANALYSIS OF DIPYRIDAMOLE IN BIOLOGICAL SAMPLES

Reference	Analytical method	Sample	Sensitivity (ng/ml)	Reproducibility* (range)	Comments
1	Spectrofluorometry, spectrophotometry	Serum, urine, bile, feces	Not given	Not given	Lacks specificity
2	Spectrofluorometry	Plasma, urine, bile, tissues	5	Not given	Lacks specificity
3	Spectrofluorometry	Serum, urine, bile, tissues	1	Not given	Lacks specificity
4	HPLC—spectrophotometric detection	Serum	8	$r = 0.9999$ (calibration curves of 0.1—10 μg/ml)	Lacks sensitivity; uses external/internal standard; relatively time-consuming
5	HPLC—fluorescence detection	Plasma	5	1.8% (replicates of 4 different concentrations between 100 and 580 ng/ml)	Alkaline aqueous phase in solvent mixture may cause premature deterioration of column; uses internal standard.
This paper	HPLC—fluorescence detection	Plasma, whole blood	1	6.1% (calibration curves of 1—500 ng per sample of plasma or whole blood)	Ion-pair chromatography; uses internal standard.

*r = correlation coefficient; % = coefficient of variation.

low concentrations. These procedures use indomethacin as an internal or external standard, respectively, and the method uses spectrophotometric detection. This method has two disadvantages: (1) the use of two different procedures for sample preparation makes the method inconvenient and time-consuming, and (2) the method suffers from the relative lack of sensitivity due to its use of spectrophotometric detection. The other HPLC method [5] involves a single extraction of an alkalinized sample with dichloromethane, followed by filtration and evaporation of the organic phase and spectrofluorometric detection. This method uses an analogue of dipyridamole, compound RA-764-BS, as an internal standard. Although rapid and sensitive, this method has the disadvantage of using an alkaline aqueous phase (pH 8.6) in the solvent mixture which is likely to result in premature deterioration of the reversed-phase column.

This paper describes a rapid, sensitive, specific, and accurate HPLC method for the analysis of dipyridamole which is based on ion-pair chromatography and fluorescence detection and which is applicable to both plasma and whole blood.

EXPERIMENTAL

Reagents and materials

Dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-*d*)-pyrimidine] was supplied by Boehringer Ingelheim (Ridgefield, CT, U.S.A.), and the internal standard, RA 433 [2,4,6-trimorpholinopyrimido-(5,4-*d*)pyrimidine] was a gift from Dr. Karl Thomae GmbH (Biberach an der Riss, G.F.R.) (see Fig. 1). Stock solutions are prepared in methanol from which dilutions were made in deionized water to produce standard solutions. These solutions are stored at 4°C for approximately one month with no detectable decomposition. The methanol used in chromatography is of high purity and is purchased from MC/B-EM (LiChrosolv; Cincinnati, OH, U.S.A.). The sodium salt of 1-heptanesulfonic acid is obtained from Eastman Kodak (Rochester, NY, U.S.A.). All other solvents and reagents are of reagent grade.

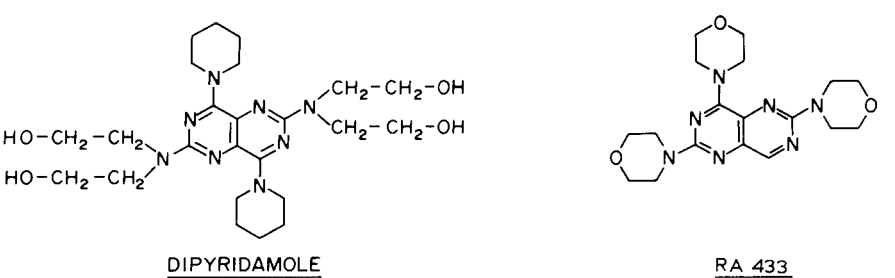

Fig. 1. Structural formulae of dipyridamole and the internal standard, RA 433.

Sample preparation

Whole blood or plasma (0.1—1.0 ml) is placed in a 15-ml capacity culture tube, fitted with a PTFE-lined screw cap, and the internal standard solution (25 μl containing 25 ng of RA 433), 1.0 ml of 1 *N* sodium hydroxide, and 5 ml of diethyl ether are added. The samples are immediately extracted using a

Vortex-Genie mixer at high speed for 30 sec, followed by centrifugation at 1000 *g* for 5 min to separate the aqueous and organic phases. The lower aqueous phase is frozen by immersing the tube in a dry ice—acetone bath, and the organic phase is decanted into another tube. The diethyl ether is evaporated under purified nitrogen at room temperature using an N-Evap (Organomation Assoc., Northborough, ME, U.S.A.). The residue is dissolved in 100 μl of the mobile phase, and all or part of this volume is injected into the chromatograph depending on the expected amount of dipyridamole present.

Chromatography

The high-performance liquid chromatograph consists of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A high-pressure solvent delivery system which is equipped with a Model U6K injector and fitted with a Waters Assoc. μBondapak C_{18} reversed-phase column (30 × 0.39 cm I.D.; particle size 10 μm). The mobile phase is a mixture of methanol—water (65:35), both containing 1-heptanesulfonic acid sodium salt (0.005 *M*) with 0.1% acetic acid. The flow-rate of the solvent mixture is 2 ml/min, with a column input pressure of 170 atm (2500 p.s.i.). The column is insulated with sponge rubber to minimize ambient temperature variations. Fluorescence is measured with a Schoeffel (Kratos, Westwood, NJ, U.S.A.) Model FS 970 fluorometer. An excitation wavelength of 285 nm was selected in conjunction with a 470-nm emission filter. Chromatograms are recorded on a Linear Instruments (Irvine, CA, U.S.A.) Model 585 dual-pen recorder.

Calibration and accuracy

Calibration curves were constructed for each series of unknown samples by adding standards of 1, 5, 10, 50, 100, 250, and 500 ng of dipyridamole (in 100 μl of deionized water) and the internal standard (25 ng of RA 433) to control samples, which were assayed concurrently with the unknowns. The peak height ratio (PHR) of dipyridamole to the internal standard for each standard sample was divided by the amount of dipyridamole added to yield the normalized PHR. The mean normalized PHR was then used to calculate the amount of dipyridamole in the unknown samples, and the standard deviation of the normalized PHR was used to determine the accuracy of the method over the range of dipyridamole standards used. The method was standardized for 0.1 and 1.0 ml of both whole blood and plasma.

The reproducibility of the method was examined by submitting five replicate samples of plasma and whole blood, 0.1 and 1.0 ml, containing 500 ng of dipyridamole per sample to the entire procedure. The total recoveries of dipyridamole and the internal standard were estimated by comparing the peak heights of analyzed samples containing known amounts to the respective peak heights obtained by injecting equal amounts directly into the chromatograph.

The effect of the size of sample volume on the analysis of dipyridamole in whole blood and plasma was examined by adding 50 ng of dipyridamole and 25 ng of the internal standard in duplicates to 0.1-, 0.5-, and 1.0-ml samples, and using the same volumes of base and organic solvent as previously described. The effect of adding water to the 0.1- and 0.5-ml samples of whole blood in order to produce equal volumes of aqueous phase for all samples (1.0 ml) prior to extraction was also examined.

Application of the method

A healthy female volunteer received a single oral dose of 50 mg of dipyridamole (two tablets of Persantine, 25 mg, Boehringer Ingelheim). Samples of venous blood were collected at varying intervals during the first 8 h after administration and also the next two mornings. The blood was collected in glass tubes and anticoagulated with heparin (10 units of sodium heparin per ml of whole blood). Aliquots (1 ml) of whole blood were removed from each sample and stored in the extraction tubes at −70°C until analyzed. The remaining blood was centrifuged and the plasma transferred to glass vials and stored at −70°C until analyzed.

RESULTS AND DISCUSSION

The present HPLC method for the quantitative analysis of dipyridamole in plasma and whole blood involves a simple extraction of an alkalinized sample with diethyl ether, followed by evaporation of the organic solvent and ion-pair chromatography using fluorescence detection. The use of diethyl ether as the extraction solvent offers several advantages, i.e., it has a lower specific gravity than water and has a low freezing point; thus it can be separated easily from the aqueous phase by freezing. Also, it is readily evaporated and, because of its lower polarity, it is less likely to extract interfering compounds than many chlorinated solvents. Ion-pair chromatography for the analysis of dipyridamole which is a base with pK_a of 6.4, avoids the use of a precise pH control [4] and the use of an alkaline aqueous phase in the solvent mixture [5] which may cause premature deterioration of the reversed-phase column. Optimal fluorescence response under the present chromatographic conditions was achieved by using an excitation wavelength of 285 nm and a 470-nm emission filter.

The total recovery of dipyridamole from plasma samples averaged 87.2% and was independent of the volumes of plasma used. The total recovery of dipyridamole from whole blood averaged 93.9% for 0.1-ml volumes and 76.4% for 1.0-ml volumes. The total recovery of the internal standard was essentially 100% for both plasma and whole blood and was independent of the volumes used.

Figs. 2 and 3 show chromatograms for blank plasma and plasma containing 10 ng/ml of dipyridamole, and blank whole blood and whole blood containing the same added amounts of dipyridamole, respectively. With the chromatographic conditions previously described, the retention times for the internal standard and dipyridamole are 3.5 and 5.1 min, respectively.

Estimates of accuracy for the method are shown in Table II. The average mean normalized PHR for dipyridamole to internal standard obtained from calibration curves from plasma and whole blood had a mean coefficient of variation of 6.1% for a total of 16 such calibration curves using two different volumes. This estimate of accuracy covers the entire range of the assay procedure, from 1 to 500 ng of dipyridamole per sample. Reproducibility studies on replicates containing 500 ng of dipyridamole per sample yielded an average coefficient of variation of 3.1% for plasma and 2.6% for whole blood. As it may be necessary to use variable volumes of plasma or whole blood for dipyridamole measurement, the effects of volume on the method (see Experimental)

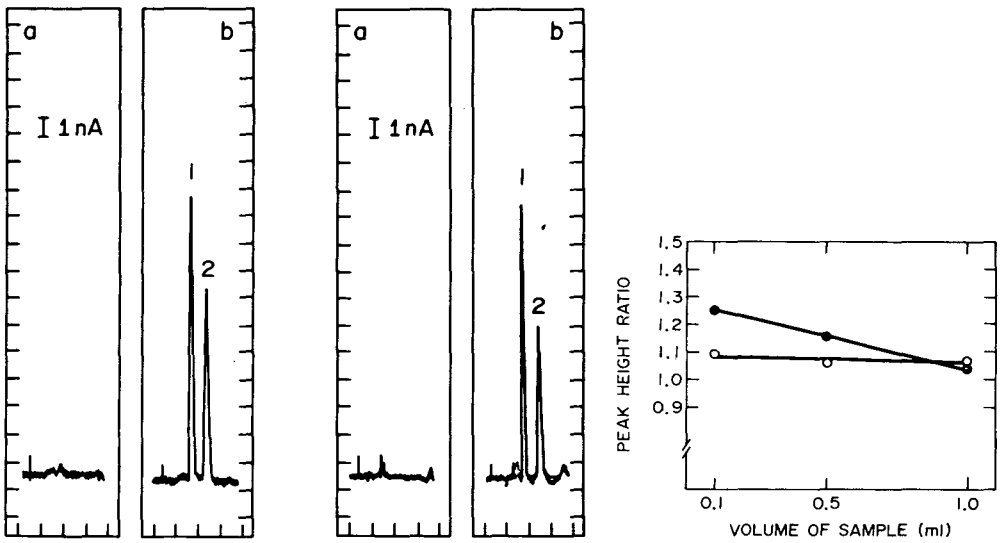

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing (1) internal standard and (2) 10 ng/ml of dipyridamole. For visual clarity, only one tracing of the dual pen recording is shown.

Fig. 3. Chromatograms of (a) control whole blood and (b) whole blood containing (1) internal standard and (2) 10 ng/ml of dipyridamole. For visual clarity, only one tracing of the dual pen recording is shown.

Fig. 4. Effect of sample volume on the peak height ratio of dipyridamole to internal standards in plasma (○) and whole blood (●).

TABLE II

ESTIMATES OF ACCURACY OF THE METHOD FROM CALIBRATION CURVE DATA

Concentrations 1—500 ng per sample.

Biological fluid (volume)	Average mean normalized peak height ratio	Number of studies	Coefficient of variation (%)	
			Average	Range
Plasma (1 ml)	6.56	6	4.5	3.1—6.1
Plasma (0.1 ml)	5.20	1	4.5	
Whole blood (1 ml)	4.98	7	7.2	4.1—11.1
Whole blood (0.1 ml)	5.35	2	7.8	7.6—8.1

were examined as judged by the PHR. The results of those studies are summarized in Fig. 4 which shows that the PHR for plasma is independent of the volume (between 0.1 and 1.0 ml) used, while the PHR for whole blood varies with the volume used. This indicates that variable volumes of plasma can be used with each calibration curve, whereas for whole blood the samples containing the unknown concentrations of dipyridamole have to be of the same volume as the samples used for the calibration curve. The reason for the declining PHR with increasing volume of whole blood used was found to be decreasing

recovery of dipyridamole (see above). Adding water to whole blood in order to produce equal volumes of aqueous phase for all whole blood samples prior to extraction did not eliminate this volume-dependent recovery of dipyridamole and resulted in erratic PHR.

Application of the method to the determination of dipyridamole in plasma and whole blood from a healthy female volunteer after oral administration of 50 mg is demonstrated in Fig. 5. It can be seen that the concentrations of dipyridamole in whole blood are lower than those in plasma over the first few hours following drug administration after which the concentrations in whole blood are higher than those in plasma, suggesting time-dependent blood/plasma concentration ratio. It should also be noted that detectable amounts of dipyridamole are still present in whole blood and plasma on the third day after drug administration, suggesting much longer terminal half-life of dipyridamole in man than the published range of about 0.5—2.5 h [1, 6]. These observations indicate that the pharmacokinetics of dipyridamole are still unsettled.

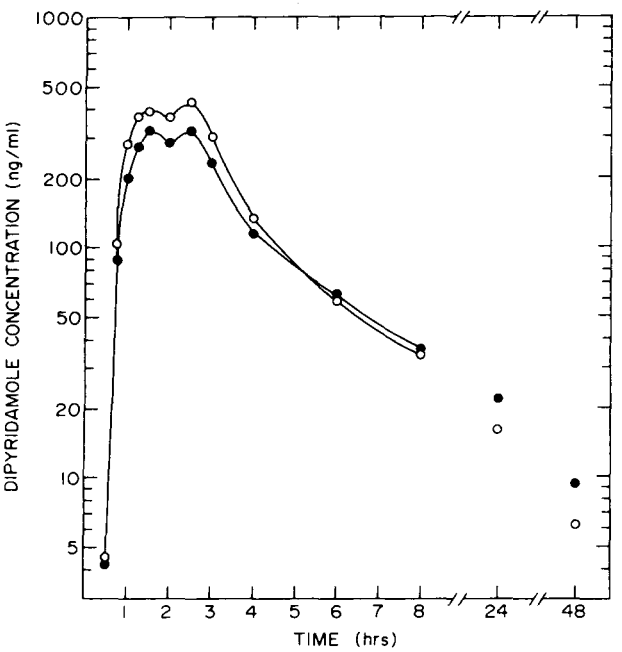

Fig. 5. Semi-logarithmic plot of dipyridamole concentrations in plasma (○) and whole blood (●) in a healthy female subject after a single oral dose of 50 mg of dipyridamole.

The HPLC method described here for the quantitative determination of dipyridamole in plasma and whole blood is simple and rapid. It only involves a single extraction step followed by evaporation and chromatography. By using the techniques described, 40—50 samples can easily be assayed in a day.

ACKNOWLEDGEMENTS

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CHROMBIO. 582

SIMULTANEOUS ANALYSIS OF DISOPYRAMIDE AND QUINIDINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new reversed-phase high-performance liquid chromatographic method allowing simultaneous measurement of plasma concentrations of disopyramide and quinidine is described. Disopyramide and quinidine were separated on a reversed-phase column using 0.05 *M* phosphate buffer (pH 3.0)—acetonitrile (73:27, v/v), as mobile phase and the peaks were monitored by UV absorbance at the wavelengths of 254 and 325 nm. The drugs were extracted from alkaline plasma with chloroform containing the internal standard. The organic phase was evaporated to dryness and the residue was redissolved in a small volume of the mobile phase before analysis by high-performance liquid chromatography. The method is convenient and reliable in routine monitoring of both drugs.

INTRODUCTION

Both quinidine and disopyramide are used in the treatment and prophylaxis of cardiac arrhythmias. Because of a relatively narrow therapeutic index and intersubject variability in the pharmacokinetics of these drugs, monitoring the serum concentration of these drugs may assist in individualizing the dosage requirements and in assessing patient compliance with the dosage regimen. Methods used previously for measuring disopyramide and quinidine have included fluorometric [1, 2], gas—liquid chromatographic (GLC) [3, 4] and high-performance liquid chromatographic (HPLC) [5—8] assays. The fluorometric assays have proven non-specific and subject to interference by background fluorescence [3]. GLC methods for measuring disopyramide have suffered from frequent lack of linearity at lower concentrations [5]. The most

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promising technique for reliably measuring these drugs in biological fluids appears to be HPLC. Methods capable of measuring groups of drugs simultaneously, e.g. the simultaneous monitoring of drugs such as the anticonvulsant drugs, have been shown to be efficient and desirable, because they provide information on the plasma levels of a number of drugs at the same time with a minimum delay [9]. None of the previously described methods for the measurement of anti-arrhythmic drugs allows the simultaneous measurement of disopyramide and quinidine, both of which may be encountered in the plasma of the same patient. This paper describes an HPLC method for simultaneously measuring plasma concentrations of disopyramide and quinidine.

MATERIALS

Disopyramide [4-diisopropylamino-2-phenyl-2-(2-pyridyl)-butyramide], its mono-N-dealkylated metabolite [4-isopropylamino-2-phenyl-2-(2-pyridyl)-butyramide] and the internal standard [4-diisopropyl-amino-2-*p*-chlorophenyl-2-(2-pyridyl)-butyramide] were supplied by Roussel (Castle Hill, Australia). Quinidine was obtained from ICN K & K Labs. (Cleveland, OH, U.S.A.). Lignocaine and tocainide were from Astra Chemicals (North Ryde, Australia) and procainamide was from E.R. Squibb & Son (Melbourne, Australia). The chloroform used for extraction was analytical grade obtained from E. Merck (Darmstadt, G.F.R.) and the acetonitrile used for the chromatography was HPLC grade obtained from Waters Assoc. (Eagle Farm, Australia). All other chemicals were analytical grade products available commercially.

METHODS

Sample preparation

One ml of plasma was made alkaline by adding 100 μl of 5 *M* sodium hydroxide. The extraction was performed with 5 ml of chloroform containing the internal standard at the concentration of 2 mg/l. The extraction was carried out in tubes fitted with PTFE-lined screw caps using a rotary mixer at 32 rpm for 20 min. After centrifugation at 600 *g* for 10 min, the upper aqueous layer was aspirated and 3 ml of the remaining chloroform layer were transferred into tapered centrifuge tubes. The chloroform was evaporated under a stream of air. The residue was redissolved in 250 μl of the mobile phase used for the chromatography and 20 μl of this were injected into the high-performance liquid chromatograph.

Absorbance spectra

To determine suitable detection wavelengths the absorbance spectra of disopyramide, its mono-N-dealkylated metabolite and quinidine were recorded using Cary 118-C (Varian Instrument Division, Palo Alto, CA, U.S.A.). The compounds were dissolved in the mobile phase at concentrations of 10 mg/l. The solvent alone was placed in the reference light path.

High-performance liquid chromatography

The HPLC system used consists of a solvent delivery system (Model

M-6000A pump, Waters Assoc., Milford, MA, U.S.A.) and a fixed-volume loop injector, Rheodyne 7120 (Rheodyne, Berkeley, CA, U.S.A.). The eluent was monitored continuously for absorbance changes at 254 nm using a Model 440 detector (Waters Assoc.) and at 325 nm using a Schoeffel SF 770 Spectroflow detector (Schoeffel, Westwood, NJ, U.S.A.). These detectors were connected in series. The output of the detectors was recorded by a dual-pen recorder. LiChrosorb RP-8 column, 10-μm particle size (stainless steel, 250 mm × 4.6 mm I.D.) were made by Brownlee Labs. (Activon Scientific Products, Granville, Australia).

Samples were eluted isocratically with 0.05 *M* sodium phosphate buffer (pH 3.0)—acetonitrile (73:27, v/v) at a constant flow-rate of 1.8 ml/min. The column temperature was maintained at 40°C. The method was calibrated by adding known amounts of disopyramide, 1.0—8.0 mg/l, and quinidine, 1.25—10.0 mg/l into drug-free plasma; these concentrations cover the therapeutic ranges of the drugs. To construct the standard curves the peak height ratios of disopyramide and quinidine to the internal standard were plotted against the amounts of drugs added.

Possible interference by drugs administered to patients having anti-arrhythmic treatment was checked. The following compounds were chromatographed using the same method: lignocaine, procainamide, tocainide and the N-dealkylated metabolite of disopyramide.

RESULTS

Under our chromatographic conditions good separation of disopyramide and quinidine was achieved (Fig. 1). The retention times of quinidine, disopyramide

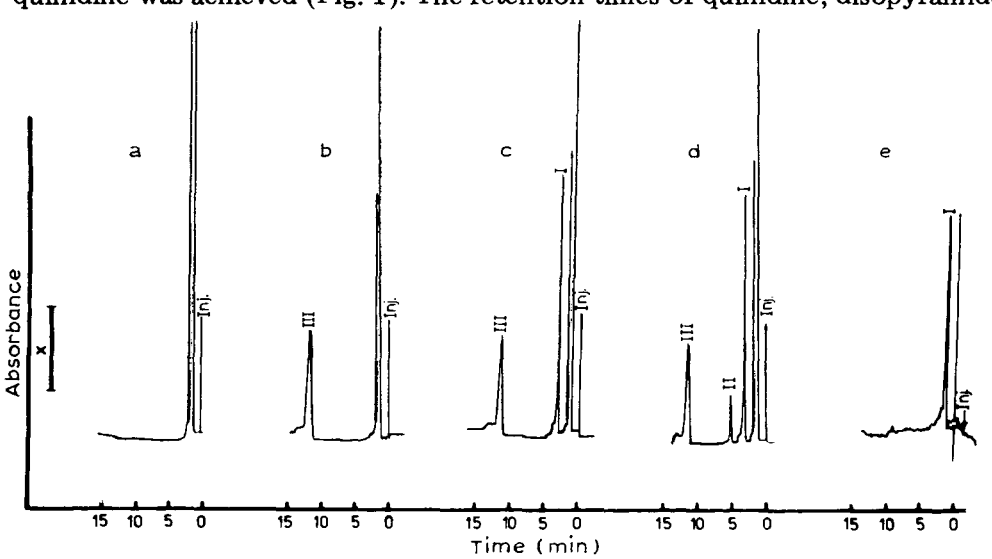

Fig. 1. HPLC tracings obtained for drug-free plasma used for all standards (a); patient plasma prior to quinidine administration (b); plasma from the same patient 2 h after quinidine administration (c); the same plasma after spiking it with disopyramide (d). Peaks: I, quinidine; II, disopyramide; III, internal standard. The tracings a—d were obtained by monitoring the eluent at 254 nm and × corresponds to 0.002 absorbance units. (e) Simultaneous tracing of Fig. 1d monitored at 325 nm and in this case × corresponds to 0.001 absorbance units.

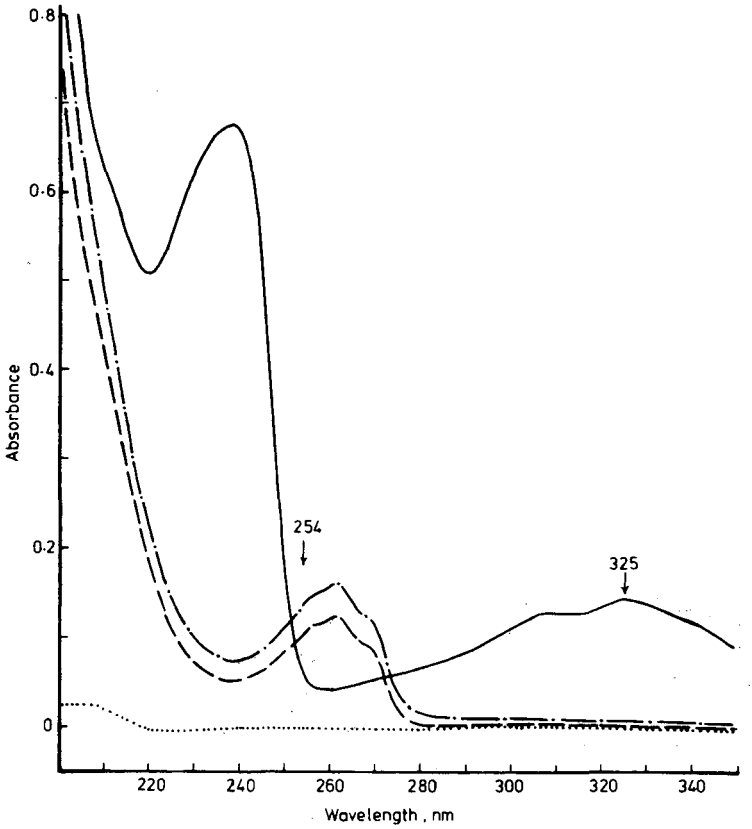

Fig. 2. Absorbance spectra of quinidine (——), disopyramide (— — —) and the mono-N-dealkylated metabolite of disopyramide (—·—·—). In each case the sample cuvette contained the compound in question at a concentration of 10 mg/l dissolved in the mobile phase used for the HPLC analysis. The reference cuvette was filled with the mobile phase. (. . . . . .) denotes the absorbance spectrum when both the sample and the reference cuvettes contained the mobile phase.

TABLE I

RETENTION TIMES OF VARIOUS COMPOUNDS AND THEIR ABSORBANCE RATIOS

	Retention time (min)	Absorbance ratio 325 nm/254 nm
Procainamide	1.9	N.D.*
Tocainide	2.6	N.D.
Mono-N-dealkylated metabolite of disopyramide	3.5	N.D.
Quinidine	3.6	0.248 ± 0.005 (mean ± S.D., n = 29)
Lignocaine	3.9	N.D.
Disopyramide	6.0	N.D.
p-Chloro-disopyramide	12.8	N.D.

*N.D.: none of the test compounds was detectable at 325 nm.

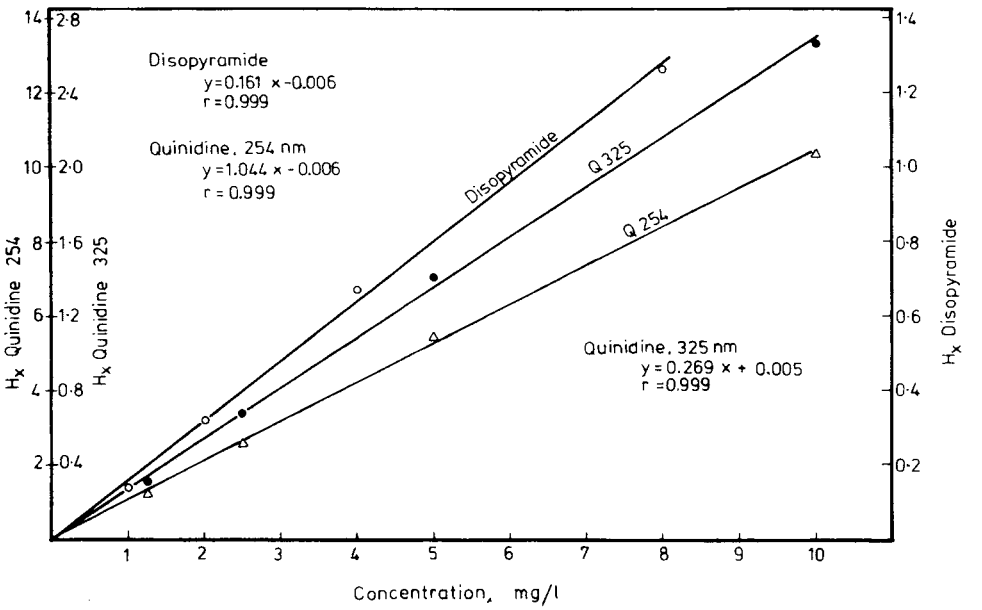

Fig. 3. Standard curves obtained for disopyramide monitored at 254 nm and quinidine monitored at both 254 (Q254) and 325 nm (Q325). H_x is the peak height ratio of the drug in question obtained by dividing the peak height of the drug in question by the peak height of the internal standard. The equations shown were obtained for each set of data by regression analysis.

TABLE II

EXTRACTION EFFICIENCY EXPRESSED AS PER CENT OF PEAK HEIGHT OF AQUEOUS STANDARDS

	n	Mean ± S.D.
Disopyramide	4	76.2 ± 3.4
Quinidine	4	93.0 ± 5.9

TABLE III

ANALYTICAL PRECISION OF THE EXTRACTION AND HPLC ANALYSIS

	X ± S.D. (mg/l)	C.V. (%)	*n*
Disopyramide	1.1 ± 0.1	4.9	10
	2.6 ± 0.2	4.6	9
	4.2 ± 0.2	5.7	10
	8.7 ± 0.4	3.6	10
Quinidine, 254 nm*	1.4 ± 0.1	3.7	10
	3.6 ± 0.1	2.6	9
	5.6 ± 0.2	2.5	10
	11.3 ± 0.6	5.3	10
Quinidine, 325 nm**	1.3 ± 0.04	4.5	10
	3.5 ± 0.1	3.2	9
	5.3 ± 0.1	2.3	10
	10.8 ± 0.5	5.0	10

*Results obtained by monitoring quinidine at 254 nm.
**Results obtained by monitoring quinidine at 325 nm.

and the internal standard were 3.6, 6.0 and 12.8 min respectively. Other plasma constituents did not interfere with the peaks of interest. A compound potentially interfering with quinidine determinations is the mono-N-dealkylated metabolite of disopyramide. This compound eluted immediately before quinidine with a retention time of 3.5 min, however it has essentially no absorbance at 325 nm (Fig. 2) and is not detectable at this wavelength. Dual wavelength monitoring was used routinely and wavelength ratioing results are shown in Table I. The retention times of disopyramide, quinidine, *p*-chloro-disopyramide and some potentially interfering compounds that could be encountered in patient samples were determined (Table I). The standard curves for disopyramide at 254 nm and for quinidine at both 254 and 325 nm were linear as shown in Fig. 3.

The efficiency and the reproducibility of the extraction was acceptable (Table II). The precision of the method was determined at four concentrations of disopyramide and quinidine. In all cases the coefficient of variation is less than 6% (Table III). The total assay time for a single sample is approximately 60 min.

DISCUSSION

Using the HPLC method described in this paper we have achieved good separation of disopyramide and quinidine. The method has allowed simultaneous measurement of these drugs with good reproducibility.

It must be noted that the mono-N-dealkylated metabolite of disopyramide runs very close to quinidine on this HPLC system. With column deterioration it would be possible to have the metabolite underlying the quinidine peak. This may not be a significant limitation, as the metabolite has been detected in only 15% of patients given single therapeutic doses of disopyramide and even then the plasma concentrations were less than 0.4 mg/l [3]. However, to completely eliminate this potential interference and to avoid similar interference going unnoticed, absorbance monitoring at two wavelengths simultaneously was carried out. The mono-N-dealkylated metabolite of disopyramide has no absorbance at 325 nm (Fig. 2) and is not detectable at this wavelength, therefore not interfering with quinidine determinations at 325 nm. It is highly recommended that dual wavelength monitoring and wavelength ratioing is utilized in clinical drug assays [10]. This will greatly reduce the possibility of erroneously high results being released if an unknown peak interferes with the drug being measured. More seriously, an erroneously low value for plasma level of a drug could be obtained if an unknown peak was underlying the internal standard peak. Simultaneous dual wavelength monitoring can be achieved by having two detectors connected in series or most conveniently and inexpensively this can be done using a dual-channel detector.

This assay method provides reliable means of measuring two important antiarrhythmic drugs, both of which could, under some therapeutic change-over situations be found in patients' plasma at combined levels that may represent a threat to the well-being of already dangerously ill patients. Therefore, it is very important to obtain plasma concentrations of these drugs as efficiently as possible. The method described here has the advantage over previous assay

methods for disopyramide and quinidine in that no more effort is required to assay these drugs simultaneously than to assay either drug separately.

ACKNOWLEDGEMENTS

We would like to thank Dr. J. Cameron of Roussel (Castle Hill, Australia) for providing us with samples of disopyramide, its mono-N-dealkylated metabolite and *p*-chloro-disopyramide. We would also like to thank Dr. Tatiana Assaykeen of Astra Chemicals (North Ryde, Australia) for providing us with a sample of tocainide.

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CHROMBIO. 567

Note

Simple method for gas—liquid chromatographic analysis of polyethylene glycol 400 in biological fluids

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Polymers of ethylene glycol (PEGs) are used extensively in the study of intestinal physiology, since their high solubility in water and low toxicity [1—6] make them useful as non-radioactive tracers. A wide range of molecular weights, 200—20,000, is available; the polymers used commonly include PEG 400, 600, 1500, 4000 and 6000 (numbers refer to the average molecular weight of commercial mixtures, which usually consist of 9—15 polymers). The PEG 4000 polymer has been found to be essentially inert in and not absorbed by the intestine [5, 6]; thus, it has been the standard aqueous marker for studies of intestinal absorption and transit [7—9]. Polymers of lower molecular weight are also useful: PEG 400, which is a common food additive, has been used in physiological studies as a marker of intestinal permeability [10, 11].

We have previously described [10] a method for the analysis of PEG 400 by gas—liquid chromatography (GLC) in biological fluids, and have applied the technique to the study of human physiology and disease [11]. However, wide application of these approaches is restricted potentially by methods available for the analysis of PEG 400 in biological fluids, particularly urine. Our previous method [10] involved acetylation and extraction of the samples, a rather tedious and lengthy process. Also, some samples produced by this method appear to be stable for only a few hours.

Here we report a simpler method for the analysis of PEG 400 by GLC. The method does not require extensive sample preparation and, consequently, a slow and somewhat difficult assay is rendered faster and easier.

STANDARDS AND REAGENTS

Polyethylene glycol 400 (stated by the manufacturer to be of "average molecular weight 380—420") was purchased from Matheson, Coleman and Bell (Norwood, OH, U.S.A.). The internal standard used was tetraethylene glycol, molecular weight 194.23, (No. A8A) from Eastman Kodak Company (Rochester, NY, U.S.A.); it was assessed for purity by repeated GLC analysis, which always yielded a single peak. The ion-exchange resin, used for urine samples, was Amberlite MB-3 (H^+ OH^-) from Mallinckrodt (St. Louis, MO, U.S.A.).

Urine standards were used when analyzing urine samples and deionized water or saline (8.5 g of NaCl per liter) standards were used when analyzing water or saline solutions. The standards were prepared to yield concentrations of PEG 400 ranging from 2 to 16 mg/ml. All solutions were frozen until needed.

PROCEDURE

Standards and samples

After thawing, 10-ml aliquots of the standards and samples were pipetted into 50-ml Pyrex tubes containing 10 ml of MB-3 resin. Tubes were covered with Parafilm®, mixed well, allowed to stand for 5 min and mixed again. After settling, 5 ml of the deionized sample were pipetted into 20-ml scintillation vials. One milliliter of the internal standard solution (25 mg of tetraethylene glycol per ml of water) was added to both the standard and sample. The vials were capped, mixed well, frozen and then lyophilized. When dry, the samples were reconstituted in acetone and transferred to injection vials.

Gas—liquid chromatography

Two glass columns (50 cm × 2 mm) were silanized, then packed with 1% PolyS-179 on Gas-Chrom Q 100—120 mesh (Applied Science Labs., State College, PA, U.S.A.). They were conditioned overnight at 310°C with a helium flow-rate of 20 ml/min.

Analyses were performed on a Hewlett-Packard 5840A gas chromatograph in the dual column (bleed-correction) mode. The injector temperatures were 275°C and detector temperatures were 350°C. The temperature program started at 125°C and was isothermal for the first 4 min. Temperature was increased at 5°C/min until a final temperature of 310°C was reached. This higher temperature was maintained for 6 min, whereupon the analysis was considered complete and the oven cooled for the next analysis.

The data system was calibrated with duplicate standards to yield a report based on the internal standard. The absolute amount of PEG 400 in the sample was obtained from the 5840A report, and the percentage composition of the PEG 400 was calculated from the peak areas.

RESULTS

Fifteen urine standards of varying concentrations (prepared from the same lot number of PEG 400) were analyzed on different days using different columns. An average coefficient of variation of less than 10% was obtained from

these data. This compared to an average coefficient of variation of 8% for data that were generated from multiple samples analyzed on the same column on the same day. Multiple injections of the same sample onto multiple columns yielded data with a coefficient of variation of less than 5%. The graph of the percentage composition of PEG 400 is shown in Fig. 1. There is a Gaussian distribution of the components according to their molecular weights.

Table I gives the least-squares fit of the standard curves for all components relative to tetraethylene glycol internal standard. The relationships for all the PEG 400 components are linear in the range of concentrations used.

Fig. 2 shows a chromatogram of a deionized urine sample, showing the Gaussian distribution of the PEG 400 components. Also, the lack of any added peaks illustrates the specificity of the method for polyethylene glycol.

PEG 400 was administered in doses of 5 g, dissolved in 20 ml water. Urine was collected in 6-h periods following administration. An average of 23% of the PEG 400 dose was recovered in the first 6 h of urine collection, confirming earlier quantitative recoveries [10].

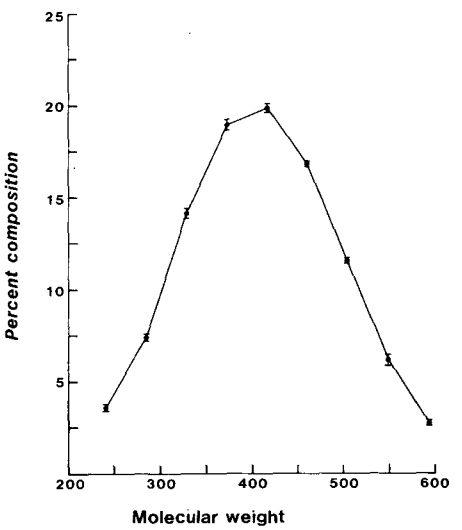

Fig. 1. The percentage composition of PEG 400, measured by GLC. Mean (± S.E.M.) for 15 estimations of samples from the manufacturer's same lot are shown, illustrating the Gaussian distribution of molecular weights.

TABLE I

LEAST-SQUARES ANALYSIS OF STANDARD CURVES FOR INDIVIDUAL PEGs

Ethylene oxide units	Correlation coefficient	Slope	Intercept
5	0.999	0.848	4.85
6	1.000	1.80	− 0.56
7	1.000	3.53	− 8.13
8	1.000	4.70	−12.3
9	0.999	4.89	−16.2
10	0.998	4.05	−21.9
11	0.994	2.63	−24.2

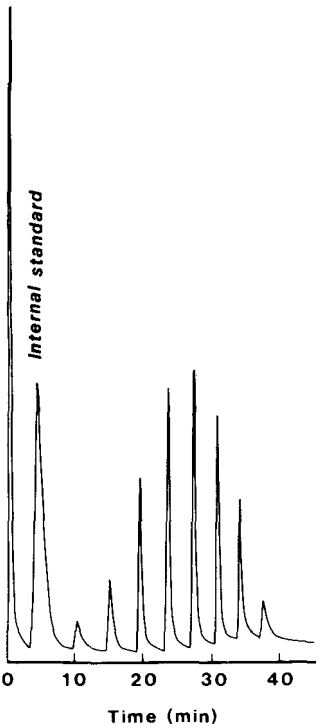

Fig. 2. Chromatogram of PEG 400 in urine using a 50 cm × 2 mm I.D. glass column packed with 1% PolyS-179. Injector temperature was 275°C, detector temperature 350°C. The initial temperature of 125°C was increased after 4 min at a rate of 5°C/min to 310°C. The internal standard was tetraethylene glycol.

DISCUSSION

PEG 400 is a clear viscous liquid that dissolves readily in water to form a colorless solution. It is tasteless in solutions of low concentration and is nontoxic [3, 4]. Moreover, PEG 400 is excreted rapidly and quantitatively in urine [10, 11], and thus recovery from urine can be used to quantify absorption after oral administration. These factors and the availability of an easy, accurate method for simultaneously quantifying several components of the mixture make PEG 400 useful as a differential permeability marker in the study of intestinal absorption.

The number and amount of each component polymer of PEG 400 varies with the batch (for example, different lot numbers). PEGs are prepared by hydroxide-initiated polymerization of ethylene oxide; therefore, there is a batch-to-batch variation in the number of PEG homomers, depending on the exact conditions under which the reaction was performed. The manufacturer labels the bottle of PEG 400 as containing "average molecular weight 380—420" because of these lot-to-lot variations. The data presented in this paper were obtained from standards and samples all containing the same lot number. However, in all experiments, it is essential to characterize the particular lot being used.

The differences in the coefficients of variation for groups of data collected from multiple samples analyzed on different columns, multiple samples analyzed on the same column, and a single sample analyzed on multiple columns, indicate that the differences in columns do not appear to affect the reproducibility of this method as much as does the sample handling. It is likely that the major variant is the degree of redissolution achieved for each sample. Since high volatility of the solvent and free solubility of PEG 400 are desired, acetone was selected to fulfill these qualifications among the solvents commonly available.

The standard curve showed linearity for each PEG 400 species in the concentration range 2—16 mg/ml based on tetraethylene glycol, the internal standard. However, the y-intercept values listed in Table I show that the standard curves of constituent molecules do not intercept the y-axis at zero; intercepts begin at 4.85 and decrease to negative values with increasing molecular weight. The possible causes for this phenomenon include decreasing detector response with increasing molecular weight, an increased boiling point with increasing molecular weight, or optimal efficiency of the column for a particular polymer (hexaethylene glycol had a y-intercept closest to zero, −0.56). Since the biological application is usually a ratiometric one, the non-zero intercept is of no practical concern. Our method is capable of detecting accurately amounts of PEG 400 as low as 1 mg/ml of urine. Samples below this limit could be handled by lyophilizing more than 5 ml of urine; we have used amounts of up to 10 ml.

The other constituents of urine do not appear to affect the GLC analysis of PEG 400. Treatment with ion-exchange resin removes most of the components that would cause unwanted peaks. The first few centimeters of column also trap other nonvolatile components and will darken upon injection of urine samples. Column life can be extended by replacing the darkened region with fresh packing when needed. As many as 75 samples have been analyzed before the column needed complete repacking.

Since we believe interest in the use of PEG 400 as an intestinal permeability marker will be stimulated by the work of Chadwick et al. [10, 11], a more convenient method for the analysis of PEG 400 in urine should have general use. The present approach appears to be much faster and more convenient, has an acceptable sensitivity and gives reproducible results.

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CHROMBIO. 566

Note

Simple method for isolating free urinary catecholamines by boric acid gel chromatography

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There has been considerable interest in methods for the quantitative analysis of catecholamines in biological fluids, in view of the pharmacological and diagnostic importance of these compounds. Spectrophotometric [1] and fluorimetric [2, 3] techniques have been applied, while isotope dilution methods [4] have been developed recently. Amperometric detection of catecholamines, separated by high-performance liquid chromatography, has also been reported [5]. Mainly due to the rather high polarity and chemical instability of the catecholamines, their isolation from biological samples has always been a problem. Adsorption onto aluminium oxide [2, 6] has been used, as well as cation-exchange chromatography [7, 8]; Higa et al. [9] have investigated the use of immobilized *m*-aminophenylboronic acid for the isolation of catechol compounds. Difficulties encountered with the cation-exchange method presently used and previous experience [10] with the boric acid gel material in our laboratories have led to further investigation of the latter method; the possibility of analysis of the urinary extract by thin-layer chromatography was also examined.

EXPERIMENTAL

Chemicals and reagents

Noradrenaline, adrenaline and dopamine were obtained from Sigma (St. Louis, MO, U.S.A.). [2-^{14}C]Dopamine (59 mCi/mmol), [7-^{14}C]adrenaline (59.4

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mCi/mmol) and [^{14}C]noradrenaline (uniformly labeled at the phenyl carbon atoms, 55 mCi/mmol) were purchased as tartrates in sterile aqueous solution from IRE, Fleurus, Belgium; stock solutions were prepared in 0.1 *N* HCl. Boric acid gel (particle size 0.1—0.4 mm) came from Aldrich Europe (Beerse, Belgium). Ethylene diamine (97%, water-free) was obtained from Fluka, Buchs, Switzerland, and was stored in the dark. All other chemicals were of analytical reagent grade and were purchased from E. Merck (Darmstadt, G.F.R.).

Phosphate buffer, pH 8.0, 0.67 *M*, contained 60 g of disodium EDTA per liter.

Spray reagent was freshly prepared before use by mixing 80 ml of methanol with 20 ml of ethylene diamine and then adding 20 ml of an aqueous 0.5% (w/v) potassium ferricyanide solution [11].

Extraction columns (6 cm × 6 mm I.D.) were prepared with boric acid gel, which was first allowed to swell in phosphate buffer (pH 8.0, 0.67 *M*) for 1 h. Glass wool plugged glass columns, with a Teflon metering valve and a 15-ml reservoir on top, were used.

Urine analysis

Urine samples (24-h volume) were collected with the addition of 200 ml of 1 *M* H_2SO_4 and kept in a refrigerator until analyzed.

Mix 10.0 ml of urine with 2.0 ml of phosphate buffer (pH 8.0, 0.67 *M*) and adjust the pH to 8.0 by addition of 1 *N* NaOH; centrifuge if necessary. Transfer the diluted sample quantitatively to an extraction column and allow the solution to pass through the resin bed. Wash the column with 20 ml of distilled water and elute the catecholamines with 10 ml of 0.05 *N* HCl. The collected eluate is used for further determinations.

Acetylation

The boric acid gel column eluate (10 ml) of 0.05 *N* HCl) is mixed with 2.5 g of $NaHCO_3$ and 1.0 ml of acetic anhydride; the mixture is then heated in a water-bath (50°C) until all the $NaHCO_3$ is dissolved and no more carbon dioxide is produced. The reaction mixture is extracted with two 10-ml portions of dichloromethane. The combined organic extracts are washed twice with 10 ml of distilled water, dried with anhydrous Na_2SO_4 and evaporated to dryness in vacuo at 45°C.

Thin-layer chromatography

The residue of the acetylated column eluate is dissolved in 50 μl of dichloromethane; a 5-μl aliquot is spotted onto a 10 cm × 10 cm Kieselgel 60 high-performance thin-layer chromatographic (HPTLC) plate (without fluorescence indicator, Cat. No. 5631, E. Merck). The plates are developed in acetone—dichloromethane—acetic acid—methanol (20:80:4:1, v/v), air-dried and sprayed with the spray reagent described. The plates were then incubated for 30 min at 65°C; the resulting fluorescence was measured with a Zeiss TLC scanner (Carl Zeiss, Oberkochen, G.F.R.), coupled to a Model A-25 10-mV range recorder (Varian Assoc., Walnut Creek, CA, U.S.A.). The excitation and emission wavelengths were 405 and 495 nm, respectively.

The HPTLC plates were pre-developed with the described solvent mixture and air-dried before use.

Recovery experiments

Urine samples, collected without preservatives over a 24-h period from a healthy volunteer, were supplemented with catecholamines to obtain the following concentrations: adrenaline: 12.0, 24.0, 36.0, 48.0 and 60.0 μg/l (as [7-^{14}C]adrenaline); noradrenaline: 36.0, 72.0, 109, 145 and 182 μg/l (5% as [^{14}C]noradrenaline); dopamine: 201, 402, 603, 804 and 1005 μg/l (1% as [2-^{14}C]dopamine). Aliquots (10.0 ml) were then taken through the extraction procedure as described. The column eluate was collected and 1.0 ml was mixed with 15 ml of liquid scintillation fluid (Instagel, Packard Instrument Co., Downers Grove, IL, U.S.A.). The radioactivity was measured in a Packard Tri-Carb 3390 liquid scintillation counter. As references, 0.5-ml aliquots of the original urine samples were mixed with 15 ml of Instagel and counted; dpm values were obtained using the internal standard method for quench correction.

RESULTS AND DISCUSSION

Due to their *cis*-diol conformation, the catecholamines adrenaline, noradrenaline and dopamine can form stable complexes with boric acid and consequently undergo adsorption onto immobilized *m*-aminophenylboronic acid [12] under alkaline conditions. This complex can be dissociated at lower pH and elution of the compounds is possible with dilute acid. The optimum pH value for adsorption of adrenaline on columns of boric acid gel was found to be 8.0, which is in agreement with the results of Higa et al. [9]. The two other catecholamines show an analogous behaviour. The recovery values, obtained with urine samples spiked with ^{14}C-labeled adrenaline, noradrenaline and dopamine in a concentration range including physiological values, are given in Table I. Furthermore, linear relationships were observed when plotting the radioactivity recovered (dpm) against the urinary concentration (μg/l). The proposed isolation method, using small boric acid gel columns, is practical and allows simultaneous treatment of several samples. The acidic eluates obtained are clean enough to permit the estimation of urinary catecholamine levels by differential fluorimetry using the trihydroxyindole technique [2, 8], since no salts or urinary pigments are retained on the column. Column eluate blanks were lower and less interference was encountered than when using the cation-exchange method [8].

TABLE I

RECOVERY OF ADRENALINE, NORADRENALINE AND DOPAMINE FROM HUMAN URINE SAMPLES AS DETERMINED BY LIQUID SCINTILLATION COUNTING

Adrenaline		Noradrenaline		Dopamine	
μg/l	Recovery (%)	μg/l	Recovery (%)	μg/l	Recovery (%)
12.0	80.6	36.0	81.5	201	81.5
24.0	73.4	72.0	76.1	402	84.0
36.0	76.2	109	81.4	603	84.3
48.0	70.1	145	85.9	804	85.1
60.0	74.2	182	84.2	1005	87.3
Mean	74.9	Mean	81.8	Mean	84.4
S.D.	3.9	S.D.	3.7	S.D.	2.1

However, we preferred a method wherein internal standardisation would be possible; therefore, the possibility of TLC analysis of the acidic eluate was investigated. The method published by Geissler et al. [11] proved to be suitable: the acetylation of the catecholamines protects them from degradation and allows further concentration of the sample, application to an HPTLC plate and subsequent separation. The R_F values of the triacetyl derivatives of noradrenaline, adrenaline and dopamine were 0.41, 0.56 and 0.68, respectively. The in situ condensation reaction with ethylene diamine yields fluorescent compounds [11] suitable for quantitative estimation by a TLC scanner. A chromatogram, obtained by analysis of a urine sample from a healthy volunteer, is illustrated in Fig. 1. These preliminary results indicate that quantitative analyses are possible, provided a suitable internal standard can be found. This compound could then be added to the urine sample and compensate for losses in the isolation and derivatisation steps; its triacetyl derivative should be well separated from the other catecholamines by TLC and yield sufficient fluorescence for detection by the spray reagent used.

We conclude that the use of the boric acid gel material for the isolation of free catecholamines affords a good recovery and yields extracts clean enough to be analyzed by quantitative fluorimetric or TLC techniques.

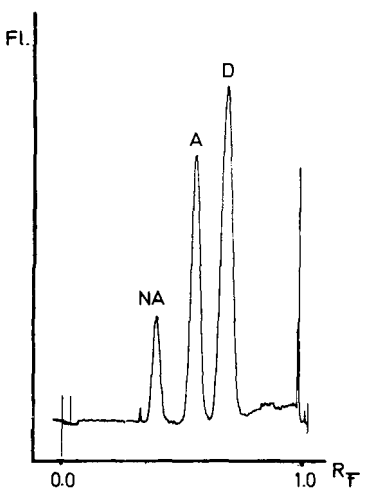

Fig. 1. Fluorescence TLC scan of a derivatised urine sample from a healthy volunteer.

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Note

Analysis of *Z* and *E* isomers of urocanic acid by high-performance liquid chromatography

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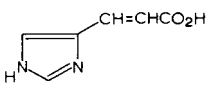
There has recently been a substantial effort devoted to the chromatographic detection of urocanic acid (UA), a major metabolite of histidine, and a naturally occurring sunscreen [1—7]. Of the several reported high-performance liquid chromatographic (HPLC) methods [8—11], none has described the resolution of UA into its *E* (*trans*) and *Z* (*cis*) isomers, though such isomerization is critical to the function of UA as a sunscreen [12]. We report here on two HPLC systems that resolve UA isomers, one an ion-paired, reversed-phase (for recent reviews, see refs. 13 and 14) procedure (method A) with a detection limit better than those reported for unresolved UA, and the other a "normal"-phase procedure (method B).



Urocanic acid (UA)

EXPERIMENTAL

Instrumentation and procedures

HPLC was performed using a composite system consisting of a Waters Model 6000A solvent delivery system, a Rheodyne 70-10 sample injection valve with a 20-μl loop, a Laboratory Data Control UV monitor with 254-nm detector and a Hewlett-Packard 3380A electronic integrator. Method A employed a 250 × 4.2 mm I.D. Waters μBondapak C_{18} (10 μm particle size) column: the eluent was 24 mg of tetrabutylammonium sulfate, 3.36 g of $NaH_2PO_4 \cdot H_2O$ and 8.00 g of Na_2HPO_4 in 1 l of distilled (from $KMnO_4$) water. The flow-rate of the pH 7.3—7.4 solution was 2.0 ml/min. Method B used a 250 × 4.2 mm I.D. Whatman

0378-4347/80/0000-0000/$02.25

Partisil (11 μm particle size) column with 2% conc. (58%) ammonia in 1-propanol at 1 ml/min as eluent.

Chemicals

E-UA was from Aldrich (Milwaukee, WI, U.S.A.) and was recrystallized from water to give crystals of the dihydrate. After washing with acetone and vacuum drying at 45°C for several hours, the melting point was 239°C (uncorr.) (lit. 218°C [15], 228°C [16]. L-Histidine · HCl was from Sigma (St. Louis, MO, U.S.A.).

Z-UA was prepared by irradiating a slurry of 5.4 g of *E*-UA in 2 l of water brought to pH 9 with potassium hydroxide. The slurry was photolyzed in a Rayonet Reactor (New England Ultraviolet Company) at 310 nm for 4 days at 30°C with occasional stirring. During this time the excess potassium salt of *E*-UA slowly went into solution. The resulting UA mixture (*E*/*Z* = 3:7) is virtually at the photostationary state [12]. The solution was lyophilized, the residue dissolved in a little water, and the pH adjusted to 9 with glacial acetic acid. This solution was chromatographed on a 23 × 3.5 cm column of Bio-Rad AG 1-X8 (acetate), 200—400 mesh, in 0.0125 *M* acetic acid [7]. Elution with 300 ml of 0.0125 *M* acetic acid was followed by 500 ml of 0.025 *M* and 1200 ml of 0.1 *M* acetic acid. *Z*-UA was eluted first and appeared after ca. 1000 ml of eluent. (In order to avoid $Z \rightarrow E$ isomerization by the slightly acidic eluent, the eluent was trapped in receiving flasks cooled with methanol—dry ice.) The combined fractions were lyophilized to yield a fluffy white solid which was washed with hexane and dried over phosphorus pentoxide at 65°C. The yield was 2.4 g (45%), m.p. 178—180°C (uncorr.) (lit. 175—176°C [15]); elemental analysis confirmed that this *Z*-UA is *not* a hydrate (compare *E*-UA above).

A sample of sweat was obtained by diluting several drops of tension-produced sweat from the epidermis on the back of the hand with water.

RESULTS AND DISCUSSION

The separation of an artificial mixture of *Z*- and *E*-UA using method A is illustrated in Fig. 1 (curve a). Retention times are *E*-UA 3.0 min, and *Z*-UA 4.7 min. Fig. 1 (curve b), a chromatogram of human sweat, illustrates the sensitivity of the technique. We determined that using a 20-μl injector volume, the detection limit for *E*-UA is 1 ng and for *Z*-UA, 3 ng (3×10^{-7} *M* and 1×10^{-6} *M*, respectively).

Our quantitative analyses were for solutions 6×10^{-4} *M* to 6×10^{-3} *M* in UA. The response of the UV detector was found to be linear over most of this range (6×10^{-4} *M* to 1.5×10^{-3} *M*) for both the *E* and *Z* isomers (correlation coefficients were 0.924 and 0.997, respectively). The calibration curves should be freshly determined or checked, however, since they are quite susceptible to small changes in flow-rate, column deterioration, etc. At higher concentrations there are large deviations from the Beer—Lambert Law within the detector, so that solutions of more than 2×10^{-3} *M* are best diluted before analysis. Alternatively, one may use known concentrations of *E*- or *Z*-UA which are within 5% of the unknown. The precision of multiple HPLC analyses was usually 1—2%. Areas, rather than peak heights, should be used for quantitation be-

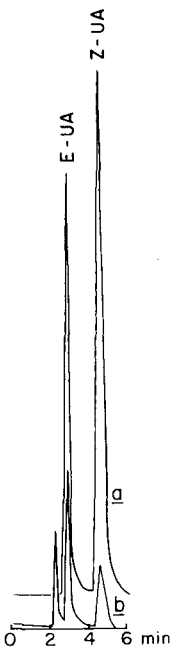


Fig. 1. HPLC analysis of *E*- and *Z*-UA using method A: (a) synthetic mixture; (b) sweat from the back of the hand.

cause the shapes of the peaks are dependent on the solvent used to dissolve the sample (for example, ^2H_2O vs. H_2O).

Method B complements method A in that the elution sequence is reversed; the retention times are *Z*-UA 2.5 min, and *E*-UA 3.8 min. Method A successfully resolves histidine from UA at a flow-rate of 1 ml/min. Retention times are histidine 3.3 min, *E*-UA 4.5 min, and *Z*-UA 8.9 min. The limit of detection for histidine is 0.5 μg. Reversed-phase columns are subject to deterioration when used with buffer salts; for appropriate precautions, see ref. 17. When the column deteriorates partially, a soap chromatography [18] technique using hexadecyltrimethylammonium bromide in place of tetrabutylammonium sulfate will give increased resolution: *E*-UA 2 min, *Z*-UA 8 min, at 2 ml/min.

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CHROMBIO. 580

Note

Rapid quantitative assay of plasma 11-deoxycortisol and cortisol by high-performance liquid chromatography for use in the metyrapone test

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Metyrapone stimulates corticotropin secretion through inhibition of the negative feedback mechanism by blocking the adrenal 11β-hydroxylase, i.e. the conversion of 11-deoxycortisol to cortisol. The single-dose version of the metyrapone test, first introduced by Jubiz et al. [1] forms a convenient substitute for the time-consuming classical metyrapone test [2]. In a study on a large number of patients Spiger et al. [3] reported that the estimation of plasma 11-deoxycortisol after a single dose of metyrapone is sufficient for testing the integrity of the pituitary-adrenal axis. In case of subnormal plasma 11-deoxycortisol levels these workers recommend the measurement of plasma cortisol levels to assure that the administered dose of metyrapone effectively blocked the 11β-hydroxylase.

Plasma levels of cortisol and 11-deoxycortisol are usually determined by a competitive protein binding (CPB) [1, 4—7] or a radioimmunoassay (RIA) method [3, 8—12]. The specificity of these methods is limited by the presence of cross-reacting material. Levels of interfering material can increase after administration of metyrapone. High-performance liquid chromatography (HPLC) does not suffer from this disadvantage. It has been demonstrated that HPLC is suitable for the determination of plasma cortisol [13—16]. Recently a method has been reported for the determination of cortisol and 11-deoxycortisol by means of reversed-phase HPLC [17]. However the accuracy of this method is questionable because no internal standard is used for quantification. We describe a rapid quantitative assay of 11-deoxycortisol and cortisol by means of HPLC on a silica gel column. A simple extraction procedure and the possibility of immediate quantification make this method very useful in the single-dose metyrapone test.

EXPERIMENTAL

Apparatus and materials

A Varian liquid chromatograph Model 8500 was used, equipped with a stop-flow injector, a LDC Model 1203 254-nm single-wavelength UV detector and a 250 × 3 mm SI-10 column (silica gel, particle size 10 μm, Chrompack, Middelburg, The Netherlands). Samples were injected with a 25-μl Hamilton syringe. All solvents were of analytical reagent grade.

Standard stock solutions of 1 mg steroid per ml ethanol were diluted to working solutions of 1 μg steroid per ml ethanol.

Charcoal-stripped plasma was prepared by mixing 5 g charcoal with 100 ml plasma pool for 1 h at room temperature. Charcoal was then removed by centrifugation.

Administration of metyrapone

Twenty healthy subjects without evidence of pituitary-adrenal disease were studied by the single-dose metyrapone test. All subjects received approximately 30 mg of metyrapone per kg body weight at midnight. Blood samples were taken at 8 a.m. on two consecutive days, before and after metyrapone administration. Plasma was separated by centrifugation and stored at −20°C.

Determination of steroids

11-Deoxycortisol. To 0.6 ml plasma 0.05 ml 3 *N* sodium hydroxide solution and 100 ng prednisone as internal standard were added. Extraction was carried out by mixing for 5 min with 4 ml dichloromethane. After centrifugation the water phase was discarded and the extract was washed once with 0.1 *N* hydrochloric acid and once with water. After evaporation to dryness of 3 ml of the extract the residue was dissolved in 50 μl chloroform and 20 μl of this solution were injected onto the column. The eluent used in HPLC was chloroform—isooctane—methanol—water (48.5 : 48.5 : 2.9 : 0.12, v/v). The flow-rate was 65 ml/h. The ratio of peak heights of 11-deoxycortisol and prednisone was used as the basis for quantification.

Cortisol. For the determination of cortisol the method as described for 11-deoxycortisol was modified by using prednisolone as internal standard and chloroform—isooctane—methanol—water (71 : 25 : 3.75 : 0.25, v/v) as eluent.

Estimation of the recovery

The recovery of 11-deoxycortisol was estimated as follows: 100 ng 11-deoxycortisol was added to 0.6 ml charcoal-stripped plasma. Samples were then analyzed as described, except that prednisone was added after the extraction procedure. Recovery was calculated by comparing the ratio of peak heights to the ratio of peak heights of a reference mixture of the same amounts of 11-deoxycortisol and prednisone.

For the estimation of the recoveries of cortisol, prednisone and prednisolone the same procedure was carried out on the understanding that instead of prednisone, prednisolone, 11-deoxycortisol and cortisol respectively were used.

RESULTS

Plasma concentrations of 11-deoxycortisol were determined in 20 subjects. The amount of 11-deoxycortisol in all plasma samples before administration of metyrapone was below the detection limit of 25 nmol/l. Mean values of 11-deoxycortisol in plasma after metyrapone were 290 nmol/l ± 78 (S.D.), range 190—450 nmol/l.

In addition, in pre- and postmetyrapone plasma samples of six unselected subjects cortisol was also measured. Values of 11-deoxycortisol and cortisol of these samples are given in Table I.

Chromatograms of plasma extracts before and after metyrapone are shown in Figs. 1 and 2.

Sensitivity

The minimal detectable amount for both cortisol and 11-deoxycortisol was 4.5 pmol. This amount corresponds to a concentration of 25 nmol/l when 0.6 ml plasma is analyzed according to our method.

Reproducibility

The precision was estimated from duplicate assays, the relative standard deviation was 8.0% for 11-deoxycortisol (n=20) and 4.6% for cortisol (n=12).

Linearity

To 0.6 ml of a charcoal-stripped plasma pool 11-deoxycortisol was added in amounts equivalent to 55—1100 nmol/l. A constant amount of 100 ng prednisone as internal standard was added to each sample, which was then processed as described. The same procedure was followed for cortisol with prednisolone as internal standard. Concentrations and peak height ratios were linearly related over this whole range for 11-deoxycortisol as well as cortisol.

Recovery

Recoveries of cortisol, 11-deoxycortisol, prednisone and prednisolone were estimated as described. Results are shown in Table II.

TABLE I

CONCENTRATIONS OF 11-DEOXYCORTISOL AND CORTISOL IN PLASMA SAMPLES OF 6 NORMAL SUBJECTS BEFORE AND AFTER ADMINISTRATION OF METYRAPONE

Subject	11-Deoxycortisol (nmol/l)		Cortisol (nmol/l)	
	Before metyrapone	After metyrapone	Before metyrapone	After metyrapone
1	< 25	410	285	45
2	< 25	210	325	80
3	< 25	200	420	215
4	< 25	210	550	145
5	< 25	270	325	150
6	< 25	280	385	85
mean ± S.D.	< 25	265 ± 72	380 ± 87	120 ± 56

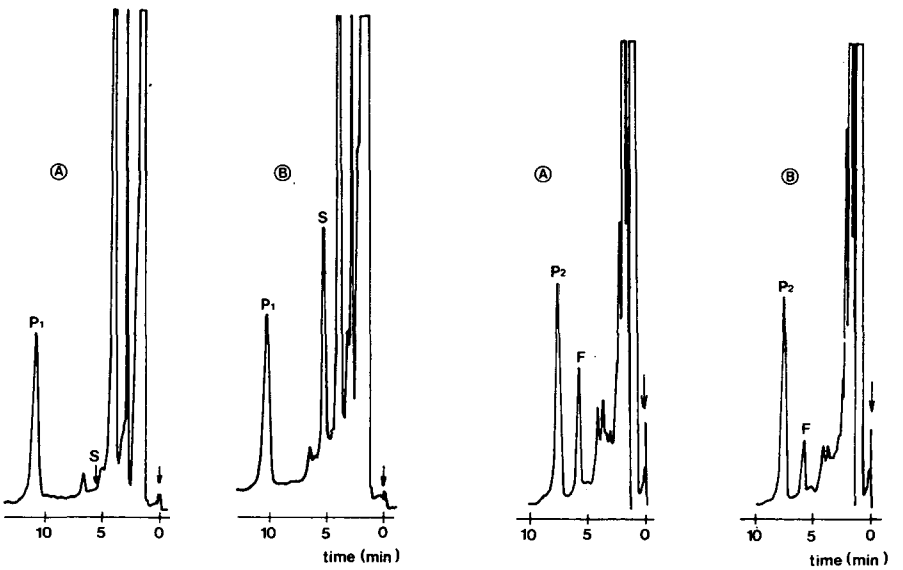

Fig. 1. Chromatograms of plasma extracts before (A) and after (B) administration of metyrapone. S = 11-deoxycortisol, P_1 = prednisone (internal standard). Column SI-10, eluent chloroform—isooctane—methanol—water (48.5 : 48.5 : 2.9 : 0.12, v/v). Flow-rate 65 ml/h; UV detection 254 nm, 0.008 a.u.f.s.

Fig. 2. Chromatograms of plasma extracts before (A) and after (B) administration of metyrapone. F = cortisol, P_2 = prednisolone (internal standard). Column SI-10, eluent chloroform—isooctane—methanol—water (71 : 25 : 3.75 : 0.25, v/v). Flow-rate 65 ml/h; UV detection 254 nm, 0.008 a.u.f.s.

TABLE II

RECOVERIES OF 100 ng STEROIDS ADDED TO CHARCOAL-STRIPPED PLASMA PRIOR TO EXTRACTION

Steroid	Recovery (%)	*n*
Cortisol	95.2 ± 0.8	4
11-Deoxycortisol	97.6 ± 0.7	4
Prednisone	100.7 ± 1.9	4
Prednisolone	96.2 ± 1.2	4

DISCUSSION

Concentrations of 11-deoxycortisol in plasma before and after a single dose of metyrapone as determined by our HPLC method are in good agreement with those estimated by CPB or RIA [4, 7, 11, 12]. The possibility of a rapid quantification of 11-deoxycortisol however is in favour of the HPLC method.

Concentrations of cortisol in plasma before metyrapone as determined by our HPLC method correlate well with those estimated by CPB or RIA [4, 11]. After a single dose of metyrapone however there is less agreement. For in-

stance, Schöneshöfer et al. [11] and Spark [4] reported postmetyrapone cortisol values of 248 ± 100 nmol/l and 236 ± 83 nmol/l respectively. These values are significantly higher than our postmetyrapone cortisol values of 120 ± 56 nmol/l. The difference is probably due to non-specificity of CPB and RIA [17]. It is obvious that levels of cortisol after metyrapone cannot be assessed correctly by means of CPB or RIA without additional purification. In our opinion HPLC is the advisable method to determine postmetyrapone concentrations of cortisol.

In conclusion: the method described offers a simple, rapid and reliable determination of 11-deoxycortisol and cortisol for use in the single-dose metyrapone test.

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Note

Determination of metoprolol metabolites in plasma and urine by electron-capture gas—liquid chromatography

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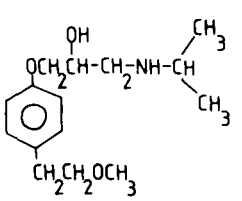
(First received November 29th, 1979; revised manuscript received February 11th, 1980)

Metoprolol (Fig. 1, I) is a cardioselective β-adrenoceptor-blocking drug widely used in the treatment of hypertension [1] and angina [2]. Four metabolites have been identified [3], and the two to be discussed in this paper have been shown to possess some β-blocking activity in the cat. The structures of these two metabolites, designated by Hässle as H119/66 and H105/22, are given in Fig. 1 as (I-A) and (I-B), respectively. They are both five to ten times less potent than the parent drug. Methods for the determination of the metabolites are available [4] but require the use of gas chromatography—mass spectrometry (GC—MS), a technique beyond the reach of many workers. Alternative methods utilising only gas—liquid chromatography (GLC) are presented here.

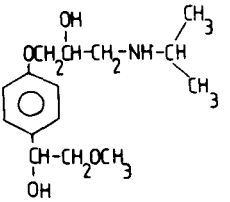
MATERIALS AND METHODS

Gas—liquid chromatography

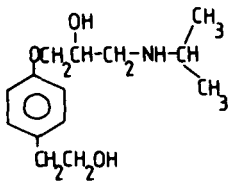
A Hewlett-Packard Model 5700A gas chromatograph fitted with a ^{63}Ni detector was used. A 6 ft. × 2 mm I.D. glass column, packed with 3% OV-1 on Gas-Chrom Q, 100—120 mesh (Field Instruments, Surrey, Great Britain) was used for the (I-A) analysis and 3% OV-17 on Gas-Chrom Q, 80—100 mesh (Pierce Chemicals, Rockford, IL, U.S.A.) for (I-B). The carrier gas was 5% methane in argon at a flow-rate of 60 ml/min. Injection port and detector temperatures were 250°C and 350°C, respectively. The temperature of the oven, however, depended on the method used. For (I-A) in plasma, the column was



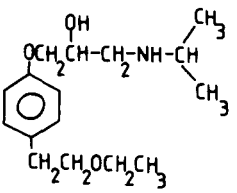
[I]



[I-A]



[I-B]



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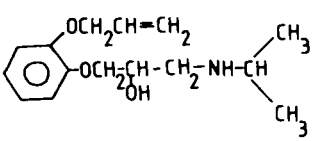
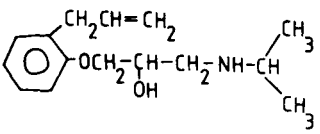


[III]



[IV]

Fig. 1. Structure of metoprolol (I), two pharmacologically active metabolites (I-A) and (I-B), and a chemically related compound (II), used for internal standardisation. III = Oxprenolol; IV = alprenolol.

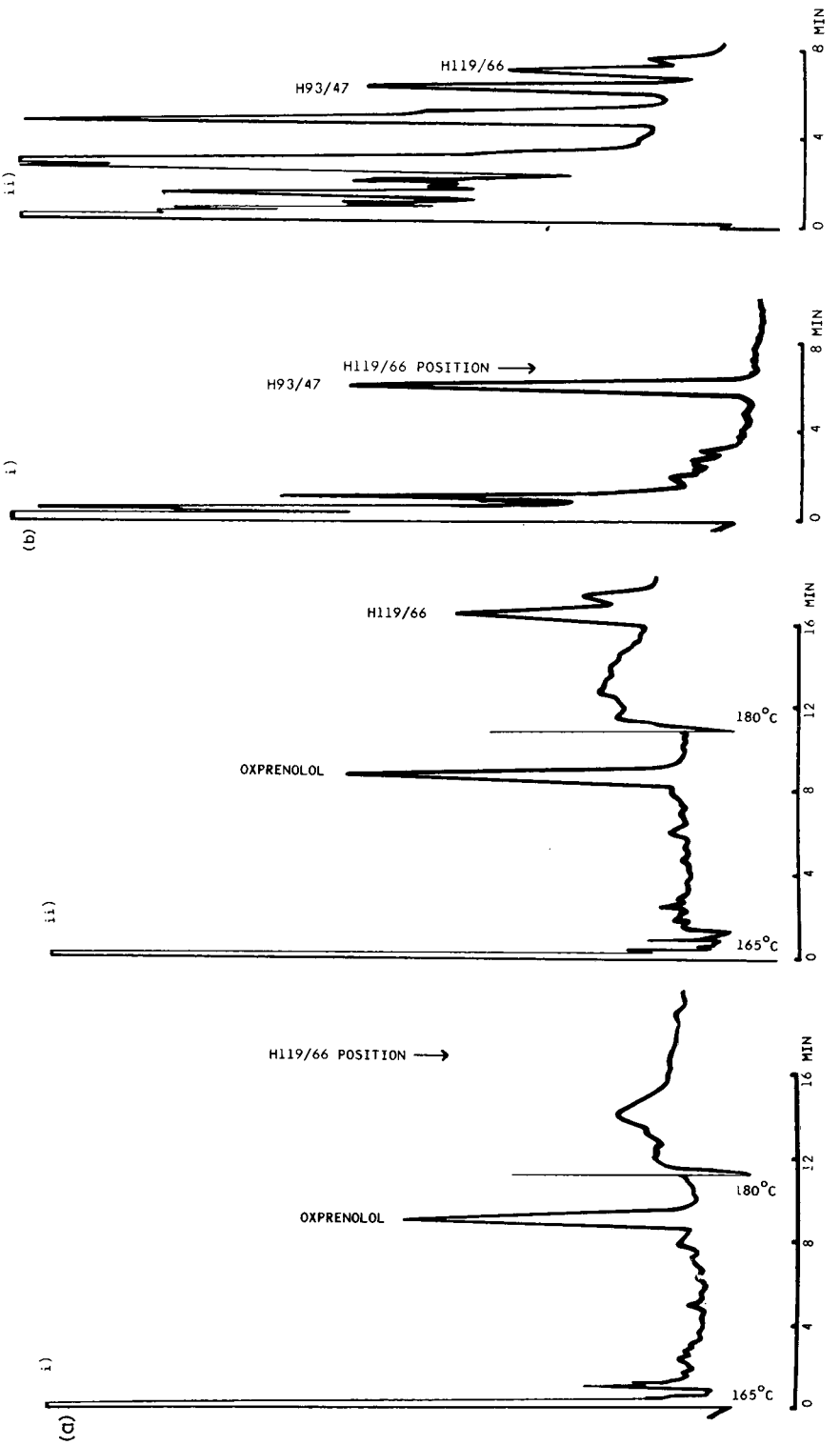
operated at 165°C until the internal standard eluted, then the temperature was increased to 180°C. For (I-A) in urine, the column was maintained at 185°C throughout. For (I-B), the column was operated at 180°C until the internal standard eluted, then it was increased to 195°C. This procedure was used for both plasma and urine.

Reagents and materials

(I-A) and (I-B), in the form of their *p*-hydroxybenzoic acid salts, and the internal standards H93/47 (II) and alprenolol (IV) as the hydrochlorides, were supplied by Hässle (Mölndal, Sweden). Oxprenolol hydrochloride (III) was obtained from Ciba-Geigy (Basle, Switzerland), trifluoroacetic anhydride (TFAA) from Sigma Chemicals (Poole, Great Britain) and N-heptafluorobutyrylimidazole (HFBI) from Pierce. All organic solvents were AR grade and redistilled before use.

Extraction procedure

The procedure for the extraction of each metabolite was similar but different internal standards were used: (II) and (III) were used for determination of


H119/66
H93/47
ii)
0
4
8 MIN
H119/66 POSITION
H93/47
i)
(b)
0
4
8 MIN
H119/66
180°C
OXPRENOLOL
ii)
165°C
0
4
8
12
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H119/66 POSITION
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i)
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(a)
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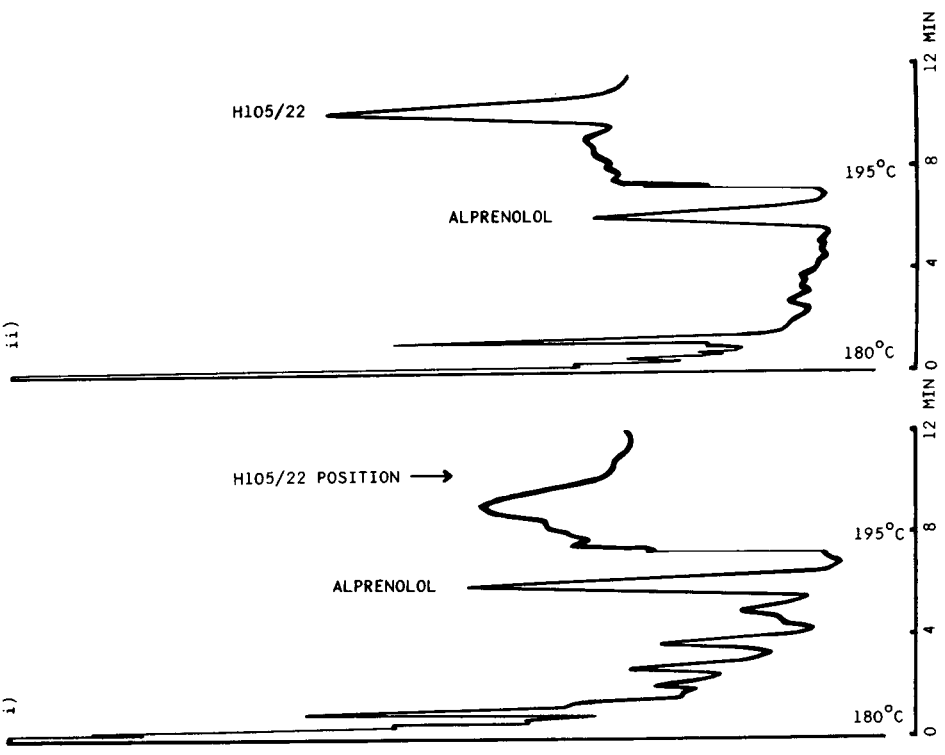

Fig. 2. (a) Typical chromatograms obtained from plasma samples containing 100 ng of internal standard and (i) no (I-A) (H119/66), or (ii) 100 ng of (I-A) (H119/66). (b) Typical chromatograms obtained from urine samples containing 100 ng of internal standard and (i) no (I-A) (H119/66), or (ii) 200 ng of (I-A) (H119/66). (c) Typical chromatograms obtained from urine samples containing 100 ng of internal standard and (i) no (I-B) (H105/22), or (ii) 100 ng of (I-B) (H105/22).

(I-A) in plasma and urine, respectively, and (IV) as standard in the assay of (I-B).

A 0.1-ml volume of urine was diluted with 1.0 ml water for (I-A) analysis but 1.0 ml of undiluted urine was used for (I-B) determination because its concentration in urine is very low. Determination of the metabolites in plasma also required a 1.0-ml sample. The appropriate internal standard (100 ng) was then added to the sample followed by 1.0 ml of saturated aqueous sodium chloride solution. The sample was then made alkaline (pH > 10) with 0.5 ml of 1 *N* NaOH and extracted twice with 5 ml of dichloromethane—diethyl ether (2:3). After centrifugation at 300 *g* for 10 min, the combined organic extracts were evaporated to dryness at 40°C.

Derivatization

Metabolite (I-A). To the dried extract, 100 μl of HFBI in benzene (1:3) were added and derivatization allowed to proceed for 1.5 h at room temperature. The sample was then evaporated to dryness, redissolved in 200 μl of toluene and washed with 0.5 ml of 1 *N* NaOH; 3 μl of the toluene phase were injected into the chromatograph.

Metabolite (I-B). The extract was redissolved in 500 μl of toluene and derivatized with 200 μl of TFAA for 1.5 h at room temperature. After evaporation to dryness, the derivative was redissolved in 200 μl of toluene and 3 μl were injected into the chromatograph.

RESULTS AND DISCUSSION

The recovery of each metabolite was greater than 90%. Typical chromatograms obtained are shown in Fig. 2a, b and c. After dosing with (I), (I-A) can be measured in plasma and urine but levels of (I-B) in plasma are below the limit of detection so that this metabolite can only be determined in urine.

By extracting known amounts of each metabolite calibration curves were constructed and shown to be linear within the ranges used. The equations for

TABLE I

ACCURACY AND PRECISION OF METOPROLOL METABOLITE METHODS

Values are given as the mean ± S.D. (n = 6)

Actual concentration (ng/ml)	Observed concentration (ng/ml)		
	Plasma (I-A)	Urine (I-A)	Urine (I-B)
10	11 ± 2.3	11 ± 4.8	12 ± 4.6
20	23 ± 3.6	18 ± 3.2	22 ± 5.7
40	39 ± 3.6		
100	100 ± 6.3	94 ± 9.8	102 ± 4.8
120	115 ± 10.6		
200	196 ± 12.3	203 ± 8.3	190 ± 12.4
300		307 ± 6.3	315 ± 9.0
400		408 ± 10.2	396 ± 10.9

the calibration curves are as follows:

(I-A), plasma, $y = 0.00385\,x + 0.01570$
(I-A), urine, $y = 0.00563\,x - 0.01125$
(I-B), urine, $y = 0.00618\,x + 0.01407$

Restandardisation was carried out each day in order to maintain precision, although day-to-day variation in detector response was small. Table I demonstrates the accuracy and precision obtained with each method over the range of

TABLE II

CONCENTRATION OF METOPROLOL METABOLITES IN SPIKED URINE AND IN PATIENT PLASMA AND URINE SPECIMENS AS DETERMINED BY GLC AND GC—MS

Sample		Plasma (I-A) concentration (ng/ml)		
		GC—MS	GLC	
Plasma	1	42	77	
	2	106	146	
	3	54	68	
	4	127	176	
	5	96	143	
	6	26	93	
		Correlation coefficient, $r = 0.92$		
		Urine (I-A) concentration (ng/ml)		
		GC—MS	GLC	Added
Spiked	1	1692	1586	1698
	2	552	593	566
	3	62	77	57
Urine	1	9169	12925	
	2	8688	9397	
	3	5236	5856	
	4	1019	1720	
		Correlation coefficient, $r = 0.98$		
		Urine (I-B) concentration (ng/ml)		
		GC—MS	GLC	Added
Spiked	1	76	80	77
	2	17	16	15
	3	3	<6	3
Urine	1	213	220	
	2	129	69	
	3	96	84	
	4	38	<6	
		Correlation coefficient, $r = 0.95$		

concentrations used. The coefficients of variation at the 100 ng/ml level for plasma (I-A), urine (I-A) and urine (I-B) are 6%, 10% and 5%, respectively. The methods were capable of measuring 10 ng of salt per ml, corresponding to 6 ng of free base per ml.

The accuracy of each method was verified by the parallel analysis of metabolite concentrations in both spiked samples and samples taken after metoprolol dosing using the GC—MS method of Hässlę. Each set of results is presented in Table II, and it can be seen that there is a reasonable overall agreement. It should be noted that the few discrepancies may well have been due to sample deterioration during storage prior to analysis. It has been observed that, for some urine specimens, storage can result in a reduction in (I-A) level and, whereas the GLC determinations were carried out immediately, GC—MS analysis by Hässle was, for technical reasons, delayed by more than four months. The methods presented here, however, offer a useful alternative to GC—MS.

The method has been used in a study in which eight healthy volunteers received an oral dose of 100 mg of metoprolol tartrate. Plasma levels of metabolite (I-A) were measured and the urine excretion of metabolites (I-A) and (I-B) determined over a period of 24 h. A summary of the results is presented in Table III.

TABLE III

PLASMA AND URINE LEVELS OF METOPROLOL METABOLITES AFTER ORAL ADMINISTRATION OF 100 mg METOPROLOL TARTRATE TO EIGHT HEALTHY VOLUNTEERS

Metabolite (I-A)	Plasma:	Mean half-life (h)	6.7
		C_{max} (ng free base per ml)	67
	Urine:	Mean percentage dose excreted in 24 h	6.1
Metabolite (I-B)	Urine:	Mean percentage dose excreted in 24 h	0.14

ACKNOWLEDGEMENT

We wish to thank M. Ervik and his colleagues at Hässle, for helpful discussion during this work.

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Note

Sensitive gas chromatographic method for the determination in plasma of FM 24, 1-(2-*exo*-bicyclo[2,2,1]hept-2-ylphenoxy)-3-[(1-methylethyl)amino]-2-propanol, a new β-adrenoceptor blocking agent

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FM 24, 1-(2-*exo*-bicyclo[2,2,1]hept-2-ylphenoxy)-3-[(1-methylethyl)amino]-2-propanol, is a new and very potent β-adrenoceptor blocking agent whose duration of action in animal experiments has been shown to be two to five times longer than that of propranolol [1—3]. The evaluation of the pharmacokinetic profile of such a long acting drug appeared important for the better understanding of its mechanism of action, and for more efficient therapeutic application. Thus, in the course of clinical investigation of this drug, it became necessary to develop a highly sensitive and specific method for the determination of plasma levels of FM 24.

EXPERIMENTAL

Standards and reagents

FM 24 and FM 25 used as internal standard were generously supplied by Pharmindustrie (Gennevilliers, France). Their structural formulae are shown below.

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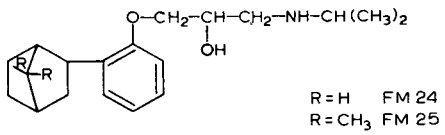

The following reagents (all purchased from Merck, Darmstadt, G.F.R.) were used: toluene, *n*-hexane, methanol, ethyl acetate, 1 *N* sodium hydroxide, 0.1 *N* hydrochloric acid (the organic solvents were distilled before use, then washed with sodium hydroxide and hydrochloric acid). Dimethylchlorosilane (DMCS) and pentafluoropropionic anhydride (PFPA) were obtained from Pierce (Rockford, IL, U.S.A.).

Glassware

All glassware was cleaned in chromic acid. Conical and round-bottomed glass centrifuge tubes were silanized with 5% DMCS in toluene, then rinsed with methanol.

Gas chromatographic conditions

Analyses were performed under isothermal conditions on a Hewlett-Packard Model 5710A gas chromatograph equipped with a ^{63}Ni linear electron-capture detector. The glass column (2 m × 3 mm I.D.) was packed with Chromosorb W HP (100—120 mesh) coated with 3% OV-17 (Pierce), and conditioned for 1 h at 260°C (argon—methane (90:10) carrier gas flow-rate, 30 ml/min), 3 h at 320°C (no gas flow) and 24 h at 270°C (carrier gas flow-rate 40 ml/min). The column temperature was 215°C, injection port temperature 250°C, detector temperature 300°C and carrier gas (argon—methane) flow-rate 40 ml/min.

Mass spectrometric conditions

A Micromass VG organic 305 gas chromatograph—mass spectrometer with a VG Data System M8 3R was used. Spectra were obtained under the following conditions in the electron-impact mode: electron energy 70 eV, trap current 200 μA, accelerating voltage 4 kV, ion source temperature 200°C and molecular separation temperature 260°C. In gas chromatographic—mass spectrometric (GC—MS) analyses, the same column was used as described under gas chromatographic conditions, except that the helium flow-rate was reduced to 20 ml/min.

Sample preparation

Extraction from plasma. In a glass-stoppered centrifuge tube was placed 0.5 ml of internal standard solution (1 μg/ml in methanol) and evaporated to dryness under a gentle stream of nitrogen, then 1 ml of plasma sample, 0.5 ml of 1 *N* sodium hydroxide and 7 ml of toluene were added. The tube was placed on a rotating mixer (60 rpm) for 15 min and then centrifuged at 4°C for 15 min at 1200 *g*. The organic phase (6.5 ml) was transferred to another centrifuge tube and back-extracted with 5 ml of 0.1 *N* hydrochloric acid by shaking for 15 min. After centrifugation (10 min), 4.5 ml of acidic aqueous layer were transferred to another centrifuge tube, alkalinized with 1 ml of 1 *N* sodium hydroxide and extracted with 6 ml of hexane by shaking for 15 min. After

centrifugation (10 min), 5 ml of organic phase were transferred to a conical test-tube and evaporated to dryness in a water-bath at 50—60°C under nitrogen. The residue was submitted to derivatization followed by analysis.

Derivatization. A 50-μl volume of PFPA (1:1 solution in ethyl acetate) was then added to the dry residue. The tube was capped and heated at 65—70°C for 1 h, then the excess of reagent was evaporated to dryness at 50—60°C under a gentle stream of nitrogen. When the residue was dried, the nitrogen flow-rate, was increased for a further 5 min in order to remove compounds derived from the reaction mixture which would appear as interfering peaks on the chromatogram. After dissolution in 150 μl of hexane, 5 μl of this solution were injected into the gas chromatograph.

Standard curves

Varying quantities of FM 24 (5—500 ng) and 500 ng of the internal standard were added to 1 ml of human plasma control and carried through the analytical procedure. The ratio of the peak areas of FM 24 to FM 25 were plotted against the concentration of FM 24.

RESULTS AND DISCUSSION

Typical chromatograms obtained with blank plasma before and after spiking with a known amount of FM 24 and with a plasma sample in a patient receiving FM 24 by the oral route are shown in Fig. 1. The peaks of the two compounds were well resolved with retention times of 4.5 and 6.8 min for FM 24 and FM

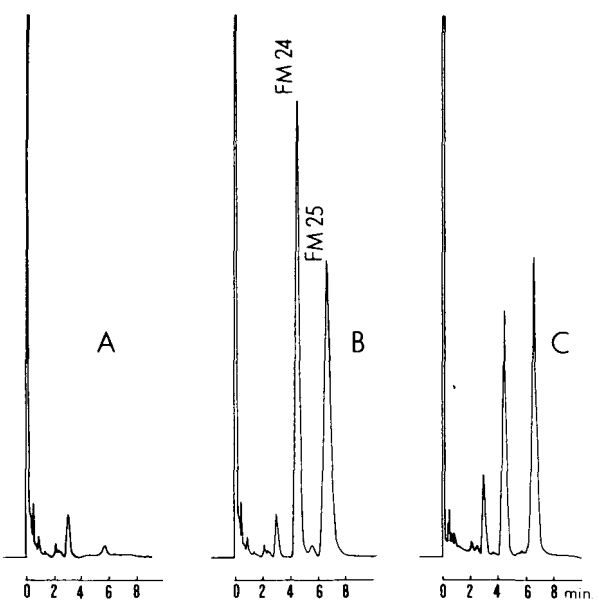

Fig. 1. Gas chromatograms of plasma extracts. (A) Plasma free from drug; (B) plasma spiked with FM 24 (100 ng/ml) and FM 25 (500 ng/ml); (C) plasma from a patient receiving 40 mg of FM 24 orally.

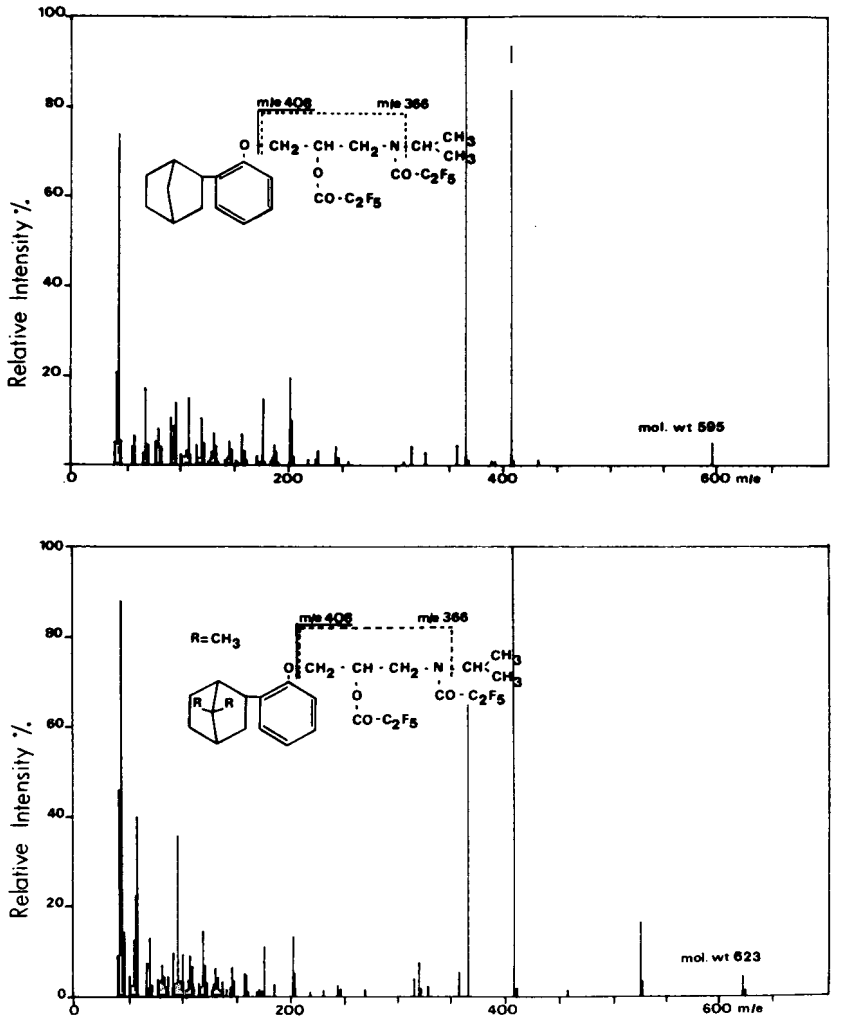

Fig. 2. Mass spectra obtained during GC—MS analyses for dipentafluoropropionates of FM 24 (top) and FM 25 (bottom).

25, respectively. No interferences were observed from endogenous substances.

The GC—MS analysis confirmed the identity of the GC peaks. Those due to the reaction products of FM 24 and FM 25 with PFPA showed molecular ions at *m/e* 595 and 623 respectively in the mass spectra corresponding to the formation of the dipentafluoropropionates (Fig. 2). Their structure was confirmed by the observation of ions at *m/e* 408 and *m/e* 366, the formation of which from molecular ion is specific for a β-blocker aryloxy group [4].

A standard calibration graph obtained after extraction of FM 24 from plasma was linear in the range of concentration studied ($y = 0.0042\ x + 0.00645$; $r = 0.9998$). The reproducibility of the method is reported in Table I. The recovery of the extraction procedure determined with ^{14}C-labelled FM 24 was found to be 72.5%.

TABLE I

ACCURACY OF DETERMINATION OF FM 24 IN HUMAN PLASMA

Amount added (ng/ml)	Average of 6 assays ± S.D. (ng/ml)	Coefficient of variation (%)
5	6.0 ± 0.6	9.9
10	10.8 ± 0.5	4.6
20	19.7 ± 0.9	4.6
50	51.1 ± 1.2	2.3
100	101.4 ± 2.5	2.5
200	194.2 ± 4.6	2.4
500	503.7 ± 13.3	2.6

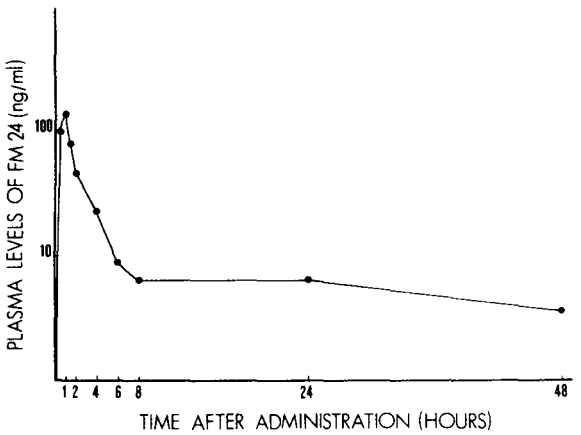

Fig. 3. Plasma level—time curve obtained for FM 24 following a single oral administration of 40 mg of the drug to a patient.

The absolute sensitivity of the electron-capture detector was about 30 pg for FM 24; this means that under the prescribed conditions, it is possible to detect 1 ng/ml of FM 24 in plasma.

The method has been applied to the determination of FM 24 in plasma of patients treated with this β-blocker. Fig. 3 illustrates the time course of plasma levels of FM 24 observed in a patient after a single oral administration of 40 mg of the drug. The observation of the last part of the curve clearly indicates that the method described above allows the pharmacokinetic study of FM 24 even in the case of a single oral administration of a very small dose in man.

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Note

Analysis of piroxicam in plasma by high-performance liquid chromatography

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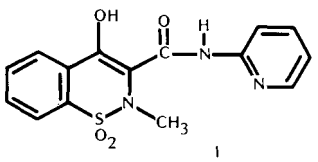
Piroxicam, a non-steroidal anti-inflammatory agent of the benzothiazine family, has demonstrated efficacy in man in the treatment of rheumatoid arthritis and other inflammatory disorders [1]. During pharmacokinetic studies in man, mean peak piroxicam plasma concentrations of 1.5 μg/ml were measured after the administration of single 20-mg doses. The mean steady-state level after chronic 20-mg daily doses for 14 days was reported to be 4.5 μg/ml. Alteration of the dose level resulted in a near linear relationship between the amount of drug administered and plasma concentration [2].

The quantitative determination of piroxicam in plasma during these pharmacokinetic studies was accomplished by degradative fluorometric wet chemistry methods involving strong acid hydrolysis of drug to generate 2-aminopyridine and measurement of this species after various purification steps. Concentrations were calculated from the fluorescence measured at specific excitation and emission wavelengths as compared to externally standardized samples. The high-performance liquid chromatographic (HPLC) method reported here and recommended for future measurement of piroxicam plasma levels has a number of analytical advantages. It is exceptionally reproducible and accurate, does not require chemical modification of the drug, uses small amounts of plasma (0.1 ml), is simple and rapid with assay steps performed in disposable glassware, obviates the need for quantitative transfers by the the incorporation of an internal standard, and is compatible with automated analytical equipment.

EXPERIMENTAL

Reagents and materials

Piroxicam [4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] (I) and the internal standard, isoxicam [4-hydroxy-2-methyl-N-(5-methyl-3-oxazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] (II), were prepared as aqueous solutions in 0.1 *N* NaOH at a concentration



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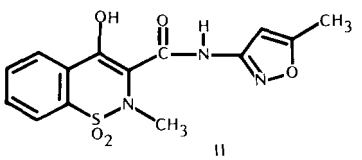

II

of 1.0 mg/ml and stored in the dark at 4°C. No degradation was noted for at least one month. Appropriate dilutions of these solutions were made weekly with water to produce working standards containing 50 μg/ml and 5 μg/ml for piroxicam and 100 μg/ml for isoxicam. Acetonitrile was glass-distilled (Burdick and Jackson, Muskegon, MI, U.S.A.) as was the water used in preparing the reagents and the chromatographic mobile phase. Diethyl ether, acetic acid, sulfuric acid, and tris(hydroxymethyl)aminomethane were of analytical reagent grade.

Sample preparation

In disposable 16 mm × 100 mm culture tubes, 0.1 ml of plasma was fortified with 10 μg of internal standard, mixed with 0.5 ml of 0.1 *N* sulfuric acid, extracted with 4 ml of diethyl ether on a vortex mixer for 30 sec and centrifuged. The solvent layer was transferred to another tube and evaporated to dryness using a vortex evaporator. The residue was reconstituted in 1 ml of 0.05 *M* tris(hydroxymethyl)aminomethane and aliquots subjected to HPLC. Samples at this stage could be held for at least seven days at room temperature as demonstrated by the same peak area count ratio of piroxicam to the internal standard measured on days 1, 3, and 8.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) analytical liquid chromatograph equipped with a 300 mm × 3.9 mm I.D. 10-μm μBondapak cyano column (Waters Assoc., part No. 84042) was fitted with a Waters Model 440 UV detector using low-dead-volume hardware. A mobile phase of acetonitrile–water–acetic acid (25:70:5) was filtered, degassed, and used at a flow-rate of 1.2 ml/min. The effluent stream was monitored while employing a 365-nm

filter on the detector. The range setting was fixed at 0.02 a.u.f.s., with the signal monitored by a 10-mV strip chart recorder (0.5 cm/min) interfaced with a Spectra Physics System IV electronic integrator. Samples were automatically injected at 11-min intervals using an HPLC autosampler (Micromeritics Model 725) equipped with a 100-μl sample loop. Column effluent was recycled back into the mobile phase reservoir (800 ml). The mobile phase was replaced every three days (approximately 350 samples).

Calibration and specificity

Calibration curves were constructed by determining the response from known amounts of piroxicam and the internal standard added to control plasma. Assay linearity was demonstrated over the range 0.5—20.0 μg/ml; the amount of the internal standard was held constant at 10 μg/sample. For daily validation, six samples fortified at mid-range (5.0 μg/ml) were processed with each group of test samples, two at the beginning of the run, two at mid-run, and two at the end of the run. The mean ratio of piroxicam to the internal standard (IS) integrator area counts was determined and the piroxicam concentration in test samples calculated from the expression

$$\text{Concentration } (\mu g/ml) = \frac{\text{area counts drug}}{\text{area counts IS}} \times \frac{C}{\text{mean ratio}}$$

where C represents the concentration (μg/ml) of piroxicam in the fortified samples.

To assess assay specificity, control plasma samples were fortified at 100 μg/ml with all known animal and human metabolites of piroxicam [3, 4] and processed through the assay. Salicylate at concentrations of 100 μg/ml of plasma was also tested.

RESULTS AND DISCUSSION

Reproducibility was determined at each fortification level of the calibration curve (Table I). Relative standard deviations (n = 4) ranged from 0.6 to 5.6%. A

TABLE I

PIROXICAM CALIBRATION CURVE FROM HUMAN PLASMA

Piroxicam level (μg/ml)	Piroxicam/IS* mean ratio (n = 4)	S.D.	R.S.D.** (%)
0	0	—	—
0.5	0.0355	0.0019	5.4
1.0	0.0873	0.0029	3.3
2.0	0.175	0.0043	2.5
5.0	0.528	0.0034	0.6
10.0	1.098	0.061	5.6
20.0	2.242	0.022	1.0

*Internal standard concentration was 100 μg/ml. Ratio values were derived from electronic integrator area count units.

**Relative standard deviation.

regression analysis of data pairs in Table I (concentration vs. area count ratio) resulted in a regression coefficient of 0.999916, indicating exceptional linearity over the assay range. Assay recovery was determined by comparing the response from known amounts of drug and the internal standard with processed fortified plasma samples (n = 6). Recoveries for piroxicam and the internal standard were 73.6 and 77.3%, respectively. In experiments designed to assess assay specificity, salicylate at plasma concentrations of 100 μg/ml did not respond. Of all the known animal and human biotransformation products of piroxicam, only the 5′-hydroxylated metabolite yielded a chromatographic peak; retention times, however, were sufficiently different so as not to interfere with piroxicam measurements. Additionally, the extraction conditions specified result in a relatively poor recovery of this metabolite (<20%). Retention times in the system as described were: piroxicam, 4.8 min; 5′-hydroxypiroxicam, 5.3 min; and isoxicam (internal standard), 7.0 min.

The chromatograms in Fig. 1 demonstrate the lack of interference and the specificity of the assay procedure for the measurement of piroxicam in plasma.

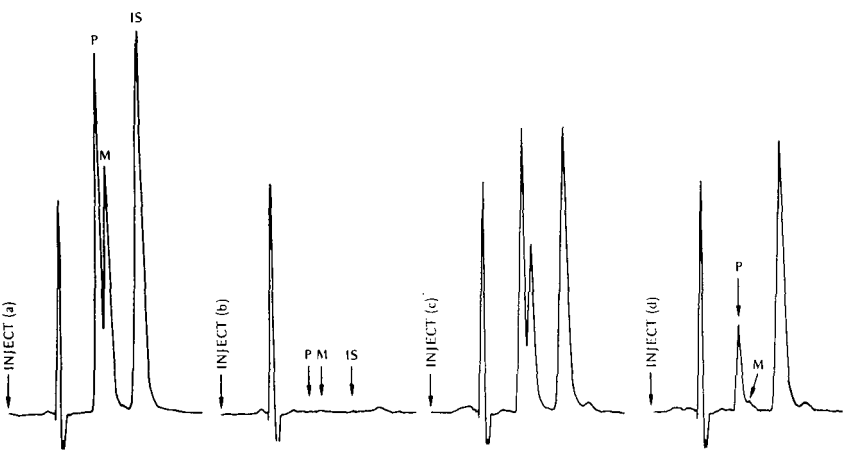

Fig. 1. Chromatograms of: (a) a standard mixture of 50 ng of piroxicam (P), 50 ng of 5′-hydroxypiroxicam (M), and 1 μg of isoxicam (IS); (b) control plasma extract; (c) extract from plasma fortified with P at 5 μg/ml, M at 20 μg/ml and IS at 100 μg/ml; (d) plasma extract from a sample taken 8 h after a volunteer received 20 mg of piroxicam.

Latitude to increase assay detection limits where necessary is evident and can be approached by using larger plasma aliquots, larger injection volumes, increased detector sensitivity settings, or any combination of these parameters; a ten-fold improvement of the lower detection limit (from 0.5 to 0.05 μg/ml) has been demonstrated in preliminary experiments.

ACKNOWLEDGEMENTS

The authors greatly appreciate the contributions and technical expertise of Ms. Diane Farrell and of Dr. Joseph G. Lombardino who provided samples of piroxicam, 5′-hydroxypiroxicam, and isoxicam.

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Note

High-performance liquid chromatographic assay for tocainide in human plasma: comparison with gas—liquid chromatographic assay

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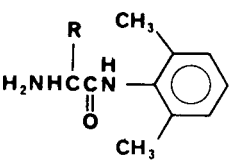
(Received December 27th, 1979)

Tocainide (Astra Pharmaceutical Products, Sydney, Australia) (I) is currently undergoing clinical trial in this country and may become established as useful alternative therapy in the treatment of arrhythmias. Plasma levels of the drug have been assayed by gas–liquid chromatographic (GLC) methods [1–3]



I: R = CH_3
II: R = H
III: R = CH_2CH_3

which have involved lengthy sample extraction and derivatization steps. A high-performance liquid chromatographic (HPLC) assay employing dansylation and fluorescence detection has been reported [4] and recently a simplified HPLC assay involving UV detection of the underivatized drug has also been reported [5].

In the present study a precise HPLC assay for tocainide has been developed and is compared with a published GLC assay [3]. Advantages of the new HPLC method over published methods are discussed.

EXPERIMENTAL

Reagents

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Dichloromethane was Nanograde from Mallinckrodt,

0378-4347/80/0000—0000/$02.25

St. Louis, MO, U.S.A. Specially purified acetonitrile (210 nm cut-off, Unichrom from Ajax Chemicals Melbourne, Australia) was used for HPLC. Tocainide hydrochloride (I) and the internal standards (II and III) were generously provided by Astra Pharmaceutical Products.

Standards

Stock solutions of tocainide hydrochloride (I) and the internal standards (II and III) were prepared in water (200 μmol/l each) and were stable for at least one month at 4°C. The tocainide solution was used to prepare the appropriate plasma standards for each assay run. Peak area ratios of tocainide to the internal standard were determined for plasma standards and unknowns, and quantification was performed by reading unknown values from a plotted standard curve.

HPLC tocainide assay

A 1-ml aliquot of plasma was pipetted into a 15-ml glass stoppered tube. The internal standard solution (300 μl) was added, followed by 2 ml of 1.0 *N* sodium hydroxide—0.6 *M* sodium tetraborate (pH 10), and 5 ml of dichloromethane; the mixture was then shaken on a horizontal shaker for 10 min at 100 opm. The phases were separated by centrifugation (1100 *g* for 10 min) and the aqueous layer was removed by vacuum aspiration and discarded. The organic layer was poured into autosampler tubes (diSPo tubes from Scientific Products, State College, PA, U.S.A.) (75 mm × 12 mm) and evaporated under a stream of pure nitrogen at 45°C. The tubes were removed from the heating block as soon as they were dry and the residue was reconstituted in 0.5 ml of mobile phase [1.5 m*M* phosphoric acid—acetonitrile (95:5)]. Fifty microlitres of this solution were injected into the chromatograph.

High-performance liquid chromatography

A chromatograph (Spectra-Physics Model SP 8000) equipped with a ternary solvent system, helium degass and automatic data reduction facilities was used. Files for the instrument operation and integration were stored on disc (Spectra-Physics Model SP 4010 disc module). The reversed-phase column used measured 300 mm × 4 mm and was packed with alkyl phenyl bonded to 10-μm silica (μBondapak/Phenyl from Waters Assoc., Milford, MA, U.S.A.). Column oven temperature was 50°C. The mobile phase was automatically mixed by the instrument and consisted of 1.5 m*M* aqueous phosphoric acid—acetonitrile (95:5) at a flow-rate of 1 ml/min. The instrument was operated in the constant-flow mode and all solvent lines from the column to the detector were carefully thermally insulated. The column effluent was monitored at 230 nm with a variable-wavelength UV detector (Schoeffel Model 770). The detector absorbance setting was 0.04, an attenuation of 2 was used (0.08 a.u.f.s.) and time constant 9.0 sec. Samples were injected automatically using a 50-μl sample loop and an autosampler (Spectra-Physics Model 8010).

Recovery and reproducibility

Recovery of the HPLC assay was determined at concentrations of 10, 20, 40 and 60 μmol/l in plasma by comparison of the tocainide peak area with that

obtained for an aqueous solution containing a known concentration of tocainide (200 μmol/l) injected directly into the chromatograph.

Intra-assay reproducibility of the HPLC and GLC assays was determined at concentrations of 20, 40 and 60 μmol/l by assaying five plasma samples at each concentration. This was repeated using the HPLC assay where 1 *N* sodium hydroxide replaced the 1 *N* sodium hydroxide—0.6 *M* sodium tetraborate. Inter-assay reproducibility was determined by assaying a single plasma sample containing added tocainide (40 μmol/l) in each assay run. Five determinations were made using the HPLC assay and its modification and twenty determinations using the GLC assay.

Comparison of HPLC and GLC assays

Thirty-one venous blood samples were collected from eight patients at steady-state on oral tocainide and plasma levels of the drug assayed by the HPLC and a published GLC method [5].

Steady-state plasma levels

Blood samples were taken hourly over the 8-h dosage interval from each of three subjects taking oral tocainide (600 mg, 8-hourly) at steady-state and assayed for tocainide by the HPLC method.

Interference by other drugs

Samples of the drugs and metabolites listed in Table I were dissolved in mobile phase (200 μmol/l of each) and injected into the high-performance liquid chromatograph. The retention times were obtained if a peak was observed.

TABLE I

RETENTION TIMES OF DRUGS AND METABOLITES IN THE HPLC ASSAY FOR TOCAINIDE

Drug	Retention time (sec)
Tocainide (I)	520
Internal standard (II)	434
Internal standard (III)	670
N-Acetylprocainamide	360
Caffeine	> 1000
Carbamazepine	> 1000
N-Desisopropylpropranolol	> 1000
Dihydroquinidine	> 1000
Disopyramide	> 1000
Ethosuximide	506
4-Hydroxypropranolol	> 1000
Lidocaine	730
Phenobarbitone	> 1000
Phenytoin	> 1000
Primidone	967
Procainamide	391
Propranolol	> 1000
Quinidine	900
Theobromine	467
Theophylline	556

RESULTS AND DISCUSSION

The use of an alkyl phenyl reversed-phase column at 50°C with 1.5 m*M* phosphoric acid—acetonitrile (95:5) as mobile phase resulted in an efficient separation of tocainide, the internal standard and plasma peaks (Fig. 1).

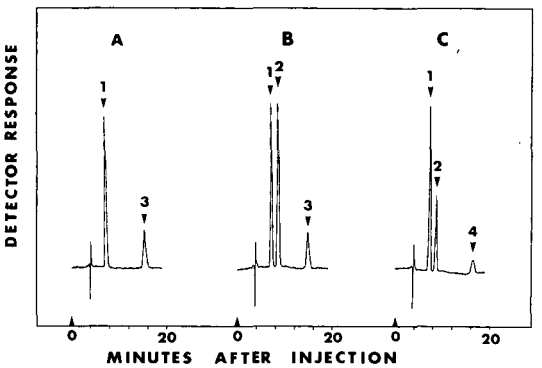


Fig. 1. Chromatograms obtained for the tocainide HPLC assay of (A) blank plasma, (B) plasma standard containing tocainide (60 μmol/l), and (C) plasma from a patient at steady-state containing tocainide (28 μmol/l). Peaks: 1 = internal standard, 2 = tocainide, and 3 and 4 = plasma peaks.

Recovery (69%) was linear over the concentration range 10—60 μmol/l (2—11.5 μg/ml). Monitoring the effluent at 230 nm allowed sufficient sensitivity for the detection of low plasma levels of tocainide with a detection limit of 1 μmol/l (0.2 μg/ml) (determined at a peak height of twice the noise level after injecting one-tenth of the total plasma extract). The assay was simple and rapid to perform requiring approximately 2 h for the processing of twenty patient samples and standards to the injection stage. The retention times of other drugs and metabolites directly injected into the chromatograph are listed in Table I. An alternative internal standard III was not used because of potential interference by lidocaine which had a similar retention time.

Using sodium hydroxide—borate buffer excellent inter- and intra-assay precision was obtained for the HPLC method (Table II). When the sodium hydroxide—borate buffer was replaced by sodium hydroxide in the HPLC assay, both inter- and intra-assay precision were considerably poorer (Table II)

TABLE II

COEFFICIENTS OF VARIATION (CV%) FOR THE TOCAINIDE ASSAYS

		HPLC*	HPLC**	GLC
Intra-assay CV%:	60 μmol/l	1	8	3
	40 μmol/l	3	6	3
	20 μmol/l	3	6	3
Inter-assay CV%:	40 μmol/l	5	19	15

*Using 1 *N* sodium hydroxide—0.6 *M* borate buffer.
**Using 1 *N* sodium hydroxide only.

without a change in recovery. Although the intra-assay reproducibility of the GLC tocainide assay was excellent, the inter-assay variation was poor. The relatively poor correlation obtained (Fig. 2) (r^2 = 0.81) for the HPLC and GLC assays is probably a reflection of the high inter-assay variability of the GLC method. The plasma level—time courses obtained for the HPLC assay of plasma samples from three patients taking tocainide (600 mg, 8-hourly) at steady-state are shown in Fig. 3. The method reported has adequate sensitivity and reproducibility for pharmacokinetic studies in man.

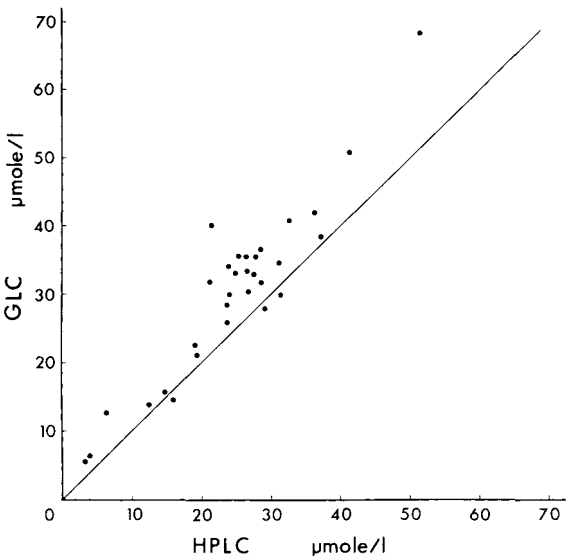


Fig. 2. Comparison of HPLC and GLC tocainide assays for 31 plasma samples taken from eight patients at steady-state. r^2 = 0.847, slope = 1.147, intercept = 1.491.

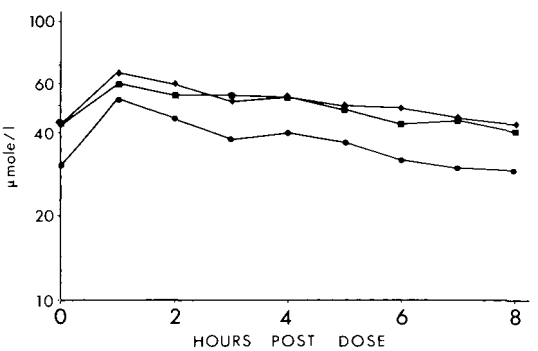


Fig. 3. Steady-state tocainide plasma levels obtained for the HPLC assay of samples from three subjects administered oral tocainide (600 mg, 8-hourly).

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CHROMBIO. 571

Note

Nachweis von Glibornurid im Serum durch Hochleistungsflüssigkeitschromatographie mit umgekehrten Phasen

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(Eingegangen am 13. November 1979; geänderte Fassung eingegangen am 4. Februar 1980)

Die oralen Antidiabetika, Derivate von Sulfonylharnstoff, sind eine Alternative zur Behandlung mit Insulin bei Altersdiabetes [1]. Zur Bestimmung von Blutspiegeln werden verwendet für Glibornurid die Flüssigkeitsscintillation [2], für Glibenclamid eine radioimmunologische Methode [3]. Schlicht et al. [4] beschrieben eine gaschromatographische Methode für die Antidiabetika Chlorpropamid, Tolbutamid, Carbutamid, Tolazamid und Glykodiazin. Auch die Hochleistungsflüssigkeitschromatographie wurde für den Nachweis von Tolbutamid und Chlorpropamid eingesetzt [5].

Unsere Problematik war die Spiegelbestimmung von Glibornurid im Serum von Patienten mit Altersdiabetes bei eingeschränkter Nierenfunktion. Für die Lösung dieses Problems haben wir die Hochleistungsflüssigkeitschromatographie verwendet.

MATERIAL UND METHODEN

Hochleistungsflüssigkeitschromatographie

Gerät: Hewlett-Packard Hochleistungsflüssigkeitschromatograph 1084B mit variablem UV-Detektor HP 79875 und automatischem Probengeber HP 79842A.

Säule: Hibar-Fertigstahlsäule (25 cm × 3 mm) mit C_8 Reverse-Phase-Material LiChrosorb (10 μm). Laufmittel: Methanol—Wasser (7:3).

Sonstige Bedingungen: Detektion bei 230 nm; Flussgeschwindigkeit 1.5 ml/min; Temperatur des Säulenraumes: 50°C.

Probenmaterial

Serum von Patienten, die therapeutisch Glibornurid erhalten haben. 1—3 ml werden mit Phosphatpuffer pH 3.0 (23.7 g $Na_2HPO_4 \cdot 2\ H_2O$ und 12 ml H_3PO_4 gelöst in 1 l Wasser) auf 20 ml aufgefüllt und dann auf eine Extraktionssäule Extrelut^R [6] gegeben. Die Säule wird mit 40 ml Dichlormethan—Isopropanol (85:15) eluiert. Die organische Phase wird eingedampft, in 200 µl Methanol aufgenommen und je nach Konzentration 10—50 µl eingespritzt, wobei von jeder Probe eine Doppelbestimmung durchgeführt wurde.

Eichung

Die quantitativen Bestimmungen wurden über einen externen Standard durchgeführt. Hierfür wurden Eichlösungen von 0.5 µg/ml und 2 µg/ml Glibornurid in Rinderserum hergestellt, die analog aufgearbeitet wurden. Mit dieser Art der Auswertung haben wir gute Erfahrungen gemacht. Sollte ein interner Standard benötigt werden, kann hierfür Gliquidon verwendet werden.

ERGEBNISSE

Fig. 1 zeigt Leerserum, Fig. 2 einen Zusatz von Glibornurid und Gliquidon zu Serum. Fig. 3 zeigt das Serum eines Patienten mit 1.16 µg/ml Glibornurid (bestimmt über externe Standardisierung) nach Einnahme von 25 mg Glutril^R

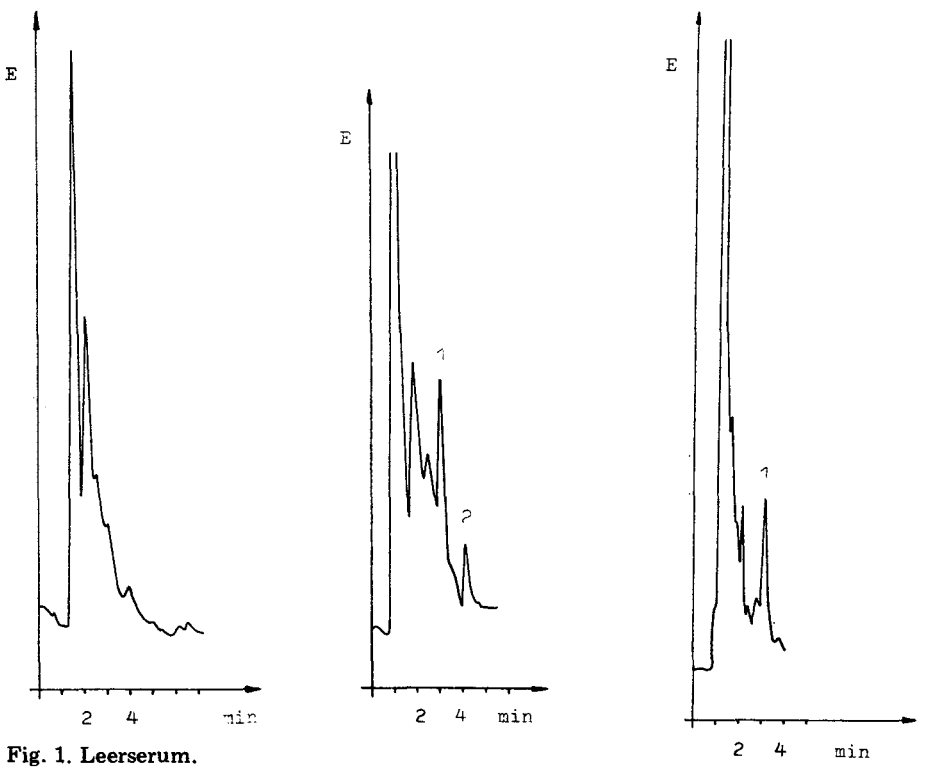

Fig. 1. Leerserum.

Fig. 2. Zusatz von 2.5 µg/ml Glibornurid (1) und 2.6 µg/ml Gliquidon (2) zum Serum.

Fig. 3. Patientenserum mit 1.16 µg/ml Glibornurid (1).

ca. 8 Stunden zuvor. Die Erfassungsgrenze für Glibornurid im Serum betrug unter den beschriebenen Bedingungen 0.5 μg/ml.

Die Eichgerade war von 0.5 μg/ml bis 5 μg/ml linear. Die Standardabweichung betrug 3% (n = 6).

DISKUSSION

Mit der Methode können ohne weiteres therapeutische Spiegel von Glibornurid erfasst werden, die zwischen 1.6 und 3.4 μg/ml liegen [2]. Das Maximum der UV-Extinktion in Methanol von Glibornurid liegt bei 212 nm. Trotz der dadurch bedingten geringeren Empfindlichkeit wurde die Wellenlänge 230 nm gewählt, da bei niedrigen Wellenlängen Störpeaks mit derselben Retentionszeit wie Glibornurid auftreten können.

DANK

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CHROMBIO. 574

Note

Determination of the diuretic bumetanide in biological fluids by high-performance liquid chromatography

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Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamoylbenzoic acid, Fig. 1) is a potent high-ceiling diuretic which is, on a weight for weight basis forty times more potent than frusemide [1]. Since the usual therapeutic dose of bumetanide is between 1 and 3 mg per day, resulting in peak serum levels of 10—120 ng ml^{-1}, a sensitive assay procedure is required to examine its pharmacokinetics in man.

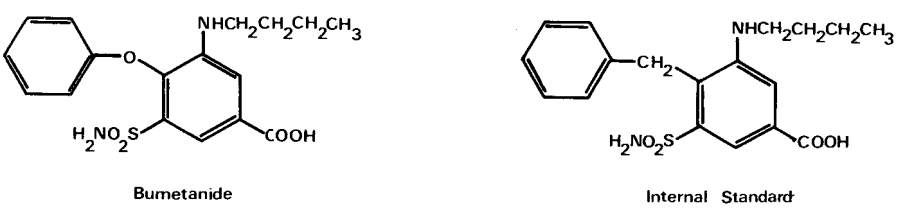

Fig. 1. Structural formulae of bumetanide and 4-benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard).

Fluorimetric [2], gas—liquid chromatographic [3], radiometric [4] and radioimmuno [5] assays have been reported for the determination of bumetanide in biological fluids. However, all these methods have inherent disadvantages. Since bumetanide is partially metabolised in man [4] a specific and sensitive quanti-

tative method is required for measuring its concentration in biological fluids.

The paper describes a quantitative high-performance liquid chromatographic (HPLC) method for the determination of bumetanide in biological fluids using a fluorescence detector and an internal standard.

MATERIALS AND METHODS

Chemicals and reagents

Bumetanide was supplied by Leo Labs. (Hayes, Great Britain). 4-Benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard, Fig. 1) was supplied by Leo Pharmaceutical Products (Copenhagen, Denmark). All reagents and solvents were AnalaR grade (BDH, Poole, Great Britain) except the methanol which was HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Standard solutions of bumetanide (10—120 ng ml^{-1}) and internal standard (100 ng ml^{-1}) were prepared in water.

High-performance liquid chromatography

A Waters Assoc. high-performance liquid chromatograph equipped with a Model 6000A pump, UK6 injector and a Model 420 fluorescence detector was used. The column (30 cm × 0.39 cm I.D.) was prepacked with 10-μm ODS-silica (μBondapak C_{18}, Waters Assoc.). The mobile phase was methanol—water—acetic acid (70:30:1), pH 2.9 delivered isocratically at the rate of 1.5 ml min^{-1} at ambient temperature. The excitation filter wavelength was 340 nm and the emission filter wavelength, 425 nm. A pre-column packed with Bondapak C_{18}/Corasil (Waters Assoc.) was used to increase the life of the column.

Extraction procedure and sample preparation

Serum (1 ml) was placed in a glass stoppered test-tube along with internal standard (0.3 ml), phosphate—citrate buffer pH 5 (2 ml) and potassium chloride (0.5 g). For the calibration standards 1 ml of the buffer was replaced with a standard bumetanide solution (1 ml, 30 ng ml^{-1}). The mixture was extracted with diethyl ether (6 ml) by mechanically shaking for 2 min. The resultant mixture was centrifuged for 10 min at 1060 *g*, and the diethyl ether phase transferred to a tapered test-tube. A second volume of diethyl ether (5 ml) was added to the aqueous phase, and the extraction step repeated. The combined diethyl ether phases were evaporated to dryness on a water-bath at 50°C. The residue was reconstituted with mobile phase (120 μl), centrifuged and injected on to the column.

Similarly, urine (0.2 ml) was placed in a stoppered test-tube along with internal standard (0.6 ml), phosphate—citrate buffer pH 5 (2 ml), water (0.5 ml) and potassium chloride (1 g), and extracted as for serum. For the urine calibration standard 1 ml of buffer was replaced with standard bumetanide solution (1 ml, 60 ng ml^{-1}).

Calibration curves and extraction yields

For the quantitative determination of bumetanide in serum and urine, standard curves were prepared by adding either bumetanide (10—60 ng) and internal standard (30 ng) to blank serum or bumetanide (20—120 ng) and internal

standard (60 ng) to blank urine. The samples were extracted and chromatographed as described above and the peak height ratios plotted against the bumetanide concentration.

The recovery of bumetanide and internal standard after either a single extraction or a double extraction with diethyl ether was obtained by adding a constant amount of each to either blank serum or blank urine and treating them in the same way as the calibration standards.

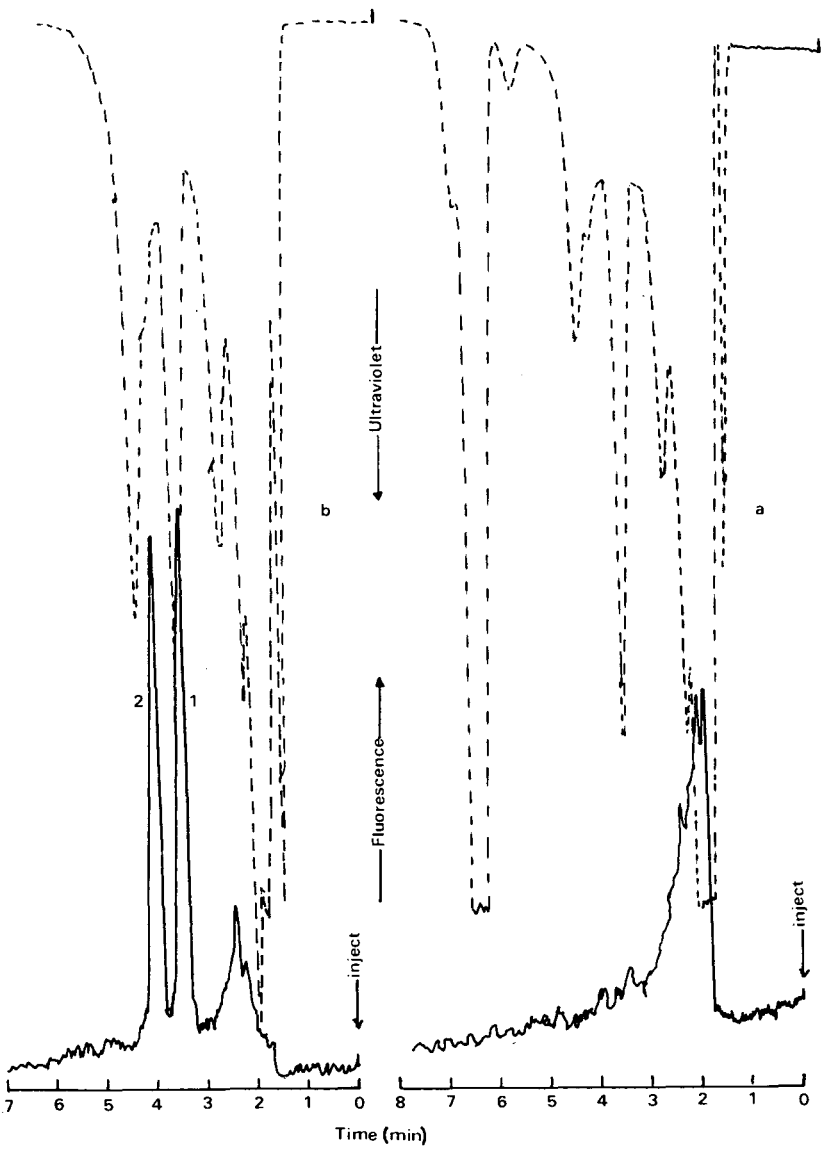

Fig. 2. HPLC chromatograms of (a) serum blank and (b) bumetanide (1) and internal standard (2) recovered from serum. Conditions as described in Materials and Methods. Detection: UV — — —; fluorescence ——.

RESULTS AND DISCUSSION

The advantage of fluorescent detection over UV detection in liquid chromatography is increased specificity, resulting in increased sensitivity since UV absorbing endogenous materials which are co-extracted with the drug and internal standard are not detected with the fluorescence detector (Fig. 2). Although the emission maxima for bumetanide and 4-benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard) obtained with an excitation wavelength of 340 nm are 446 nm and 442 nm respectively, the wavelengths used in the method were dictated by the choice of filters available with the instrument.

Using a reversed-phase C_{18} system, bumetanide and the internal standard are well resolved from the void volume peak in extracts from either serum (Fig. 2) or urine (Fig. 3d). The retention times for bumetanide and internal standard are 3.6 and 4.3 min respectively, and it has been demonstrated that the desbutyl and aliphatic acid metabolites [4] are eluted in the void volume (unpublished results) with the HPLC system described in this paper.

An improved extraction yield was obtained for both bumetanide and internal standard from serum with a second extraction step (Table I) thus increasing the sensitivity of the method. It was not necessary to carry out a double extraction from urine since the extract yield for bumetanide was 95%. The reproducibility of the method based on ten replicate analyses of bumetanide (9 ng

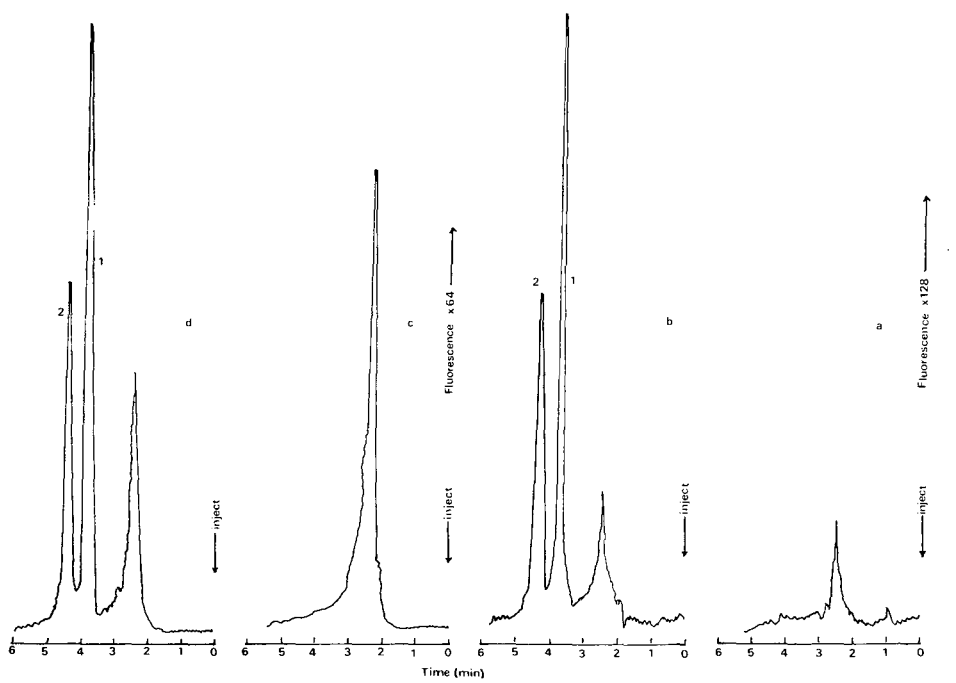

Fig. 3. Chromatograms of serum and urine samples from a subject who received bumetanide (1 mg) orally. (a) Control serum extract; (b) test serum extract; (c) control urine extract; (d) test urine extract. Peaks: 1 = bumetanide; 2 = internal standard. Conditions as described in Materials and Methods.

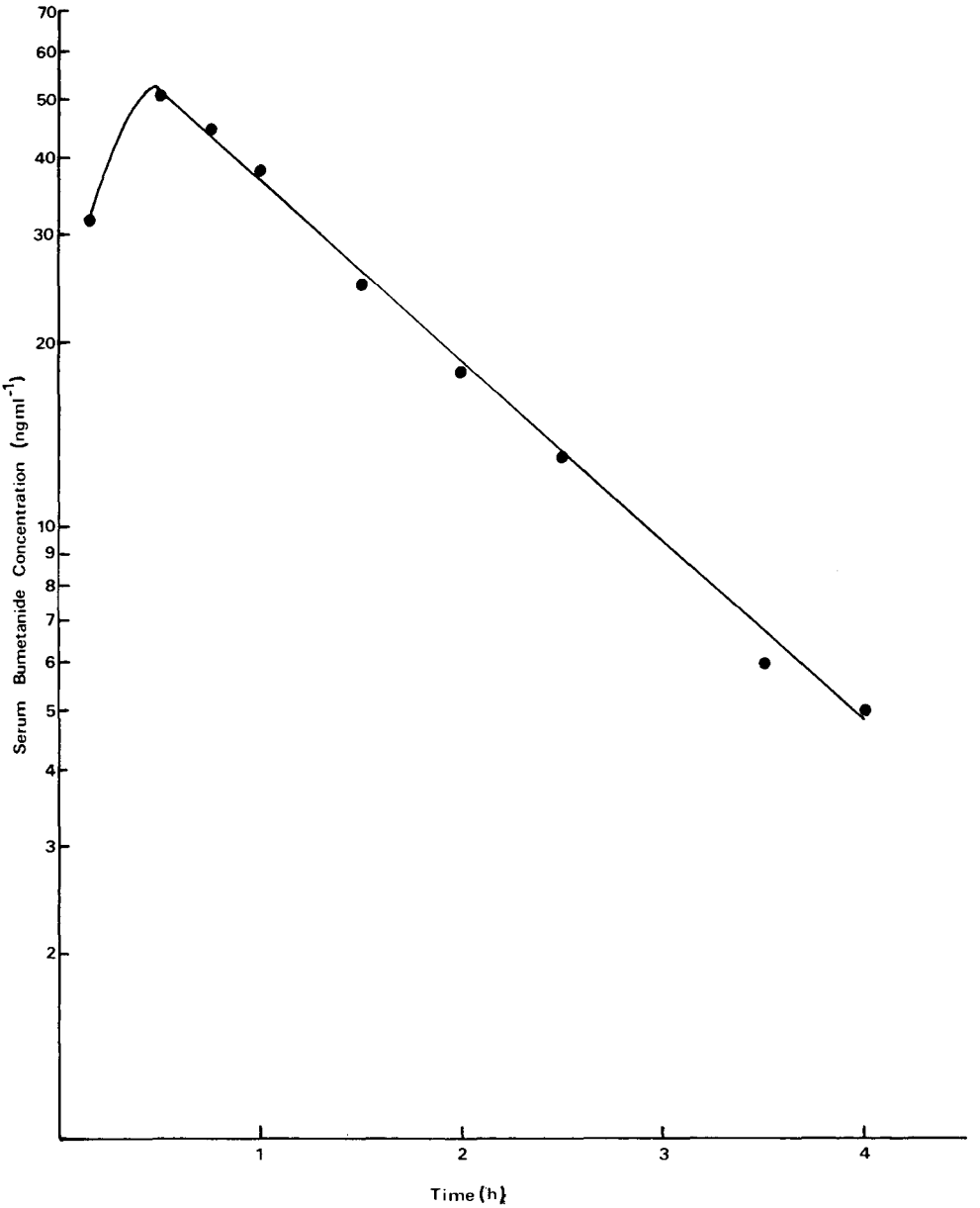

Fig. 4. Serum concentration—time curve for bumetanide assayed in duplicate.

ml^{-1}) in serum and bumetanide (66 ng ml^{-1}) in urine was 5.2% and 3.2% respectively. There was a rectilinear relationship between the peak height ratio and bumetanide concentration up to 60 ng ml^{-1} in serum and 120 ng ml^{-1} in urine.

Fig. 3 shows typical chromatograms obtained from serum and urine extracts from a subject who had received 1 mg of bumetanide orally. The biological half-life for bumetanide for this subject calculated from the serum concentration—time curve was 1 h (Fig. 4). Of the dose administered 52% was excreted in 24 h.

TABLE I

RECOVERIES OF BUMETANIDE AND 4-BENZYL-3-*n*-BUTYLAMINO-5-SULPHAMOYL-BENZOIC ACID (INTERNAL STANDARD) FROM SERUM AND URINE

$n = 6$.

	Extraction yield (%) (mean ± S.D.)	
	Bumetanide	Internal standard
Serum		
single extraction	71 ± 7	73 ± 7
double extraction	91 ± 7	86 ± 6
Urine		
single extraction	95 ± 6	89 ± 5

ACKNOWLEDGEMENTS

We are grateful to Dr. Ross Dixon of Hoffman-La Roche, Nutley, NJ, U.S.A. for his advice on establishing conditions for the assay.

We would like to thank Leo Laboratories, Middlesex for their financial support and for supplying bumetanide, and Dr. P.W. Feit of Leo Pharmaceutical Products, Denmark for supplying 4-benzyl-3-*n*-butylamino-5-sulphamoyl-benzoic acid. We are indebted to Dr. W.R. Murdoch for obtaining specimens of body fluids for analysis.

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CHROMBIO. 577

Note

Fluorimetrische Bestimmung von Xipamid in biologischem Material mit einem neuen Fluoreszenzreagenz

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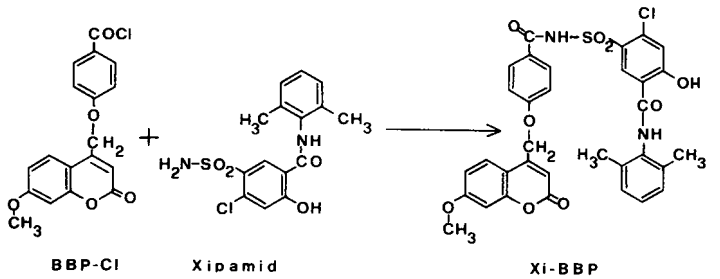
Für die Bestimmung von Xipamid (4-Chlor-5-sulfamoyl-2,6-salicyloxylidid), einem Saluretikum mit thiazidartiger Wirkung aus der Sulfonamidreihe, in biologischem Material setzten Hempelmann und Dieker [1] bei pharmakokinetischen Untersuchungen ^{35}S-markiertes Xipamid ein. Da nur die Aktivität des ^{35}Schwefels gemessen wird, ist eine sorgfältige und aufwendige Abtrennung der Metaboliten notwendig.

Andere Verfahren für die Xipamid-Bestimmung sind in der Literatur bisher nicht beschrieben. Da die oben genannte Methode bezüglich ihrer Anwendbarkeit nicht voll befriedigt und die Verwendung von radioaktivem Material nur in der Einführungsphase eines Arzneistoffs möglich ist, versuchten wir eine neue Bestimmungsmethode für Xipamid zu entwickeln. Dazu wurde ein neues, von uns synthetisiertes Fluoreszenzreagenz [4-(7-Methoxy-2-oxo-2H-benzopyran-4-ylmethoxy)-benzoylchlorid] verwendet, über dessen Synthese und Eigenschaften an anderer Stelle [2] berichtet wird. Dieses bildet mit Xipamid ein Benzoesäure-benzolsulfonylamid, dessen Struktur durch NMR-Untersuchung gesichert wurde. Die Reaktion in Aceton—Kaliumcarbonat führt zu einem fluoreszierenden Produkt, welches nach dünnschichtchromatographischer Abtrennung quantitativ direkt auf der Platte gemessen wird. Das Verfahren lässt sich sowohl bei Plasma als auch Urin anwenden.

Die Vorteile der Methode liegen in einem geringen Verbrauch an Analysenmaterial (0.1—1 ml), einer niedrigen Nachweisgrenze (< 10 ng/ml), der chromatographischen Abtrennung störender Bestandteile sowie in der einfachen Handhabung.

*Teilergebnisse der Dissertation M. Sobel, in Vorbereitung.



METHODIK

Geräte

Chromatogramm-Spektralfotometer KM 3 (Carl Zeiss, Oberkochen, B.R.D.), mit Perkin-Elmer-Recorder 56 und Perkin-Elmer-Integrator M3-B; Linomat III Camag; NMR-Perkin-Elmer R 32 90 MHz; IR Acculab 2, Beckmann, KBr; Schmelzpunkt-Bestimmungsapparat Büchi.

Chemikalien

Xipamid stellte freundlicherweise die Fa. Beiersdorf, Hamburg, B.R.D., zur Verfügung. Das Reagenz [4-(7-Methoxy-2-oxo-2H-benzopyran-4-ylmethoxy)-benzoylchlorid; BBP-Cl] wurde wie in Lit. 2 beschrieben synthetisiert. Die restlichen Chemikalien einschliesslich der Dünnschicht-Fertigplatten (Kieselgel 60, 20 × 20 cm, ohne Fluoreszenzindikator) wurden von E. Merck, Darmstadt, B.R.D., bezogen. Als Lösungsmittel für die Reagenzlösung wurden Toluol Uvasol^{R} und Aceton Uvasol^{R} verwendet. Die anderen Chemikalien hatten den Reinheitsgrad p.a.

Synthese des Xipamidderivates (Xi-BBP)

200 mg Xipamid (M.G. 354.83), 100 mg BBP-Cl (M.G. 344.8) und 500 mg $NaHCO_3$ werden in 100 ml trockenem Aceton bei Raumtemperatur bis zur völligen Umsetzung des Säurechlorids gerührt [3]. Wenn die Reaktion beendet ist, wird zum Sieden erhitzt, abgesaugt, mit heissem Aceton nachgewaschen und bis zur ersten Trübung eingeengt. Die entstehenden weissen Kristalle werden mehrmals aus Aceton, Toluol und Methanol—Wasser (7:3) umkristallisiert.

Fliessmittel: Toluol–Essigsäureethylester (1:1, v/v) R_F = 0.45. F.P. = 226°C.

$C_{33}H_{27}N_2O_9ClS$: Ber.: C 59.7, H 4.1, N 4.2; Gef.: C 59.85, H 4.36, N 4.4%.

^{1}H-NMR: DMSO-d6, δ ppm, TSP; 2.18 (s, 6H, $(-CH_3)_2$); 3.90 (s, 3H, -O-CH_3); 5.42 (m, 2H, -CH_2-); 6.40 (s, 1H, -CO-NH-); 6.46 (m, 1H, =CH-); 6.76 (m, 1H, Xipa.-C-3); 6.90—7.32 (m, 7H, -C_6H_4-, -C_6H_3); 7.75—8.03 (m, 3H, Benzopyran 5-,6-,8-H); 8.48 (s, 1H, Xipa.C-6); 13.25 (s, 1H, -OH).

IR (cm^{-1}): 3290 (-OH); 1725 (-CO-O-C); 1650 (-CO-NH-); 1165 (-SO_2-N).

Reagenzlösung

Stammlösung: 5 mg BBP-Cl werden in 50 ml Toluol gelöst. Die Lösung ist im Kühlschrank vier Wochen haltbar. Diese Lösung wird zur Anwendung 1:5 mit Aceton verdünnt. Liegt der Gehalt der Probe unter 100 ng, wird diese

Lösung nochmals 1:5 mit Aceton verdünnt. Die verdünnte Lösung ist jeweils frisch zu bereiten. Die Konzentration beträgt 20 bzw. 4 μg Säurechlorid (gleich 58 bzw. 11.6 nmol) pro ml.

Probenaufarbeitung für Plasma und Urin

In einem verschliessbaren Zentrifugenglas werden 1 (0.1) ml Plasma oder Urin mit 1 ml Pufferlösung pH 5 (Citrat—Natronlauge-Puffer, Merck) und 4 ml Essigester [4] versetzt, 1 h maschinell geschüttelt und scharf zentrifugiert. Mit einer Pipette werden von der organischen Phase 1.0—3.0 ml abgenommen und in einem zweiten verschliessbarem Glas unter Stickstoffbegasung zur Trockne eingedampft.

Der Rückstand wird in 1.0 ml Reagenzlösung gelöst, mit einer Spatelspitze wasserfreiem Na_2CO_3 versetzt und 3 h unter Vermeidung von direktem Lichteinfall bei Raumtemperatur geschüttelt. Liegt der Gehalt der Probe über 1000 ng, so ist entsprechend zu verdünnen.

Wiederfindungsgerate

Um Fehler bei unterschiedlicher Raumtemperatur durch die Flüchtigkeit des Essigesters auszugleichen, wird bei jeder Probenserie die Wiederfindungsrate bestimmt. Zu 1 ml gepooltem Leerplasma (Leerurin) wird 1 μg Xipamid (0.1 ml einer Lösung 1 mg auf 100 ml Wasser) zugesetzt. Die Bestimmung erfolgt wie oben beschrieben.

Chromatographie

Mit dem Linomaten III werden 30 μl der Probe strichförmig (10 mm) auf eine DC-Platte aufgetragen. Pro Platte werden acht Proben und, als Standard, dreimal 30 ng Xi-BBP (= 16.05 ng Xipamid), in Aceton gelöst, aufgetragen. Die Chromatographie erfolgt mit dem Fliessmittel Toluol—Essigester (1:1, v/v) unter Kammersättigung bei einer Laufstrecke von 12 cm. Die entwickelten Chromatogramme werden mit Warmluft getrocknet. Der R_F-Wert beträgt 0.45. Fluoreszierende Begleitstoffe, überschüssiges Reagenz und die teilweise entstandene Carbonsäure sind vollkommen abgetrennt.

Messung

Die Fluoreszenzmessung erfolgt in der Anordnung: Lampe—Monochromator—Probe—Sekundärfilter—Empfänger.

Exzitation: Linie 313 nm der Hg-Mitteldrucklampe ST 41; Spaltgrösse 1.5 × 8 mm.

Emission: Monochromatfilter M 405 als Falschlicht-Filter; Verstärkung 10—100, Hochspannung am Fotoelektronenvervielfacher 1.

Bei hoher Verstärkung muss das Ausgangssignal für den Integrator 1:2 oder 1:4 abgeschwächt werden. Die Messung erfolgt in Fliessrichtung des Chromatogramms bei einer Tischgeschwindigkeit von 50 mm/min, die Aufnahme der Fluoreszenzintensitäts—Ortskurve mit einem Papiervorschub von 120 mm/min. Die Bestimmung der slope—sensitivity des Integrators wird auf einem Leerfleck im unteren Teil des Chromatogramms 30 sec lang durchgeführt. Fig. 1 zeigt die Fluoreszenzintensitäts—Ortskurven der Chromatogramme eines Plasmas (A) mit 100 ng Xipamid pro ml und eines Plasmas (B) ohne Xipamid.

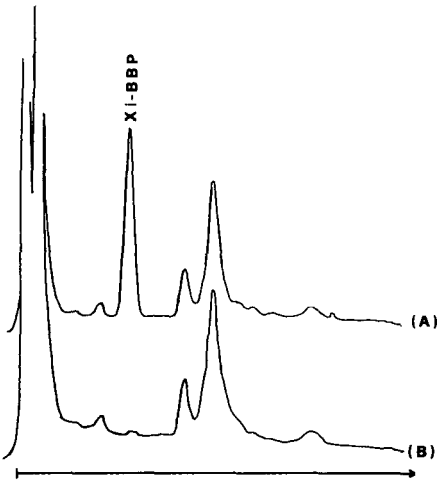


Fig. 1. Fluoreszenzintensitäts—Ortskurven der Chromatogramme eines Plasmas (A) mit 100 ng Xipamid pro ml und eines Plasmas (B) ohne Xipamid.

Auswertung

Die Auswertung erfolgt über die Flächen der Fluoreszenz—Ortskurven. Zwischen den Flächen unter den Kurven und den aufgetragenen Substanzmengen Xipamidumsetzungsprodukt besteht Linearität von 1 bis 500 ng pro Fleck. Die sich ergebende Gerade geht durch den Nullpunkt des Koordinatensystems. Um zu zeigen, dass diese Aussage auch für Plasma bzw. Urin richtig ist, wurden je dreimal 100 ng, 500 ng und 1000 ng Xipamid pro ml Plasma bzw. Urin bestimmt. Die neun Proben wurden auf einer DC-Platte bestimmt. Der Mittelwert aus drei Bestimmungen ergab eine durch den Ursprung des Koordinatensystems gehende Gerade. Daraus ergibt sich, dass die Eichgerade zur Bestimmung des Gehaltes und der Wiederfindungsrate aus dem Nullpunkt und einem einzigen Kurvenpunkt erstellt werden kann. Dieser wird zur Erhöhung der Genauigkeit aus drei Messwerten gemittelt.

ERGEBNISSE UND DISKUSSION

Der Strukturbeweis für eine Bildung des Benzoesäure-benzolsulfonylamids wurde durch NMR-Untersuchung erbracht: Nach Zusatz von ^2H_2O liessen sich die Signale zweier austauschbarer Protonen (6.40; 13.25) nachweisen. Das dritte bewegliche Proton war infolge Überlagerung durch andere Signale nicht identifizierbar. Die Flächenintegrale verhalten sich wie 1:1. Bei freier Sulfonamidgruppe müsste aber ein Verhältnis von 2:1 auftreten. Der Vergleich der Spektren von Xipamid und Xipamidmethylether zeigte, dass das Signal bei 13.25 ppm dem Proton der freien phenolischen Hydroxylgruppe von Xi-BBP zuzuordnen war. Ein weiterer Hinweis für die Substitution an der Sulfonamidgruppe und damit für das Vorliegen der unsubstituierten phenolischen OH-Gruppe war die Lage des Signals für das Proton an C-3 des Xipamidfragmentes. Dieses Proton erscheint bei ähnlicher Lage (d = 6.76 ppm) wie beim Xipamid selbst (d = 6.54 ppm). Eine Substitution der freien phenolischen Hydroxyl-

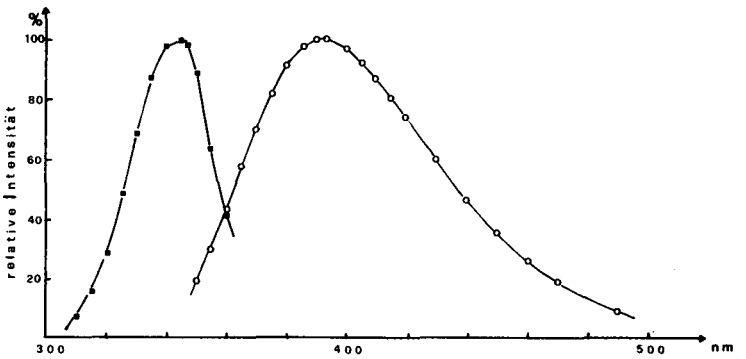


Fig. 2. Exzitations- (▪) und Emissionsspektrum (○) des Umsetzungsproduktes Xi-BBP.

gruppe des Xipamids zum entsprechenden Methylether verschiebt das Signal des Protons an C-3 zu wesentlich tieferem Feld (d = 7.50 ppm).

Zur Bestimmung der Präzision des Verfahrens wurden verschiedene Konzentrationen Xipamid gepooltem Leerplasma zugesetzt. Bei einer Konzentration von 1000 ng Xipamid pro ml Testplasma war die relative Standardabweichung 0.7%, bei 100 ng/ml 2.1% und bei 20 ng/ml 6.5% (jeweils n = 8). Die Wiederfindungsrate liegt bei 100%. Geringe Schwankungen ergeben sich durch die Flüchtigkeit des Essigesters. Deshalb sollte bei jeder Bestimmungsserie die Wiederfindungsrate mitbestimmt werden. Der Gehalt der Proben wird dann darauf bezogen.

Die Nachweisgrenze in dem beschriebenen Verfahren liegt bei < 10 ng/ml Plasma, wenn die Bestimmung mit maximal 1 ml Plasma ausgeführt wird. Um tiefer liegende Werte zu erfassen, ist Xipamid aus entsprechend mehr Plasma zu extrahieren.

Fig. 2. zeigt das Exzitations- und Emissionsspektrum des Umsetzungsproduktes (Xi-BBP). Die Maxima liegen bei 342 nm und 394 nm. Die Anregung mit der 313-nm-Linie liegt in einem relativ ungünstigen Bereich. Durch Einsatz eines Falschlichtfilters mit einer sehr steilen Flanke könnte auch mit der 365-nm-Linie der ST 41 Lampe angeregt und dadurch eine höhere Fluoreszenzausbeute erreicht werden.

Eine Überprüfung der Bestimmungsmethode wurde durch Analyse von Plasma und Urin zweier gesunder Probanden durchgeführt.

Nach einer Venenblutentnahme (Leerwert) erhielten die Probanden 80 mg Xipamid (2 Tabletten Aquaphor^{R}) oral. In den darauffolgenden 48 Stunden wurden 10 Blutproben entnommen. Das heparinisierte Blut wurde unter Zusatz von "Trennmittel Merck" zentrifugiert, das Plasma abgenommen, bei −18°C eingefroren und unmittelbar vor der Bestimmung aufgetaut. Daneben wurde über 48 Stunden Urin gesammelt.

Fig. 3 zeigt die Plasmaspiegelverläufe der beiden Probanden. Der maximale Plasmaspiegel war in 30—60 min erreicht. Fig. 4 gibt die kumulative Urinausscheidungskurve der Probanden wieder. Nach 48 Stunden (Ende der Beobachtungsdauer) sind 39% bzw. 49.8% der Dosis als unverändertes Xipamid ausgeschieden. Das gleichzeitig als Hauptmetabolit [1] ausgeschiedene Glukuronid wird bei dieser Bestimmung nicht erfasst.

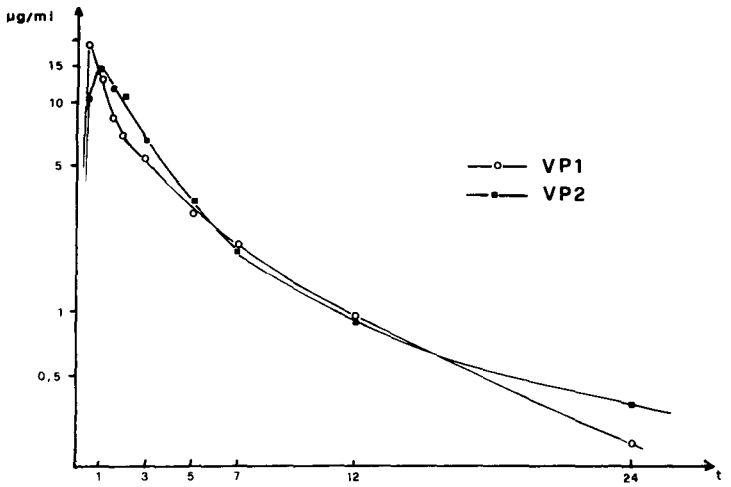


Fig. 3. Plasmaspiegelkurven nach oraler Gabe von 80 mg Xipamid; Zeit in Studen in linearer Darstellung und Konzentration in µg/ml in logarithmischer Darstellung.

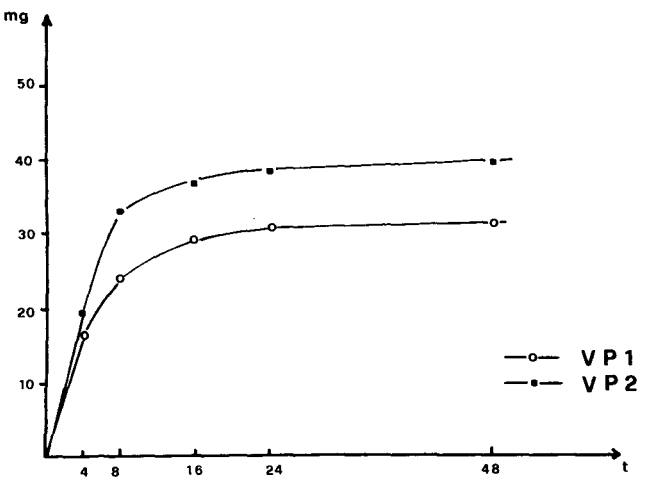


Fig. 4. Kumulierte renale Ausscheidung von Xipamid nach oraler Gabe von 80 mg; Zeit in Stunden.

Die Ergebnisse zeigen, dass die Methode geeignet ist, die Plasma- und Urinkonzentration auf einfache Weise zu bestimmen. Die Nachweisgrenze von 10 ng/ml lässt auch bei geringer Dosierung eine sichere Bestimmung zu.

DANK

H. Knauf, Freiburg, B.R.D., danken wir für die Überlassung der Proben.

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PUBLICATION SCHEDULE FOR 1980

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

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	The publication schedule for further issues will be published later.			
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2				

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