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CHROMBIO. 130

GASCHROMATOGRAPHISCHE ANALYSE VON SAUREN URINMETABOLITEN NACH TRENNUNG AUF KIESELGELFERTIGSÄULEN

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(Eingegangen am 6. September 1977; geänderte Fassung eingegangen am 25. November 1977)

SUMMARY

Gas chromatographic estimation of acidic urinary metabolites after separation on prepacked silica gel columns

The acidic ethylacetate extracts of 24-h urine specimens are evaporated and redissolved in chloroform—methanol—acetic acid. The resulting solution is transferred to a prepacked silica gel column. Elution takes 160 min using a specially designed chloroform—methanol—acetic acid gradient. The eluate is divided into fractions (16 min each) which are evaporated to dryness. The residues are silylated and determined quantitatively by gas chromatography. The capacity of the silica gel column allows analysis of 30% of a 24-h urine specimen. In consequence, metabolites can be quantitated at concentrations less than 1 mg per 24 h. The method is suitable to obtain more detailed metabolic profiles of the carboxylic acids in urine.

EINLEITUNG

Zur Diagnose einer Reihe von erblichen Stoffwechselstörungen wie Phenylketonurie, Tyrosinämie, Ahorn-Sirupkrankheit und zur Diagnose von einigen Tumoren des Nervensystems versucht man eine oder einige wenige organische Säuren aus dem Urin quantitativ zu bestimmen. Im Krankheitsfalle kann man mit extrem erhöhten Konzentrationen rechnen. Dies ermöglicht eine problemlose gaschromatographische (GC) Analyse, der meist eine Extraktion des Urins vorangeht.

Daneben gibt es Stoffwechselsituationen, in denen man nur ganz geringfügige Abweichungen des metabolischen Profils beobachtet, beispielsweise bei varianten Formen der erwähnten Aminosäurenabbaustörungen. Hier erweist es sich als zweckmässig, möglichst viele Metaboliten der betroffenen Aminosäuren quantitativ zu erfassen [1].

Für die GC-Analyse enststehen damit einige Probleme. Man muss äusserst geringe Konzentrationen erfassen (zum Teil < 1 mg/l). Die Vielzahl der Ver-

bindungen lässt selbst die GC mit der Glaskapillare oft an ihre Grenzen stossen. Man steht manchmal vor der Aufgabe subnormale Ausscheidungen im angegebenen Konzentrationsbereich nachzuweisen. Dabei hat man mit allen Schwierigkeiten der Spurenanalyse zu kämpfen. Produkte des Bakterienstoffwechsels schliesslich können das Bild noch komplizierter machen.

Konsequenterweise gehen einige Autoren dazu über, die blosse Extraktion des Urins durch wirkungsvollere Vorreinigungsschritte zu ersetzen, um so die GC zu entlasten. Horrocks et al [2] fraktionieren 5 ml Urinportionen an kleinen Ionenaustauschersäulen. Melchert und Hoffmeister [3] reinigen Essigesterextrakte von Urin auf dem lipophilen Wege LH 20 vor. Diese Arbeit setzt sich mit einem für die Urinanalyse bezeichnenden Problem auseinander: Es soll die Vanillinmandelsäure auch bei Normalpersonen bestimmt werden, deren Ausscheidungswerte liegen um 1 mg/l. Von dieser Substanz ist in keinem GC-System mit ausreichender Sicherheit die Hippursäure zu trennen. Deren Konzentration im Urin beträgt gelegentlich bis zu 1 g/l.

Die hier beschriebene Methode soll ein Beitrag zur Lösung dieser Probleme liefern. Ich habe versucht, die schnelle Vortrennung eines Urinextraktes im Praeparativmasstab zu erreichen (30% eines 24-h-Urins). Durch das gleichzeitige Verwenden zweier chromatographischer Systeme konnte hohe Nach weisempfindlichkeit zusammen mit hoher Trennleistung erreicht werden.

METHODE

Vorbereitung von Eichmessungen

Ungefähr 5 mg jeder Karbonsäure wurden in 60 ml Methanol gelöst. Zur Ermittlung einer Eichkurve wurden jeweils 2 × 8 ml, 5 ml, 4 ml, 3 ml und 2 ml abpipettiert, abgedampft und in 5 ml Methanol wieder aufgenommen. Das ganze Verfahren wurde zweimal durchgeführt.

Vorbereitung von Urinproben

24-Stunden-Urine wurden ohne Zusatz von Konservierungsmitteln gesammelt. Während der 24-h Sammlungsperiode wurden die Urine eingefroren und am folgenden Tag analysiert. Für eine spezielle Fragestellung haben wir insgesamt 40 24-Stunden-Urine analysiert. Es wurde dabei jeweils 30% des Gesamturins wie im folgenden beschrieben extrahiert. Der Urin wird auf pH 1—2 angesäuert, 1:1 mit gesättigter Natriumchlorid-Lösung versetzt und zweimal mit der doppelten Menge Essigsäureäthylester ausgeschüttelt. Die Lösung wurde über Natriumsulfat getrocknet und eingedampft. Als Kontrollbestimmung wurden bei jeder Harnanalyse 5% des Essigesterextraktes entnommen und direkt für die GC vorbereitet. Der Trockenrückstand wurde in einem 100 ml Spitzkolben mit dem unpolaren Anteil des Lösungsmittelsystems (siehe unten) versetzt und unter 2-stündigem Rühren gelöst.

Die Säulenchromatographie

Als eigentliches Trennsystem wurde eine Kieselgelfertigsäule (Grösse B) der Fa. Merck Kieselgel 60 verwendet. Dieser war eine weitere mit Kieselgel 60 (Korngrösse 0.2—0.5 mm; 30—70 mesh ASTM; Merck) gefüllte Säule vorgeschaltet (15 × 1 cm). Das Pumpsystem mit Pulsdämpfung war von der Fa.

CFG Heidelberg. Die Photometereinheit war das Uvicord-System II, der Fraktionssammler vom Typ Ultrarac 7000, beide von der Fa. LKB. Letzterer wurde so modifiziert, dass es möglich war, 10 Fraktionen mit beliebig grossen Volumina aufzufangen. Ich habe wegen der grossen anfallenden Probenzahl in unserem Experiment eine möglichst weit gehende Automatisierung mit dem LKB Gradientenmischer Ultrograd 11300 angestrebt. In einem früherem Stadium habe ich jedoch gleich gute Trennleistung mit einem selbstgebauten einfachen Gradientenmischer aus zwei Gefässen (linearer Gradient) erreicht. Betriebsbedingungen der Fertigsäule: Durchfluss, 5 ml/min; Druck, 4 atm (max); mobile Phase, (unpolarer Anteil) 600 ml Chloroform, 6 ml Methanol, 0.8 ml Essigsäure und (polarer Anteil) 400 ml Chloroform 75 ml Methanol, 25 ml Essigsäure.

Die Form des Gradienten ist Fig. 1 zu entnehmen. Die Vorsäule ist über einen magnetisch betriebenen Dreiwegehahn mit der Trennsäule verbunden. Nach 100 min wird dieser Hahn vom Ultrograd-Steuergerät umgestellt, so dass die mobile Phase nicht mehr über die Vorsäule sondern direkt auf die Fertigsäule gepumpt wird. Diese wird so auch bei sehr häufigem Gebrauch von starker Verschmutzung frei gehalten. Die Füllung der Vorsäule wird für jede Analyse erneuert.

Aufgabe der Proben

Das Probenvolumen war immer 5 ml. Die Aufgabe erfolgte mit einer 500 µl SGE-Spritze durch ein handelsübliches Septum für die GC in einen Injektionsblock aus V4A-Stahl.

Vorbereitung der Probe für die Gaschromatographie

Die einzelnen Fraktionen werden in Rundkolben aufgefangen und am Ro-

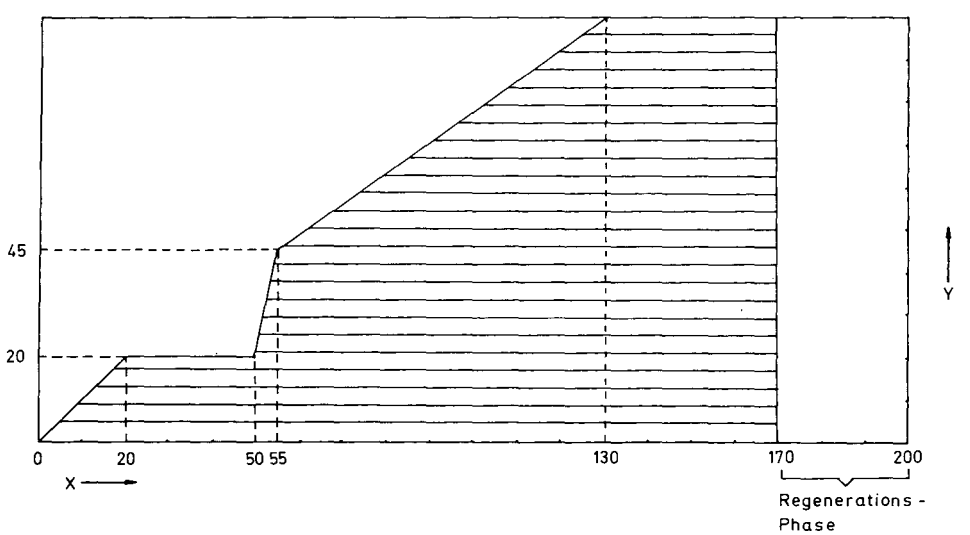


Fig. 1. Programmkarte für den Gradientenmischer. Der Verlauf der Grenzlinie zwischen schraffiertem und nicht schraffiertem Gebiet stellt die Änderung der Konzentration der mobilen Phase in der Zeiteinheit dar. x-Achse: 4 h, y-Achse: 7.5 sec.

tationsverdampfer eingedampft. Durch zwei bis dreimaliges Zusetzen von Essigsäureäthylester und anschliessendes Einrotieren gelingt es, auch Spuren von Essigsäure aus der Probe zu entfernen. Der Trockenrückstand wird mit 1 ml Reaktionslösung versetzt (10 ml Chloroform, 20 ml Acetonitril, 10 ml BSA, 60 µl Tetradecan). Die Proben wurden 1 h auf 40° erhitzt.

Gaschromatographie

Verwendetes Gerät: Pye Unicam 104, automatischer Probenaufgeber S 4, elektronischer Integrator Vidar Modell 6300. Der Gaschromatograph war mit 2 Glassäulen von 2 m Länge und 2 mm I.D. ausgestattet. Sie waren gepackt mit 3% OV-3 auf Chromosorb W HP (100—200 mesh). Durchfluss Wasserstoff, 28 ml/min; Luft, 700/ml/min; Stickstoff, 12 ml/min. Der Injektionsblock wurde bei 180°, der Detektor bei 300° Zufluss gehalten. Temperatur-Programm: 5 min bei 100°, 4°/min, 5 min bei 250°; Abschwächung, 8×10^2

Behandlung des Kieselgels

Verwendete man das Kieselgel G 60 in der Vorsäule ohne Vorbehandlung, so zeigten alle Substanzen eine erhöhte Retention, darüberhinaus erniedrigte sich die Ausbeute für Benzoesäure, Phenylessigsäure, Mandelsäure und Vanillinmandelsäure auf rund die Hälfte. Zu den unten angegebenen Ausbeuten kommen wir, wenn wir 200 g Kieselgel G 60 in 1 l des polaren Anteils der mobilen Phase waschen und anschliessend mit der unpolaren Komponente auf den Anfangszustand des Chromatographiesystems einstellen. Um reproduzierbare Trenneigenschaften zu erreichen, mussten wir in ähnlicher Weise für die Hauptsäule eine Desaktivierung vornehmen. Wir pumpten 3 h den polaren Anteil des Elutionsmittels durch Säule 10 und stellten dan mit dem unpolaren Anteil den Ausgangszustand her. Die Retentionsvolumina blieben so für 20 Bestimmungen von 24-h-Urinen konstant. Wechsel der Fraktionen erfolgte alle 16 min.

ERGEBNISSE

Tabelle I zeigt die Konstanten *m* und *b* der Regressionsgeraden bzw. die Korrelationskoeffizienten *r* der von uns bearbeiteten aromatischen Karbonsäuren. Es sind für jede Substanz die Variationskoeffizienten von 4 Wiederholungsmessungen bei den beiden angegebenen verschiedenen Konzentrationen gezeigt (VK 1, VK 2). Die angegebenen Wiederfindungsraten sind aus den einzelnen Messpunkten der Eichgeraden errechnet worden. Fig. 2 zeigt das Elutionsdiagramm einer Standardmischung und eines 24-h Urins.

Fig. 3 zeigt die GC der einzelnen Fraktionen im Vergleich zum nicht vorbehandelten Extrakt.

Von den ausgewählten Verbindungen sind in jedem 24-Stunden-Urin einwandfrei messbar: 4-Hydroxyphenylessigsäure, 3-Hydroxyphenylessigsäure, 2-Hydroxyphenylessigsäure, Phenylmilchsäure, Mandelsäure, Vanillinmandelsäure, Homovanillinmandelsäure, wenn man als Identifizierungsparameter das Retentionsvolumen der Kieselgelchromatographie zusammen mit der Retentionszeit der GC heranzieht. Dies sollte natürlich abgesichert werden durch massenspektroskopische Studien, womit dann auch schlüssige Betrachtungen

in Bezug auf die vielen anderen organischen Säuren angestellt werden könnten.

Auf jeden Fall erscheint es uns problematisch, wenn quantitative GC-Messungen von Karbonsäuren aus blossen Extrakten oder aus den die gesamten saure und neutrale Fraktion enthaltenen Ionenaustauschereluaten erstellt werden. Dies sei an der Vanillinmandelsäure erläutert. Diese Substanz wird in der 8. bis 10. Fraktion eluiert (siehe Fig. 3, Peak 17); in der 2. Fraktion wird mit der gleichen Retentionszeit (Peak 17') in etwa 10 mal höherer Konzentration eine Substanz eluiert, bei der es sich natürlich nicht um Vanillinmandelsäure handeln wird. Diese Verhältnisse wiederholen sich in unseren Untersuchungen auch in etwa quantitativ bei allen 24-h-Urinen.

TABELLE I

KONSTANTEN DER EICHGERADE $y = mx + b$ SOWIE KORRELATIONSKOEFFIZIENT r DER EINZELNEN SUBSTANZEN

y, Relative Impulszahl; x, Konzentration in mg pro 2 ml.

Nr.	Substanz	m	b	r	Einwaage 1 (mg)	VK 1	Einwaage 2 (mg)	VK 2	Wiederfindung %	VK-Urin (%)
1	Phenylessigsäure	39353	− 647	0.997	0.83	7.72	0.21	8.31	92.4	
2	Benzoesäure	29764	−1687	0.944	0.75	10.83	0.19	1.0	85.7	
3	Zimtsäure	53043	−1849	0.992	0.58	3.98	0.15	15.67	102.3	
4	Veratrumsäure	31764	−1480	0.996	0.70	1.90	0.18	10.52	103.3	
5	2-Hydroxyhippursäure	55040	−5267	0.989	0.60	1.41	0.15	18.76	74.9	
6	Homovanillinsäure	72361	−4387	0.998	0.65	2.88	0.16	2.29	94.8	5.6
7	2-Hydroxyphenylessigsäure	49597	−8797	0.999	0.78	1.39	0.20	1.01	89.1	9.3
8	Ferulasäure				0.66		0.17		67.0	
9	4-Hydroxyzimtsäure	42147	− 621	0.995	0.82	2.71	0.21	13.24	98.6	
10	3-Hydroxyzimtsäure	54209	−2312	0.997	0.63	3.61	0.16	1.61	94.1	
11	2,4-Dihydroxybenzoesäure	45467	− 337	0.997	0.73	2.41	0.18	7.41	83.8	
12	4-Hydroxyphenylessigsäure	68741	−2453	0.996	0.61	1.71	0.15	1.24	105.5	4.1
13	3-Hydroxyphenylessigsäure	52806	−2318	0.995	0.90	0.30	0.23	2.69	101.9	6.2
14	3-Phenylmilchsäure	62948	− 412	0.998	0.71	2.44	0.18	2.58	72.4	5.6
15	Mandelsäure	53532	−1023	0.997	0.71	3.45	0.18	1.12	79.2	10.4
16	2,6-Dihydroxybenzoesäure	54486	−3280	0.997	0.74	2.44	0.19	10.84	83.9	
17	Vanillinmandelsäure	37573	−3231	0.971	0.85	7.51	0.21	9.44	64.7	7.5

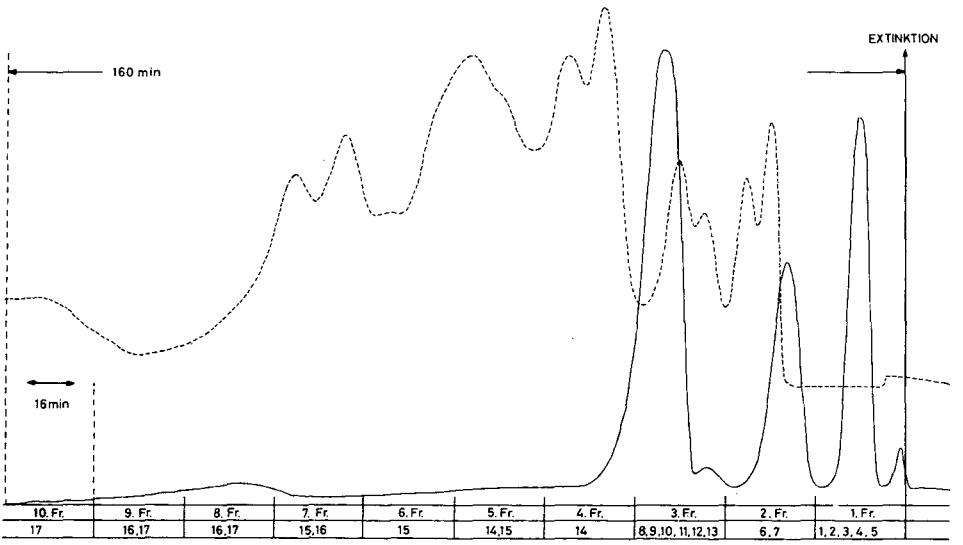


Fig. 2. —Standardgemisch der Substanzen 1—17 (siehe Tabelle I). -----30% eines 24-h-Urins. UV-Elutionsdiagram bei 280 nm.

Die Wiederholbarkeit wurde nur für die identifizierten Verbindungen im Urin ermittelt, und zwar jeweils aus 20 Doppelbestimmungen von 24-h-Urinen (siehe Tab. I).

DISKUSSION

Durch Herabsetzen der Durchflussgeschwindigkeit erreicht man in den ersten Fraktionen eine erhebliche bessere Trennung. Den gleichen Effekt erzielt man durch Verwenden unpolarer Elutionsmittel. Die Methode erscheint ebenfalls tauglich für die flüchtigeren sauren Komponenten des Urins, wenn mann entsprechende GC-Bedingungen wählt. Die Verwendung der Fertigsäule garantiert, dass man immer unter den gleichen Packungsbedingungen arbeitet. Pump- und Detektionssystem sind in dieser oder ähnlicher Ausführung in den meisten Laboratorien vorhanden. Sie sind vergleichsweise billig und technisch völlig unproblematisch. Vergleicht man das vorliegende Verfahren mit von anderen Autoren beschriebenen [2, 3], so bleibt festzustellen, dass der zeitliche Aufwand nicht grösser ist, dass aber entweder die Trennleistung höher ist oder eine vielseitigere Anwendbarkeit gegeben ist. Wegen der nicht sehr spezialisierten Ausrüstung könnte es darüberhinaus eine Alternative zu den Vorschlägen von Alibert und Morot-Gaudry [4—7] darstellen und somit einen Beitrag zur Lösung pflanzenphysiologischer Probleme liefern. Bemerkenswert erscheint weiterhin die für eine Kieselgelsäule durchweg hohe Wiederfindung bei den hier betrachteten Verbindungen.

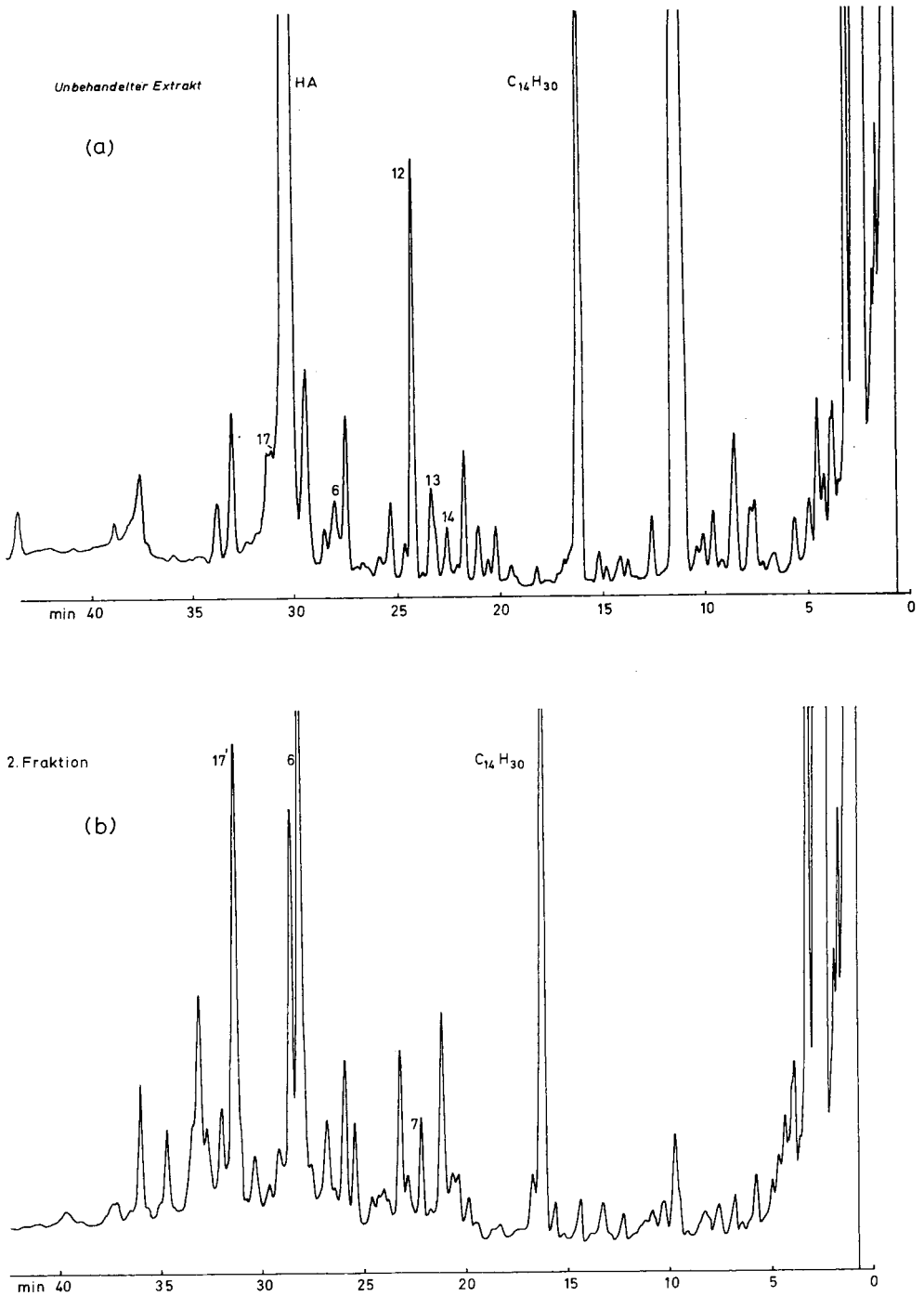
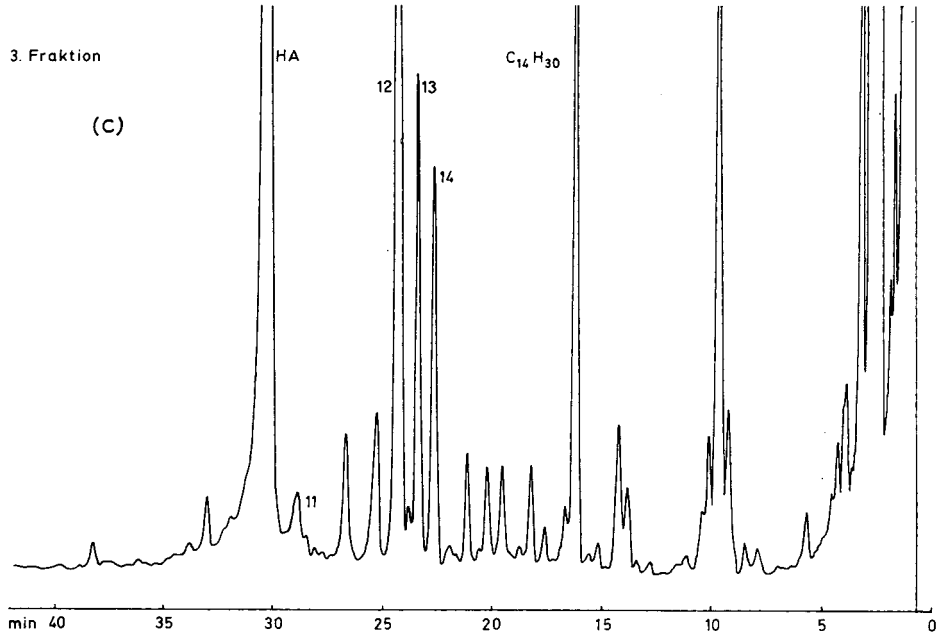


Fig. 3.

(Continued on p. 348)


3. Fraktion
HA
12
13
14
$C_{14}H_{30}$
(c)
11
min 40
35
30
25
20
15
10
5
0

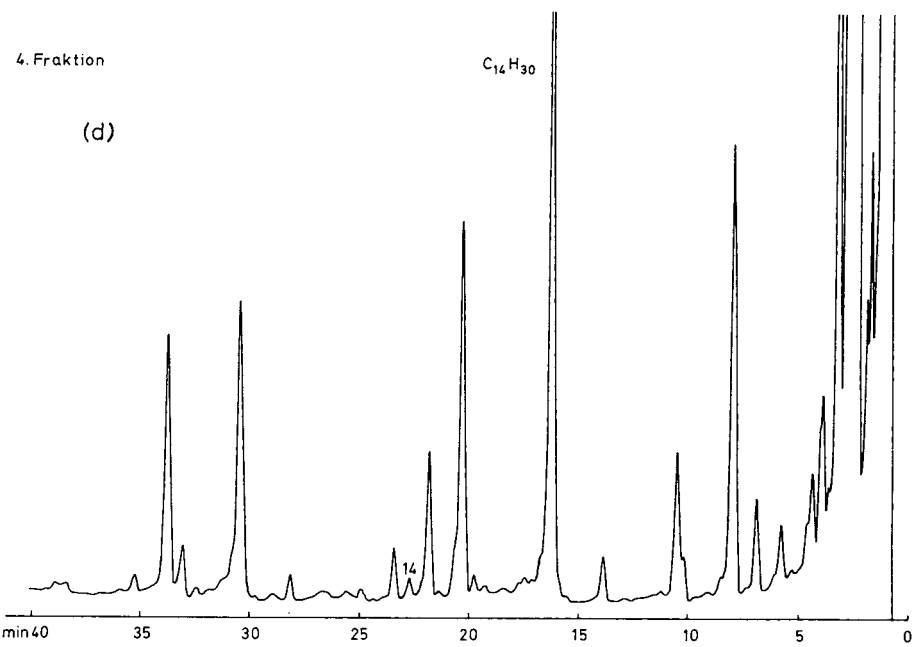

4. Fraktion
$C_{14}H_{30}$
(d)
14
min40
35
30
25
20
15
10
5
0

Fig. 3.

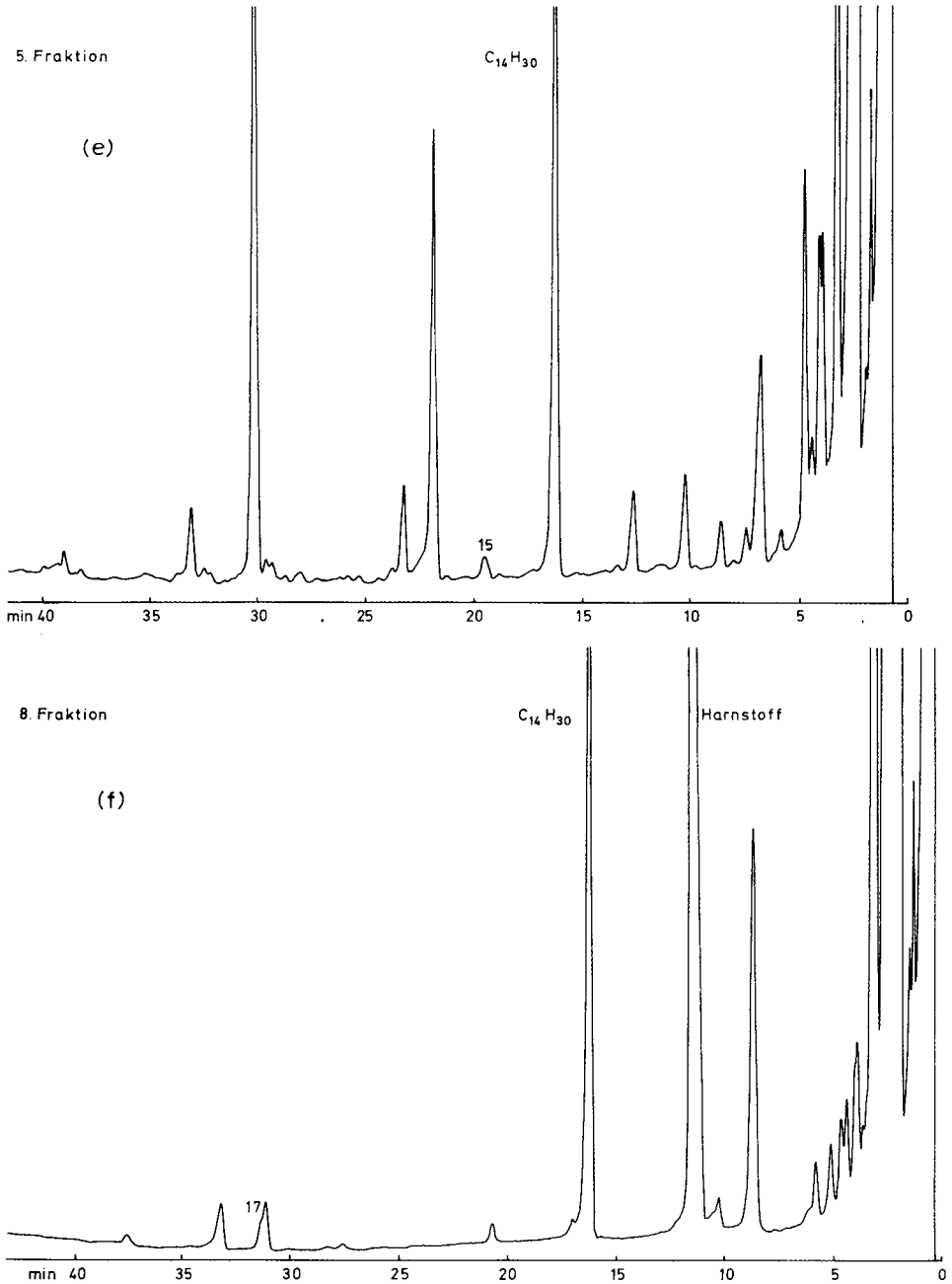


Fig. 3. Gaschromatogramme (a) von nicht vorbehandelten Urinextrakt und (b—f) von 5 Urinfraktionen in denen einige bekannte Abbauprodukte von aromatischen Aminosäuren zu erkennen waren. Die Identifizierung erfolgte nach den Retentionsvolumina der Kieselgel-Chromatographie und der Retentionszeit in der GC. HA = Hippursäure; 6 = Homovanillinsäure; 7 = 2-hydroxyphenylessigsäure; 11 = 2,4-Dihydroxybenzoesäure; 12 = 4-Hydroxyphenylessigsäure; 13 = 3-Hydroxyphenylessigsäure; 14 = 3-Phenylmilchsäure; 15 = Mandelsäure; 17 = Vanillinmandelsäure; 17′ = unbekannte Substanzen. 5% des für die Kieselgel-Chromatographie verwendeten Extraktes wurden ohne Vorbehandlung unter den angegebenen Bedingungen gaschromatographisch analysiert.

DANK

Hern G. Degadjor danke ich für die wertvolle Hilfe bei der Durchführung dieser Arbeit. Diese Arbeit wurde unterstützt von der Deutschen Forschungsgemeinschaft.

ZUSAMMENFASSUNG

Es wird eine Methode beschrieben, in der Extracte aus 24-Stunden-Urinen auf einer Kieselgelfertigsäule vorfraktioniert werden. Die eigentliche quantitative Analyse erfolgt gaschromatographisch. 10 Fraktionen werden in 160 min abgenommen. Die Kapazität der Kieselgelsäure ist so hoch, dass 30% eines 24-Stunden-Urins aufgegeben werden können. Dies ermöglicht die Bestimmung von sauren Urinmetaboliten im Bereich unter 1 mg pro 24-h-Urin. Die Anwendbarkeit für Studien von metabolischen Profilen wird diskutiert.

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Biomedical Applications

CHROMBIO. 139

MONOSACCHARIDES AND MONOSACCHARIDE DERIVATIVES IN HUMAN SEMINAL PLASMA

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(Received December 1st, 1977)

SUMMARY

Gas chromatography—mass spectrometry with an on-line data system was used to identify monosaccharides and monosaccharide derivatives in human seminal plasma. The carbohydrates were converted into the methoxime—trimethylsilyl derivatives before separation in open tubular glass capillary columns coated with SE-30. Twenty-one different compounds were detected in the seminal fluid, of which twelve have not been recognized before. Seventeen of the monosaccharides have previously been identified in urine. Similar patterns of sugars were found both in fertile and infertile individuals, including one with azoospermia. The compounds identified are, with the possible exception of D-ribose, present as free monosaccharides at the time of ejaculation, and they do not seem to be preformed by spermatozoa.

INTRODUCTION

Mann and Rottenberg [1] identified 6 monosaccharides in human seminal plasma, and later 2-acetamido-2-deoxy-hexoses [2] have also been found. It has been suggested [1, 2] that some of the sugars arise from the spermatozoa or by enzymic reactions in the seminal fluid.

Preliminary investigations [3] made it clear that several sugars in addition to those already described were present in human seminal plasma. Open tubular columns for gas chromatography attached to a mass spectrometer and an on-line data system have increased our analytical possibilities, and formed the basis for the present investigation.

MATERIALS AND METHODS

Chemicals

Reference sugars tested were: L-arabinitol, D-arabinose, erythritol, galactitol,

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D-glucitol, D-mannitol, L-rhamnose, D-ribose and L-sorbose from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.), 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, L-fucose and D-glycero-D-galacto-heptitol from Sigma (St. Louis, Mo., U.S.A.), L-arabinose, D-galactose and ribitol from Difco (Detroit, Mich., U.S.A.), D-fructose, D-glucose and glycerol from E. Merck (Darmstadt, G.F.R.), D-arabinitol and xylitol from Koch-Light (Colnbrook, Great Britain), D-mannose from Hopkins and Williams (London, Great Britain), D-xylose from BDH (London, Great Britain), threitol was a gift from Dr. Wold, Institute of Pharmacy, University of Oslo. L-Fucitol was made by reduction of L-fucose with sodium borohydride.

Methoxyamine—hydrochloride and N, O-bistrimethylsilyl-trifluoroacetamide (BSTFA) were obtained from Supelco (Bellefonte, Pa., U.S.A.).

Instrumentation

Gas chromatography—mass spectrometry (GC—MS) was performed on a Varian Model 112 combined gas chromatograph—mass spectrometer (Varian-MAT, Bremen, G.F.R.) to which an on-line data system (Spectrosystem 100, Varian-MAT), with dual discs and magnetic tape unit was attached. The gas chromatograph was a Varian Model 1400 (Varian Aerograph, Walnut Creek, Calif., U.S.A.) and the glass capillary column (LKB, Stockholm, Sweden) was 25 m × 0.28 mm I.D., and wall-coated with SE-30. The ion source of the GC detector was operated at 24 eV, and that for MS was operated at 70 eV. Injection port temperature was 250°. Samples were usually injected at an oven temperature of 80°. After elution of the solvent front the temperature control was set at 150° and the temperature programmed at 2° per minute up to 200°. Inlet pressure of helium carrier gas was 0.25 kp/cm^2, which gave a 10 cm/sec linear velocity of carrier gas. The split ratio of the injector was 1:20. The mass spectrometer scan time was 2 sec from start to reset.

A Fractovap Linea 2101 AC (Carlo Erba, Milan, Italy) gas chromatograph with a flame ionization detector and equipped with the same glass capillary column as described above, was also used. The chromatograph was operated at an injector—detector block temperature of 250°, and with the same column temperature conditions as for the GC—MS instrument. The split ratio was 1:20.

Ultrafiltration was done with a Millipore (Bedford, Mass., U.S.A.) PSAC filter 13 mm in diameter. The ultrafiltration cell was operated with a magnetic stirrer, and at 3.5 kp/cm^2 pressure exerted from a nitrogen flask.

Samples

Samples analysed were obtained through masturbation. They were received from the outpatient infertility section of the Department of Gynecology and Obstetrics. A pilot study was undertaken on 13 samples. Of these, 6 were from men without findings indicating pathology, neither in medical history nor upon routine clinical examination of semen. Of the samples, 4 showed low motility of spermatozoa, and 3 showed more pronounced pathology, including one with azoospermia.

Samples from 3 healthy donors were ejaculated into 80% ethanol to stop

enzymic action and action of spermatozoa. For comparison, the same three donors also produced samples which were left to liquefy for 30 min before separation of spermatozoa and plasma.

Methods

Semen will clot immediately after ejaculation, and then it liquefies spontaneously during the next 30 min. Seminal plasma was separated from spermatozoa through centrifugation, 30—60 min after ejaculation. A 0.5-ml volume of seminal plasma was ultrafiltered, and then passed through a mixed-bed ion-exchange resin (Dowex 2 (CO_3^{2-}) and Dowex 50 (H^+), both X8 and 100—200 mesh). The eluate was lyophilized and dissolved in 1 ml of pyridin. About 2 mg of methoxyamine—HCl was added and the mixture heated at 80° for 2 h. After addition of 100 µl of BSTFA the heating was continued for 15 min [4].

RESULTS

A gas chromatogram of the neutral fraction of pooled human seminal plasma is shown in Fig. 1. In all, 25 of the peaks have been identified, they represent 21 monosaccharides or monosaccharide derivatives.

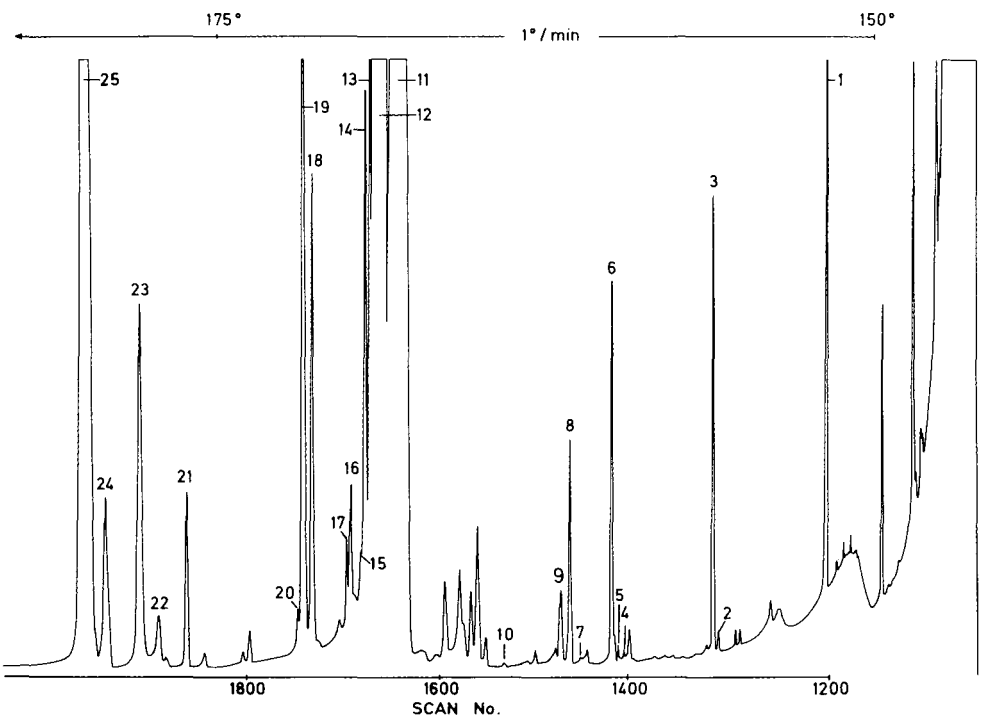

Fig. 1. Gas chromatogram of the neutral fraction of pooled human seminal plasma. A 25 m long, open-tubular, glass capillary column coated with SE-30 was used. The oven temperature at injection was 80°. After elution of the solvent front, the temperature control was set at 150° and the temperature was programmed at 1°per min to 200°. The numbering of the peaks is the same as in Table I, which also shows the identities of the compounds.

TABLE I

MONOSACCHARIDES AND ALDITOLS IDENTIFIED IN HUMAN SEMINAL PLASMA

The numbering of the peaks is the same as in Fig. 1. Retention times (SE-30 glass capillary column) are given relative to the first peak of fucose, and have been taken from a GC run with a temperature programming of 2° /min from injection at 120°.

Peak No.	Compound	Relative ret. time	Ten most abundant *m/z* fragments									
1	Glycerol	0.28	73	147	205	103	117	218	74	45	148	133
2	Threitol	0.55	73	217	147	103	205	117	189	218	204	191
3	Erythritol	0.56	73	147	217	103	205	117	204	189	191	116
4	Xylose	0.84	73	103	217	307	147	189	218	160	191	117
5	Arabinose	0.86	73	103	217	147	307	189	104	133	74	160
6	Ribose	0.90	73	103	217	147	307	189	74	104	160	218
7	Xylitol	0.98	73	103	217	205	147	307	189	148	218	117
8	Fucose, 1st peak	1.00	117	73	160	118	147	277	129	75	161	219
8	Arabinitol	1.01	pure spectrum not obtained									
9	Ribitol	1.02	pure spectrum not obtained									
9	Fucose, 2nd peak	1.03	pure spectrum not obtained									
10	Fucitol	1.18	73	117	147	217	205	219	129	103	319	133
11	Fructose, 1st peak	1.38	73	103	217	307	147	74	104	308	133	218
12	Fructose, 2nd peak	1.41	73	103	217	307	147	74	308	218	104	75
12	Mannose, 1st peak	1.42	pure spectrum not obtained									
13	Galactose, 1st peak	1.43	73	205	147	319	103	217	160	320	117	74
14	Glucose, 1st peak	1.44	73	205	319	147	160	103	217	320	157	117
15	Mannose, 2nd peak	1.45	73	103	147	205	319	217	160	74	117	129
16	Galactose, 2nd peak	1.46	73	205	147	319	103	217	160	320	129	117
17	Glucose, 2nd peak	1.47	73	147	205	103	319	160	217	74	117	157
18	Mannitol	1.54	73	205	319	147	103	217	320	117	157	206
19	Glucitol	1.55	73	205	319	147	103	217	320	307	117	206
20	Galactitol	1.56	73	217	103	147	205	319	307	218	117	74
21	Scyllo-inositol	1.77	73	217	318	305	147	191	204	319	306	103
22	2-Acetamido-2-deoxy-glucose, 1st peak	1.80	73	147	205	129	202	87	103	75	173	319
23	2-Acetamido-2-deoxy-glucose, 2nd peak	1.82	73	147	205	129	202	87	319	103	173	75
24	2-Acetamido-2-deoxy-galactose	1.87	73	147	205	129	87	319	202	103	173	75
25	Myo-inositol	1.90	73	217	305	147	191	318	204	306	218	265

Table I gives relative retention times and the 10 most abundant *m/z* fragments for the compounds identified. Identification is based on both mass spectra and retention times. Mass spectra have been compared to mass spectra of authentic sugars, and to mass spectra described in the literature. Retention times have been compared by adding authentic sugars to samples of seminal fluid.

For scyllo-inositol the retention time has not been verified. The mass spectrum, however, identifies peak 21 of Fig. 1 to be an inositol. Furthermore, the spectrum indicates that it is scyllo-inositol, since no other inositol has such a low relative abundance of *m/z* 265 [5].

Due to overlapping of neighbouring GC peaks, no pure mass spectrum could be obtained for some of the compounds in Table I. MS may, in spite of this, give data which are of importance for the identification. This is shown in Fig. 2. As can be seen from the traced fragments, the GC peaks clearly consist of two compounds each. However, no reliable order of relative ion abundance can be given.

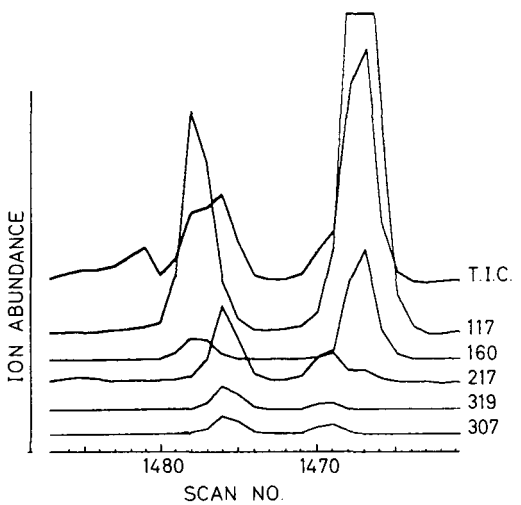

Fig. 2. Characteristic mass spectral fragments traced through peaks 8 and 9 of Fig. 1. At least two compounds are shown to be present in each peak. T.I.C. is total ion current.

Seminal fluid samples from 13 men of infertile couples were examined with gas chromatography. In some of these chromatograms the peaks for D-xylose, arabinitol, arabinose, ribitol and L-fucitol could not be detected. This might be due to the somewhat lower sensitivity of the flame ionization detector of the gas chromatograph used for these analyses, as compared to the electron impact detector of the GC—MS combination instrument. In all the samples examined, the other peaks identified in Fig. 1 were present with about the same relative heights. This was also true for the sample of the azoospermic individual. The concentrations of the sugars were, however, somewhat lower in this sample than in the others.

In order to avoid possible changes of the seminal plasma after ejaculation,

samples from three healthy donors were ejaculated directly into 80% ethanol. D-ribose was absent in two of the samples, but present in the third one. D-ribose was present in samples from the same three donors after the samples had been left to liquefy for 30 min before separation of spermatozoa from seminal plasma. The ribose-containing sample, which had been treated with ethanol, contained only small amounts of fructose. This was also true when a sample from the same donor was left to liquefy. The other compounds identified in the pooled plasma were present also in the 3 samples ejaculated into ethanol, and the GC peaks were of similar relative heights as those of the pooled sample.

DISCUSSION

A variety of derivatives have been used for the GC analysis of sugars, most of them are unsuitable when ketoses are present. The methoxime—TMS derivatives can be applied also on ketoses. This is probably the main advantage of the present method compared to others. The methoxime—TMS derivatives give one GC peak for alditols, and two peaks for most aldoses and ketoses, due to isomerism arising during methoxime formation. Without prior reaction of the carbonyl function, most other derivatization procedures, like trimethylsilylation, methylation or acetylation, will give 2—4 peaks for each sugar. Reduction of the carbonyl group will give one peak for each monosaccharide, but will not distinguish between e.g. D-glucose and D-glucitol. Aldononitriles give just one peak for each aldose and alditol, but cannot be applied to the analysis of ketoses. The oxime—TMS derivatives can be used, but they are rather unstable [6].

The methoxime—TMS derivatives are stable for months when kept at —20° in PTFE-capped vials. When destroyed by hydrolysis, the samples can easily be taken to dryness and rederivatized.

For identification of monosaccharides, mass spectra alone will not suffice. As can be seen from Table I, mass spectra of optical isomers are quite similar. Mass spectrometry is, however, well suited to the identification of classes of sugars. The literature contains mass spectra of methoxime—TMS derivatives of various sugars [4] and of 2-acetamido-2-deoxyaldoses [7], and mass spectra of TMS derivatives of inositols [5]. For identification it is, in addition, necessary to determine gas chromatographic retention times. Even then a complete identification of some of the monosaccharides is at present not possible.

Only a few of the 21 compounds identified in the present paper have previously been recognised as constituents of seminal fluid. Mann [1] showed that 6 sugars were present: D-fructose, D-glucose, D-ribose, L-fucose, D-glucitol and myo-inositol. 2-Acetamido-2-deoxy-hexoses were shown to be present in seminal fluid of many mammals, including man, by Rodger and White [2]. It was, however, not possible for them to distinguish between the different isomers. High concentrations of these sugars are found in the seminal fluid of marsupials, where they dominate as much as does D-fructose in the seminal fluid of most eutherians. Scyllo-inositol is also present in seminal fluid of different mammals [8], but has not been described in the semen of man before. This sugar is found in the same body fluids and tissues as myo-inositol, and is thought to be a part of myo-inositol metabolism [9].

It has been suggested [1, 2] that some of the sugars previously found in seminal fluid, are the result of enzymic or spermatozoic action occurring during liquefaction. The findings in the samples ejaculated into ethanol do not support this hypothesis. The results from the azoospermic sample make it, in addition, unlikely that the monosaccharides are formed by spermatozoa before ejaculation. The volume of this sample was 6 ml, which excludes retrograde ejaculation as the cause of the azoospermia. Most likely all the sugars found are present as free monosaccharides at the time of ejaculation, with the possible exception of D-ribose.

The concentrations of D-fructose and myo-inositol are known to vary within wide ranges in human seminal plasma, with means of about 12.5 mmole/l and 2.3 mmole/l, respectively. Semiquantitative calculations for the remaining 19 compounds identified give concentrations ranging from less than 5 μmole/l for xylitol to about 2 mmole/l for glycerol.

Although most of the 21 compounds have not been identified in human seminal plasma before, 17 of them are known to occur in human urine [10]. This suggests that these compounds may be part of the general carbohydrate metabolism in man.

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Biomedical Applications

CHROMBIO. 125

HARN-STEROIDPROFILE HIRSUTER FRAUEN

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SUMMARY

Urine steroid profiles of hirsute women

Steroid profiles of women suffering from idiopathic hirsutism show in more than 50% of the cases a 10—100 fold increase in the excretion of dehydroepiandrosterone (DHEA) compared with normal values.

The excretion of DHEA was reduced much more than that of other 17-ketosteroids if the adrenals (NNR) were suppressed by dexamethasone (DXM). Within one week they reached values at the compound noise level of the gas chromatograms. If the ovaries were stimulated with human chorionic gonadotropin during continued suppression of the NNR with DXM no increase of DHEA could be detected.

EINLEITUNG

Zur Diagnose von Steroidstoffwechselstörungen verwendet man neben dem Radioimmunoassay auch heute noch Gruppenbestimmungsmethoden, bei denen mit Hilfe eines Reagens alle Verbindungen erfasst werden, die eine bestimmte funktionelle Gruppe enthalten.

17-Ketosteroide werden beispielsweise im Harn nach der Methode von Vestergaard [1] analysiert. Zu dieser Gruppe von Verbindungen zählen Androsteron (A), Etiocholanolon (E), ihre 11β-Hydroxyderivate (11-HA und 11-HE), Dehydroepiandrosteron (DHEA) und sein 16α-Hydroxyderivat (16-DHEA). Diese Steroide liegen in sehr unterschiedlichen Mengenverhältnissen im Harn

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vor. Beispielsweise übertrifft der Gehalt an A und E den an DHEA in der Regel um das 10—50-fache.

Da man nicht voraussetzen kann, dass bei einer Stoffwechselstörung die Ausscheidung aller 17-Ketosteroide im Harn in gleicher Weise ansteigt oder abfällt, eignet sich diese Gruppenbestimmungsmethode eigentlich nur dann zu Diagnosezwecken, wenn eine Änderung in der Ausscheidung der Hauptsteroide A und E auftritt. Eine Veränderung in der Ausscheidung von Nebensteroidmetaboliten wird dagegen, selbst wenn diese in einer um ein Mehrfaches erhöhten Menge oder nur zu einem Bruchteil der Normalwerte ausgeschieden werden, kaum ins Gewicht fallen.

Dieser Nachteil ist radioimmunologischen Methoden nicht so zu eigen: Mit dem Radioimmunoassay kann man Steroidgruppen und auch einzelne Steroide erfassen. Der Radioimmunoassay hat aber einen anderen Nachteil: Man kann mit ihm nur solche Steroide analysieren, deren Gegenwart im Harn bekannt ist. Überdies werden aus zeitlichen Gründen nur solche Steroide erfasst, deren Bestimmung interessant und notwendig erscheint und für die daher ein spezifischer Radioimmunoassay entwickelt wurde. Steroide, die im Normalfall nicht gebildet werden oder deren Bedeutung für den Stoffwechsel als unwichtig erachtet wird und für die daher keine Bestimmungverfahren erarbeitet wurden, entziehen sich dem Nachweis.

Diese Lücke in den Nachweis- und Bestimmungsmethoden kann durch Verwendung der gaschromatographischen (GC) Analyse von Steroiden mit Glaskapillarsäulen geschlossen werden. Mit dieser Methode können alle Steroide in einer biologischen Flüssigkeit in einem Analysengang halbquantitativ erfasst werden [2—7]. Die so erhältlichen "Profile" geben ein Bild von der relativen Menge der ausgeschiedenen Steroide, so dass sich unterschiedliche Produktions- und Ausscheidungsraten erkennen lassen.

Die in den Gaschromatogrammen angezeigten Steroide können durch Messung von Retentionsindices und gleichzeitige Aufnahme von Massenspektren eindeutig identifiziert werden. Da die Ausscheidungsraten an Steroiden individuell und zeitlich unterschiedlich sind, sind Abweichungen von der Norm nur dann ein Hinweis auf Stoffwechselstörungen, wenn die Ausscheidungsrate bzw. Produktionsrate gegenüber üblichen Werten etwa um den Faktor 5 und grösser variieren. Abweichungen in dieser Grössenordnung (zwischen dem Faktor 10 und 100) haben wir in der Ausscheidung von DHEA bei Frauen mit idiopatischem Hirsutismus, der Bildung vermehrter Körperbehaarung der Frau ohne erkennbare Ursache, beobachtet. Darüber soll hier berichtet werden.

EXPERIMENTELLES

Auswahl der Patienten

Für die Untersuchung wurden 24 Patientinnen ausgewählt, die auf Grund der klinischen Hormonbestimmungen an idiopatischem Hirsutismus litten.

Durchführung des Dexamethason—Choriongonadotropin-Testes (DXM—HCG-Test)

Der DXM—HCG-Test wurde stationär in der Frauenklinik der Universität Göttingen durchgeführt, um alle Patientinnen unter gleichen Bedingungen

untersuchen und psychische Faktoren (z.B. Stress etc.) weitgehend ausschalten zu können [8].

Alle Patientinnen erhielten nach dreimaliger Bestimmung der Basiswerte vom 4. bis 9. Tag täglich 3 × 1.5 mg Dexamethason (Fortecortin®, Merck) oral und zusätzlich vom 7. bis 9. Tag 5000 I.E. Choriongonadotropin (Primogonyl®, Schering) i.v. Während des Untersuchungszeitraumes wurde täglich der 24 Stunden Urin gesammelt (Basiswert: 1. bis 3. Tag; Werte unter DXM-Medikation: 4., 5. und 6. Tag; Werte unter HCG-Behandlung und DXM-Medikation: 7., 8. un 9. Tag). Blutproben (20 ml Plasma) wurden am 2., 7., 9 und 10. Tag der Behandlung morgens um 8.00 Uhr entnommen. Alle Proben wurden bis zur Verarbeitung bei −18° aufbewahrt.

Hormonbestimmung

Die Hormonbestimmungen wurden im Hormonlabor der Frauenklinik der Universität Göttingen (Abteilung für klinische und experimentelle Endokrinologie, Leitung: Frau Prof. Dr. A. König) wie folgt ausgeführt. Urin: 17-Ketosteroide nach Vestergaard [1]; 17α-Hydroxycorticosteroide nach Appleby et al. [9]; Pregnantriol nach Fotherby und Love [10]; Plasma: 17β-Hydroxyandrogene durch Radioimmunoassay nach Ellendorff et al. [11].

Aufarbeitung der Urinproben zur GC-Analyse

Steroide liegen im Harn fast ausschliesslich in konjugierter Form vor. Die hohe Polarität der Konjugate macht eine direkte gaschromatographische Bestimmung nicht möglich, diese gelingt erst nach enzymatischer Spaltung und Derivatisierung.

Zu jeweils 50 ml Urin wurde 1.0 g Natriumacetat-Trihydrat gegeben und mit konz. Essigsäure der pH-Wert auf 4.5 eingestellt. 0.2 ml einer β-Glucuronidase-Arylsulfatase-Lösung, [Boehringer, Mannheim, B.R.D.; 1 ml enthielt 5.2 I.E. β-Glucuronidase (bestimmt bei 38° , Phenolphthaleinmonoglucuronid als Substrat) und 14 I.E. Arylsulfatase (bestimmt bei 25°, *p*-Nitrophenyl-sulfat als Substrat)] wurden zugefügt und 24 Stunden bei 37° (unter schwachem Rühren) inkubiert. Dann wurde mit einigen Tropfen konz. wässiger Natriumhydroxidlösung der pH-Wert auf 5.5 eingestellt, weitere 0.2 ml der β-Glucuronidase-Arylsulfatase-Lösung zugefügt und nochmals 24 Stunden bei 37° inkubiert [12].

Die hydrolysierten Steroide wurden nach Bradlow [13] an Amberlite XAD-2 wie folgt extrahiert. Die Säulen (600 × 20 mm) wurden mit 50 g feuchtem, frisch extrahiertem XAD-2 gefüllt und unmittelbar vor Gebrauch mit 200 ml 5% wäss. Natriumchloridlösung gewaschen. Dann wurde das Hydrolysat (50 ml) über die Säule gegeben (Tropfgeschwindigkeit 1 ml/min) und mit 75 ml Aqua dest. gewaschen; das Waschwasser wurde verworfen. Die Lipophilen Bestandteile (freie Steroide, lipophile organische Säuren etc.) wurden anschliessend mit 200 ml Methanol eluiert; Blasen wurden durch Umschütteln entfernt. Das Lösungsmittel wurde am Rotationsverdampfer bei einer maximalen Temperatur von 35° abgezogen. Der organische Rückstand wurde mit 15 ml Essigester aufgenommen und dreimal mit 10 ml Portionen wässriger 5%iger Natriumbicarbonat-Lösung—10%iger Natriumchlorid-Lösung ausgeschüttelt und

dreimal mit 10 ml gesättigter wässriger Natriumchlorid-Lösung gewaschen. Die Essigesterfraktion wurde mit wasserfreiem Natriumsulfat getrocknet und am Rotationsverdampfer eingedampft [12].

Darstellung der Trimethylsilyl-Enol-Trimethylsilyl-Ether (TMS-Enol-TMS-Ether).

Für die GC von Steroiden werden meist Methyloxim-TMS-Ether als Derivate nach Thenot und Horning [14] dargestellt. Diese Derivatisierungsmethode hat den Nachteil, dass dabei schwerflüchtiges Imidazol gebildet wird, das bei der GC-Analyse sehr breite Peaks gibt [15]. Zudem beobachtete Engel et al. [16] und Thenot und Horning [17] dass im Injektorblock und am Säulenanfang Artefakte gebildet werden können, die einen weiteren Reinigungsschritt wünschenswert erscheinen lassen [18,31]. Wir benutzten deshalb diese Derivatisierungsmethode nur in einzelnen Fällen und stellten TMS-enol-TMS-ether dar [20]. Der Rückstand des Steroid-Extraktes wurde mit 100 µl Methanol aufgenommen; 10 µl dieser Lösung wurden mit einer Mikroliterspritze in ein Glasröhrchen (2 × 50 mm) gegeben und im Vakuum eingedampft. Nach Zugabe einiger Flitter wasserfreien Natriumacetats, 10 µl N-Methyl-N-TMS-Trifluoroacetamids (MSTFA) und 10 µl wasserfreien Pyridins als Lösungsmittel [21] wurden die Röhrchen zugeschmolzen und 24 h bei 60° oder 72 h bei Raumtemperatur gehalten. Unter diesen Bedingungen wurde quantitative Trimethylsilylierung auch der 17α-Hydroxylgruppe (nicht aber der 11β-Hydroxylgruppe) und Enolisierung von Ketogruppen mit Ausnahme der in Stellung 11 beobachtet. Das Reaktionsgemisch wurde ohne weitere Aufarbeitung für die GC-Analyse verwandt.

Glaskapillargaschromatographie

Die Glaskapillargaschromatogramme wurden an einem Carlo-Erba-Gaschromatographen Modell 2300 gemessen. Als Säulen wurden Dünnfilmglasskapillarsäulen von 25 m Länge und 0.3 mm Innendurchmesser verwendet. Als stationäre Phase diente SE-30. Die Säulen wurden aus Softglas mit einem Kapillarziehgerät gezogen und nach der statischen Methode belegt. Die Säulen konnten mehrere Monate ohne merkliche Verminderung der Trennleistung und Veränderung der Methyleneinheiten verwendet werden.

GC-Bedingungen: Injektor- und Detektortemperatur, 280°, Split, 1:10; Trägergas, Helium; Flussgeschwindigkeit, 20—25 cm/sec bei 250°; Temperaturprogramm, 6 min isotherm bei 150° , dann programmiert mit 2°/min bis auf 300°, anschliessend 15 min isotherm bei 300°.

Bestimmung der Methyleneinheiten (MU-Werte)

Die MU-Werte wurden durch Koinjektion von geradzahligen *n*-Kohlenwasserstoffen (C_{16}—C_{32}) mit der Probe bei einem Temperaturprogramm von 1°/min und 200° Anfangstemperatur bestimmt. Unter diesen Bedingungen wurden die Kohlenwasserstoffe C_{24}—C_{32} in nahezu gleichen Abständen eluiert.

Massenspektrometrie (MS)

Die Strukturen aller in den Abbildungen bezeichneten Steroide wurden durch Bestimmung der MU-Werte sowie durch Aufnahme der Massenspektren und

Vergleich mit Spektren authentischer Proben (Ikapharm, Ramat-Gan, Israel und Schering, Berlin, B.R.D.) gesichert.

Die Massenspektren wurden mit einem LKB-Massenspektrometer 2091 aufgenommen, das mit dem LKB-GC—MS-Computersystem 2130 ausgerüstet war. Zur Trennung der Steroide wurden LKB-Glaskapillarsäulen (25 m, SE-30, mehr als 70,000 Böden) verwendet.

MS-Bedingungen: Separator- und Ionenquellentemperatur, 290°, Elektronenenergie, 20 eV zur Aufzeichnung des Totalionenstroms, 70 eV zur Registrierung der Massenspektren; Beschleunigungsspannung, 3.5 kV; Ionenstrom, 50 μA.

Identifizierte Steroide

Die identifizierten Steroide, ihre Abkürzungen und die MU-Werte sind in Tabelle I aufgeführt.

TABELLE I

IDENTIFIZIERTE STEROIDE UND IHRE MU-WERTE

A=Androstan; A'=Androsten; P=Pregnan; P'=Pregnen.

Abkürzung	Molekulargewicht	MU-Wert	Trivial Name	Systematischer Name
C_{24}	338	24.00	*n*-Tetracosan	
DOP	390	25.01	Dioctylphthalat	
A	434	25.34	Androsteron	5α-A-3α-ol-17-on
E	434	25.39	Etiocholanolon	5β-A-3α-ol-17-on
DHEA	432	26.10	Dehydroepiandrosteron	5-A'-3β-ol-17-on
11KA	448	26.53	11-Ketoandrosteron	5α-A-3α-ol-11,17-dion
11KE	448	26.45	11-Ketoetiocholanolon	5β-A-3α-ol-11,17-dion
11HA	450	27.04	11-Hydroxyandrosteron	5α-A-3α,11β-diol-17-on
11HE	450	26.95	11-Hydroxyetiocholanolon	5β-A-3α, 11β-diol-17-on
16DHEA	448	27.46	16α-Hydroxy-dehydroepiandrosteron	5-A'-3β,16α-diol-17-on
PD	464	27.94	Pregnandiol	5β-P-3α,20α-diol
PT	552	28,20	Pregnantriol	5β-P-3α,17α,20α-triol
P'D	462	28.49	Pregnendiol	5-P'-3β,20β-diol
A'T	522	28.67	Androstentriol	5-A'-3β,16α,17β-triol
Cl	654	30.68	Cortolon	5β-P-3α,17α,20α,21-tetrol 11-on
β-Cl	654	30.97	β-Cortolon	5β-P-3α,17α,20β,21-tetrol 11-on
THE	652	31.14	THE	5β-P-3α,17α,21-triol-11,2 dion
C	656	31.20	Cortol	5β-P-3α,11β,17α,20α,21-pentol
β-C	656	31.36	β-Cortol	5β-P-3α,11β,17α,20β,21-pentol
THF	654	31.44	THF	5β-P-3α,11β,17α,21-tetrol 20-on
α-THF	654	31.74	α-THF	5α-P-3α,11β,17α,21-tetro 20-on

ERGEBNISSE

Normalprofile

Um Steroidausscheidungen miteinander vergleichen zu können, musste man "Normalwerte" bzw. "Normalprofile" festlegen. Dies ist aber wegen der starken individuellen und zeitlichen Schwankungen (Cyclus!) in der Steroidausscheidung nicht exakt möglich. Wir haben daher ein durchschnittliches Profil (Fig. 1) willkürlich als Normalprofil festgelegt.

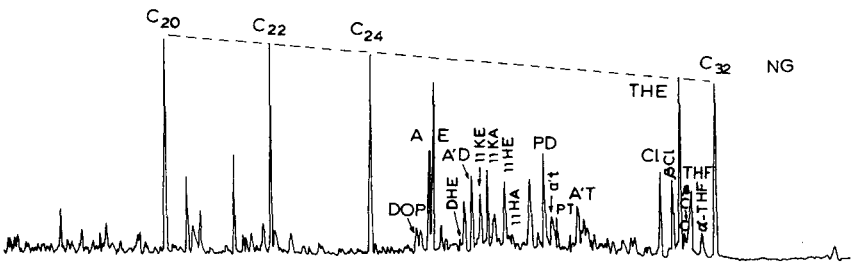

Fig. 1. Harnsteroidprofil einer gesunden Frau, 72 h Urin, 10. bis 12. Cyclustag.

Harnsteroidprofile von hirsuten Frauen

Die Steroidprofile von 24 unter idiopatischem Hirsutismus leidenden Frauen wurden vor Ausführung des Suppressions-Stimulationstestes aufgenommen. Sie unterschieden sich teilweise beträchtlich von Normalprofilen und liessen sich in drei Gruppen einteilen.

In der ersten Gruppe, die 3 Patientinnen umfasste, konnten wir keine auffallende Änderung der Profile feststellen.

Die zweite Gruppe (6 Patientinnen) zeigte gegenüber der Norm um das Doppelte erhöhte Werte in der Ausscheidung von Androstentriol und Pregnantriol, die aber entsprechend unseren Erfahrungen noch nicht als Hinweis auf Stoffwechselstörungen gewertet werden dürfen.

Die dritte Gruppe (14 Patientinnen umfassend) zeigte eine ausserordentlich stark erhöhte Ausscheidungsrate von Dehydroepiandrosteron (Fig. 2). In diesen Fällen lag die 17-Ketosteroidausscheidung, die in üblicher Weise nach Vestergaard [1] bestimmt wurde, an der oberen Grenze der Norm. Das ist verständlich, weil selbst eine gegenüber der Norm (μg-Mengen pro Tag) auf das 50—100-fach erhöhte Menge DHEA grössenordnungsmässig etwa der des Androsterons oder Etiocholanolons entspricht.

Steroidprofile hirsuter Frauen während des DXM-HCG-Testes

Zur Abklärung dieser Fälle von idiopatischem Hirsutismus mit extrem hoher DHEA-Ausscheidung untersuchten wir die Steroidprofile der Patientinnen unter Suppression der Adrenals (NNR) durch DXM und Stimulation der Ovarien durch HCG bei fortgesetzter Suppression der NNR mit DXM.

Die Fig. 2—4 zeigen die Änderungen des Steroidprofiles einer hirsuten Frau mit stark erhöhter DHEA-Ausscheidung. Als endogener Standard wurde ein Pentahydroxycholestan benutzt, dessen Ausscheidungsrate sich während des Testes nach bisherigen Beobachtungen nicht ändert.

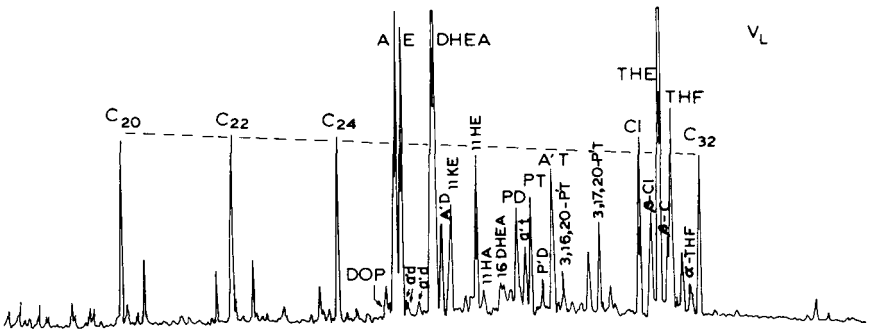

Fig. 2. Urinsteroidprofil einer 22-jährigen hirsuten Frau, Steroidausscheidung vor dem DXM—HCG-Test (10.—12. Tag des Cyclus).

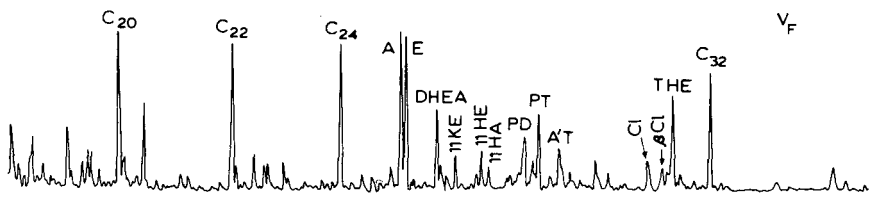

Fig. 3. Urinsteroidprofil derselben Frau (Fig. 2) bei Suppression der NNR durch DXM (3. Tag der DXM-Medikation).

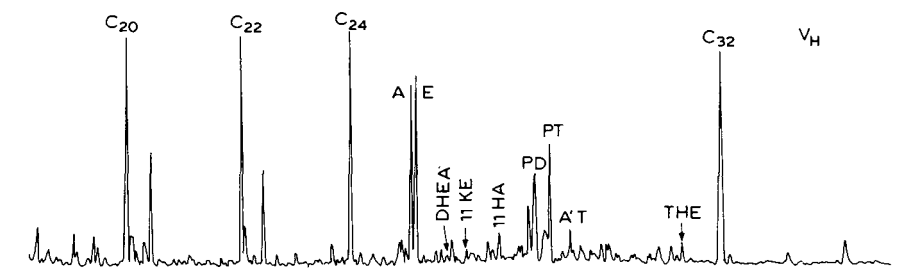

Fig. 4. Urinsteroidprofil derselben Frau (Fig. 2) bei Stimulierung der Ovarien durch HCG und gleichzeitiger Suppression der NNR durch DXM. (6. Tag der DXM, 3. Tag der HCG-Medikation).

Gegenüber dem Profil vor dem Test (Fig. 2) geht unter DXM-Bremsung der NNR (Fig. 3) die Ausscheidung von DHEA auf etwa ein Zehntel der Basalausscheidung zurück. Gleichzeitig ist ein deutlicher Abfall der Ausscheidung von Androgenen (A, E, 11-KE, 11-HA, A'T) und Corticosteroiden (Cl, β-Cl, THE, THF, β-Cl) zu sehen. Der Abfall an DHEA übertrifft den der anderen Androgene A und E beiweitem.

Die Stimulierung der Ovarien bei fortgesetzter Bremsung der NNR ist aus den Profilen schlecht erkennbar: In Fig. 4 ist die fortgesetzte Bremsung der NNR an der weiter verminderten Ausscheidung von DHEA, 11-KE, A'T und THE zu

erkennen, während die Stimulierung der Ovarien nur durch eine leichte Erhöhung von A, E, PD und PT angezeigt wird.

Beispiel eines abnormen Steroidprofils

Beschreibung der Patientin R.H. Eine 38-jährige adipöse Patientin (156 cm, 81.5 kg) beklagte sich über den in den letzten drei Jahren ständig zunehmenden Hirsutismus, Vertiefung der Stimme und Striae distensae im Bereiche des unteren Abdomens. Zusätzlich traten in den letzten 9 Monaten Regelstörungen auf.

Klinische Daten. Menarche mit 12 Jahren; 1965 Keilexcision der Ovarien (histologischer Befund: polycystische Ovarien). 1967 Fehlgeburt im 5. Monat; seit 1971 Diabetes mellitus. Bei der Röntgenuntersuchung der Sella turcica wurden keine pathologischen Veränderungen festgestellt.

Hormonspiegel (Tabelle II). Schon bei der Gruppenbestimmung der 17-Ketosteroide wurden extrem niedrige Werte gefunden, während die Ausscheidung von Corticosteroiden und die 17β-Hydroxyandrogene im Plasma im Bereich der Norm lagen.

TABELLE II

HORMONSPIEGEL

Hormon	Tag (Medikament)		
	1—3	6(DXM)	9(DXM + HCG)
Plasma-17β-Hydroxy-androgene (ng pro 100 ml)	76.2	20.0	40.0
Harn-17-keto-steroide (mg pro Tag)	1.98	0.63	0.54
Harn-17α-Hydroxy-corticosteroide (mg pro Tag)	5.20	–	1.64

Steroidprofile. Das Steroidprofil dieser Frau (Fig. 5) unterschied sich in signifikanter Weise von allen anderen: Es zeigte eine extrem niedrige Ausscheidung von Androsteron und Etiocholanolon sowie 11 KE und 11 HE und niedrigere, aber normale Ausscheidungsraten für Corticosteroide oder Corticosteroidmetaboliten (Cortolon, THE, THF), Pregnandiol und Pregnantriol. Besonders auffällig war die extrem hohe Ausscheidung an 16α-hydroxylierten Androstanen (16 DHEA und 16 βDHA), insbesondere an Androstentriol (A′). Solche Änderungen der Steroidprofile lassen sich mit konventionellen Steroidbestimmungsmethoden nicht erkennen, weil man zunächst gar nicht auf die Vermutung käme, nach derartigen Steroiden zu suchen. Sie werden erst offenkundig, wenn man die Gesamtsteroidfraktion untersucht.

Im Verlaufe des DXM—HCG-Testes ging die Ausscheidungsrate des Androstentriols sowie die der 17-Ketosteroide und Corticoide — wie erwartet — zurück (Fig. 6), während beim Stimulationstest (Fig. 7) eine geringe Steigung der Ausscheidungsrate an Pregnendiol und einiger unbekannter Stoffe, bei denen es sich nach Aussagen der Massenspektren nicht um Steroide handelte, zu beobachten war.

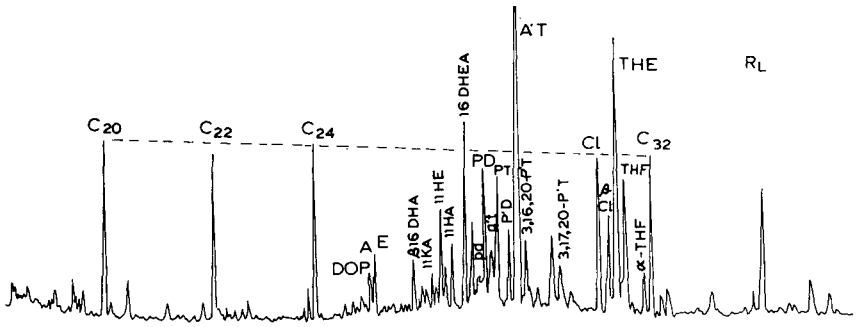

Fig. 5. Urinsteroidprofil der Patientin R.H. Steroidausscheidung vor dem DXM-HCG-Test.

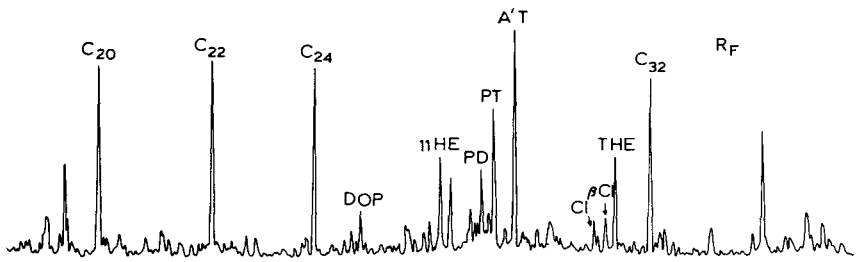

Fig. 6. Urinsteroidprofil der Patientin R.H. bei Suppression der NNR durch DXM (3. Tag der DXM-Medikation).

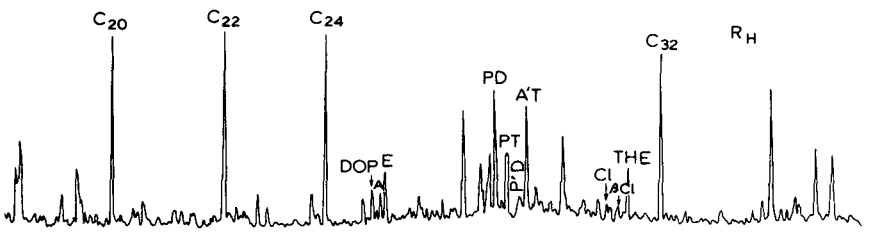

Fig. 7. Urinsteroidprofil der Patientin R.H. bei Stimulierung der Ovarien durch HCG und gleichzeitige Suppression der NNR durch DXM (6. Tag der DXM, 3. Tag der HCG-Medikation).

DISKUSSION

Die um das 10—100-fach erhöhte Ausscheidung an DHEA im Harn von mehr als 50% der von uns untersuchten Fälle von idiopathischem Hirsutismus scheint uns auf eine Funktionsstörung hinzudeuten und somit die Möglichkeit zu bieten, diese Fälle von Hirsutismus von anderen abzugrenzen. Damit könnte die Bestimmung von Dehydroepiandrosteron im Harn die Steroidbestimmungsmethoden im Blut [22] ergänzen, insbesondere deshalb, da die GC-Analyse relativ einfach ausführbar ist.

In diesem Zusammenhang erscheint es bemerkenswert, dass Gaschromatogramme, die von Harnproben hirsuter Frauen publiziert wurden, in manchen Fällen eine relativ starke Steigerung der DHEA-Ausscheidung erkennen lassen.

Diese wurden aber entweder nicht beachtet oder falls erkannt, doch nicht als charakteristisch angesehen [2, 12, 19, 23].

Die Beobachtung, dass bei Stimulierung der Ovarien und gleichzeitiger Suppression der NNR die DHEA Ausscheidung weiter stark abfällt, lässt vermuten, dass die Ovarien wohl nicht für die übermässige DHEA Produktion verantwortlich sind, obwohl nach Goldzieher [24] eine scharfe Trennungslinie zwischen der Steroid Biosynthese in Ovar und NNR nicht zu ziehen ist, wie durch Untersuchungen von Givens et al. [25] und Blichert-Toft et al. [26] belegt wurde.

Auffallend ist der starke Rückgang der DHEA-Produktion bei DXM Suppression der NNR im Vergleich zu den anderen 17-Ketosteroiden wie A und E. Dies weist möglicherweise auf eine vorzugsweise Hemmung der Bildung des Faktors hin, der die DHEA-Produktion der NNR anregt. Dieser Faktor muss nicht identisch sein mit dem ACTH [27] worauf auch die ungleiche Abnahme der Steroidproduktion hindeuten könnte.

Während GC-Untersuchungen zur Erkennung von Fällen mit hoher DHEA Ausscheidung ausreichend erscheinen, erfordert die Steroididentifizierung bei ungewöhnlichen Profilen, wie im letzten Falle (Fig. 5—7) die Anwendung der Kombination Glaskapillar-GC—MS. In diesem Falle könnte eine Fehlfunktion der NNR vorliegen, worauf die geringe Produktion von A und E hindeutet. Möglicherweise wird relativ viel DHEA produziert, jedoch extrem schnell weiter umgesetzt. Androstentriol ist nämlich ein Metabolit von DHEA, der in der Nebennierenrinde und Lebergewebe gebildet wird [30] . Als mittlere Ausscheidungsrate wurde von Jänne 424 μg pro Tag [28] angegeben. Wenn man davon ausgeht, dass die 17-Ketosteroidausscheidung bei 1.98 mg pro Tag und die der 17-Hydroxycorticoide bei 5.2 mg pro Tag gefunden wurde, ergibt sich aus der Betrachtung der Gaschromatogramme, dass die Ausscheidung an Androstentriol über 10 mg pro Tag liegen muss. Auch 16α-Hydroxydehydroepiandrosteron ist ein Metabolit von DHEA.

DANK

Wir danken der Deutschen Forschungsgemeinschaft sowie dem Fonds der Chemischen Industrie für die Unterstützung der Arbeit durch Sachbeihilfen. Ein weiterer Teil der Arbeit wurde aus Toto-Lotto-Mitteln unterstützt, dafür danken wir dem Land Niedersachsen.

ZUSAMMENFASSUNG

In über 50% der Fälle von idiopathischem Hirsutismus wurde eine im Vergleich zu Normalwerten um das 10—100fach erhöhte Ausscheidung von DHEA fesstgestellt. Die Dehydroepiandrosteronausscheidung fällt bei Suppression der Nebennierenrinde mit DXM viel stärker ab als die der anderen 17-Ketosteroide. Innerhalb einer Woche erreicht sie Werte, die unter der Nachweisgrenze liegen. Dagegen wurde bei Stimulation mit HCG unter fortlaufender Suppression mit DXM in keinem Fall ein Anstieg des DHEA beobachtet.

NOTIZ BEI DER KORREKTUR

Während der Drucklegung erschien eine Arbeit von Pal [29], in der die er-

höhte Ausscheidung an DHEA im Harn hirsuter Frauen spektralphotometrisch nachgewiesen wurde. Dadurch werden die hier niedergelegten Ergebnisse gestützt und ergänzt.

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Biomedical Applications

CHROMBIO. 140

CATECHOLAMINES AND RELATED COMPOUNDS

EFFECT OF SUBSTITUENTS ON RETENTION IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The capacity factors of 32 compounds were measured on octadecyl-silica columns by using neat aqueous phosphate buffer, pH 2.1, as the eluent. The tabulated results allow the estimation of the effect of various substituents on the retention of catecholamines and related compounds under similar chromatographic conditions.

INTRODUCTION

With the sophisticated instrumentation presently available, liquid chromatography has become a precision microanalytical tool. Recent developments in column technology gave rise to novel high efficiency columns made with so-called bonded phases, which contain an organic moiety covalently bound to the surface of 5- or 10-μm silica particles. The most widely used bonded phases are those with hydrocarbonaceous functions such as octadecyl groups and the technique in which such nonpolar stationary phases are used is commonly referred to as reversed-phase chromatography. The popularity of reversed-phase chromatography is mainly due to the simplicity of the chromatographic system as well as the wide variety of sample mixtures which can be conveniently analyzed by this technique.

Recently the method has been successfully employed for the analysis of catecholamines and related substances [1, 2]. These compounds have wide ranging physiological significance [3, 4] and their analysis is of great interest

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in the life sciences and medicine. For this reason we present the retention values of 32 compounds and attempt to quantify the effect of various substituents on their retention. The results can facilitate the prediction of their elution order in reversed-phase chromatography as well as the identification of certain peaks on the chromatogram of mixtures containing such substances.

EXPERIMENTAL

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Model 601 high-pressure liquid chromatograph with a Rheodyne (Berkeley, Calif., U.S.A.) Model 7010 sample injector and a Schoeffel (Westwood, N.J., U.S.A.) Model 770 variable wavelength UV detector was used. Chromatograms were obtained at 200 nm detector setting with a Model 54 (Perkin-Elmer) strip-chart recorder. All measurements were carried out by using 5-μm LiChrosorb RP-18 columns (Rainin, Boston, Mass., U.S.A.). The No. 316 stainless steel columns were 250 × 6.4 mm O.D. × 4.6 mm I.D.

All experiments were carried out with 0.1 *M* phosphate buffer, pH 2.1, by isocratic elution at a flow-rate of 2 ml/min. Reagent grade H_3PO_4 and KH_2PO_4 were supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.). The column temperature was maintained at 70° by using the oven of the liquid chromatograph. The column inlet pressure was 160 bar (2200 p.s.i.).

The samples were supplied by Sigma (St. Louis, Mo., U.S.A.) and Aldrich (Milwaukee, Wisc., U.S.A.). Stock solutions of the substances were made in the eluent. The amount of the individual substances in the 10–μl samples injected into the chromatograph was about 1 μg. The elution time of an unsorbed solute, t_M, was measured as described previously [5]. The retention time of the solutes, t_R, was evaluated at the peak maxima as the peaks were almost symmetrical. The capacity factors, k, were calculated by the following relationship

$$k = (t_R - t_M)/t_M$$

The relative retention values, α, were obtained as the ratios of the pertinent capacity factors.

RESULTS AND DISCUSSION

The chromatograms in Fig. 1 illustrate the speed and efficiency of the chromatographic system used in this study.

In our experience, the chromatographic conditions stated are particularly advantageous for the separation of mixtures containing compounds described here. The employment of 5-μm octadecyl-silica columns at elevated temperatures offers relatively high efficiency even with neat aqueous eluents. At elevated temperatures the column inlet pressure does not exceed the practical limits even at relatively high flow-rates. The small particle size of the stationary phase and the high linear flow-velocity of the eluent together afford high speed and efficiency.

Although most work in reversed-phase chromatography is performed with hydro-organic eluents, we have found [1, 6] that the use of neat aqueous

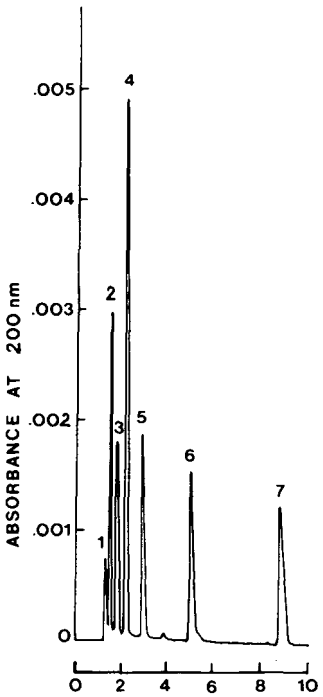

Fig. 1. Typical chromatogram of some compounds investigated. Column, 5-μm LiChrosorb RP 18, 25 × 0.64 cm O.D. × 0.46 cm I.D.; eluent, 0.1 *M* phosphate buffer, pH 2.1; flow-rate, 2 ml/min; temp, 70°, inlet pressure, 160 bar. The elution order of the substances, whose symbols are given in Table I, is as follows: 1 = NE; 2 = E; 3 = DA; 4 = TA; 5 = PEOA; 6 = PEA; 7 = DMDA.

eluents offer certain advantages in the separation of such polar biological substances. In the present study neat aqueous phosphate buffer (without any organic solvent) was used also to provide a meaningful reference framework for the retention values. At the pH of the eluent (2.1) the amino groups in the solute molecules were fully protonated, whereas for the most part the dissociation of carboxyl groups was suppressed. Under such conditions, fairly symmetrical peaks could be obtained. Octadecyl-silica was employed for two reasons. First of all, among the commercially available hydrocarbonaceous bonded phases octadecyl-silica is used most widely. Second, under otherwise fixed conditions the retention values have been found to be the greatest on such a stationary phase having a high carbon content and for the separation of such relatively polar substances a strongly-retentive column is required.

The structure of most substances under investigation can be described by the general formula given in Fig. 2. The symbols used for these compounds, the substituents of each substance according to the structure given in Fig. 2 and their capacity factors, which have been measured under conditions stated in the experimental section, are listed in Table I.

In Tables II—IV the effect of various substituents on the retention is shown.

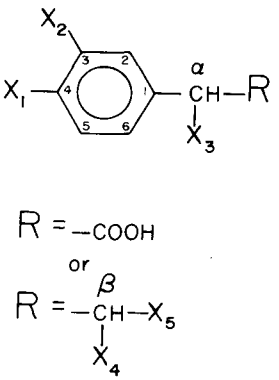

Fig. 2. Generalized structural formula of the substances investigated.

In each case the capacity factor of both the parent compound and that of a derivative, in which a hydrogen is replaced by the substituent are listed. For each solute pair the relative retention of the derivative with respect to its parent compound, α_{SP}, together with the corresponding log α_{SP} value are also given. The data offer an overview on the effect of the various substituents on the retention of this type of compound.

The results can be broadly interpreted in terms of the solvophobic theory which has been successfully applied to reversed-phase chromatography [6—8]. According to this approach, solute retention is governed by the balance of two solvent effects on the reversible association of the solute with the hydrocarbonaceous functions of the stationary phase. The reduction in the molecular surface area upon binding and the increase in surface tension of the eluent augment retention due to the free energy change associated with the cavity effect [6]. On the other hand, an increase in the energy of interaction between the solute and the solvent reduces binding. This interaction can be broken down to van der Waal's interaction, which is primarily dependent on the molecular size of the solute and solvent, and electrostatic interactions. The latter are determined by the dipole moment and/or the charges of the molecule. With our solutes of similar molecular dimensions and with a fixed eluent, the magnitude of relative retention is primarily affected by the "polarity" of the respective substituents which play a major role in determining the energy of solute interaction with water. Consequently, the degree of dissociation of ionogenic groups in the solute molecules has also a great influence on retention in such chromatographic systems. This effect has been analyzed both theoretically and experimentally [7].

As shown in Table II, the attachment of hydroxyl groups to the aromatic ring reduces retention in such a way that the effect of the first hydroxyl group is significantly greater than that of the second. On the other hand the replacement of a hydrogen on the aromatic ring by a methoxy group brings about an increase in the size of the molecule by this "unpolar" group. The effect is, of course, much more pronounced on retention when a polar hydroxyl group is replaced by a methoxy group.

Table III shows that the replacement of a hydrogen on the α-carbon atom by a hydroxyl group greatly reduced the retention. In view of the solvophobic

theory, this effect is likely to be caused by drastic changes in the molecular properties such as the dipole moment [6]. With the last four solute pairs (R = COOH) the change in relative retention is significantly greater than in the other cases. The introduction of an α-hydroxyl group into carboxylic acids

TABLE I.

STRUCTURE AND CAPACITY FACTOR OF THE SUBSTANCES INVESTIGATED

The meaning of the substituents is given in Fig. 2.

Symbol	Name	Structure				Capacity factor
		X_1	X_2	X_3	R	k
AMINES						
NE	Norepinephrine	OH	OH	OH	CH_2NH_2	0.145
OCT	Octopamine	OH	H	OH	CH_2NH_2	0.26
E	Epinephrine	OH	OH	OH	CH_2NHCH_3	0.28
NMET	Normetanephrine	OH	OCH_3	OH	CH_2NH_2	0.48
SYN	Synephrine	OH	H	OH	$CH(CH_3)NH_2$	0.51
DA	Dopamine	OH	OH	H	CH_2NH_2	0.56
MET	Metanephrine	OH	OCH_3	OH	CH_2NHCH_3	0.93
TA	Tyramine	OH	H	H	CH_2NH_2	0.96
PEOA	Phenylethanolamine	H	H	OH	CH_2NH_2	1.03
3MDA	3-O-Methyldopamine	OH	OCH_3	H	CH_2NH_2	1.86
PEA	Phenylethylamine	H	H	H	CH_2NH_2	4.06
NIE	Norisoephedrine	H	H	NH_2	$CH(OH)CH_3$	5.17
EPH	Ephedrine	H	H	OH	$CH(CH_3)NHCH_3$	6.76
DMDA	Dimethyldopamine	OCH_3	OCH_3	H	CH_2NH_2	9.09
ACIDS						
DOMA	Dihydroxymandelic	OH	OH	OH	COOH	0.51
POMA	*p*-Hydroxymandelic	OH	H	OH	COOH	0.87
MOMA	*m*-Hydroxymandelic	H	OH	OH	COOH	1.66
VMA	Vanilmandelic	OH	OCH_3	OH	COOH	1.69
DOBA*	3,4-Dihydroxybenzoic	OH	OH	—	—	2.66
DOPAC	Dihydroxyphenylacetic	OH	OH	H	COOH	4.42
MA	Mandelic	H	H	OH	COOH	5.57
POPAC	*p*-Hydroxyphenylacetic	OH	H	H	COOH	7.57
VA*	Vanillic	OH	OCH_3	—	—	9.13
HVA	Homovanillic	OH	OCH_3	H	COOH	14.67
PAC	Phenylacetic	H	H	H	COOH	47.80
AMINO ACIDS						
DOPS	Dihydroxyphenylserine	OH	OH	OH	$CH(NH_2)COOH$	0.15
DOPA	Dihydroxyphenylalanine	OH	OH	H	$CH(NH_2)COOH$	0.57
TYR	Tyrosine	OH	H	H	$CH(NH_2)COOH$	0.98
PSER	Phenylserine	H	H	OH	$CH(NH_2)COOH$	1.04
3MDOPA	3-O-Methyl-DOPA	OH	OCH_3	H	$CH(NH_2)COOH$	1.88
PHE	Phenylalanine	H	H	H	$CH(NH_2)COOH$	3.85
TRP*	Tryptophane	—	—	—	—	7.18

*The general formula in Fig. 2 is not applicable to the structure of these compounds.

generally results in a reduction of the pK_a values due to the above mentioned effect. Thus, the mandelic acid derivatives are likely to more dissociated at the eluent pH than the corresponding parent compounds, which are phenylacetic acid derivatives, and this also could contribute to the observed large decrease in relative retention.

Upon introduction of a charged ammonium group into the molecule, retention decreases considerably as shown by the examples at the top of Table IV. In the next section the retention values for zwitterionic amino acids and the corresponding amines are compared. It is seen that at pH 2.1 the relative retention of these solute pairs is very close to unity. As expected the reten-

TABLE II

EFFECT OF RING SUBSTITUTION (X_1, X_2) ON THE CAPACITY FACTORS OF THE SUBSTITUTED DERIVATIVE, S, AND THE PARENT COMPOUND, P, AND ON THE RELATIVE RETENTION, α_{SP}

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
X_1 = OH	X_1 = H				
TA	PEA	0.96	3.90	0.25	−0.61
OCT	PEOA	0.26	1.03	0.25	−0.60
TYR	PHE	0.98	3.85	0.25	−0.59
POMA	MA	0.87	5.57	0.16	−0.81
POPAC	PAC	7.57	47.80	0.16	−0.80
X_2 = OH	X_2 = H				
NE	OCT	0.145	0.26	0.56	−0.25
DA	TA	0.56	0.96	0.58	−0.23
DOPA	TYR	0.57	0.98	0.58	−0.24
DOMA	POMA	0.51	0.87	0.59	−0.23
MOMA	MA	1.66	5.57	0.30	−0.53
DOPAC	POPAC	4.42	7.57	0.58	−0.23
X_2 = OCH_3	X_2 = H				
NMET	OCT	0.48	0.26	1.85	0.27
VMA	POMA	1.69	0.87	1.94	0.29
3MDA	TA	1.86	0.96	1.94	0.29
3MDOPA	TYR	1.88	0.98	1.92	0.28
HVA	POPAC	14.67	7.57	1.94	0.29
X_2 = OCH_3	X_2 = OH				
NMET	NE	0.48	0.145	3.31	0.52
MET	E	0.93	0.28	3.32	0.52
VMA	DOMA	1.69	0.51	3.31	0.52
3MDA	DA	1.86	0.56	3.32	0.52
3MDOPA	DOPA	1.88	0.57	3.30	0.52
HVA	DOPAC	14.67	4.42	3.32	0.52

TABLE III

EFFECT OF SUBSTITUTION (X_3) AT THE α-CARBON ATOM ON RETENTION

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
X_3 = OH	X_3 = H				
NE	DA	0.145	0.56	0.26	−0.59
DOPS	DOPA	0.15	0.57	0.26	−0.58
OCT	TA	0.26	0.96	0.27	−0.57
PSER	PA	1.04	3.85	0.27	−0.57
NMET	3MDA	0.48	1.86	0.26	−0.59
PEOA	PEA	1.03	4.06	0.25	−0.60
DOMA	DOPAC	0.51	4.42	0.115	−0.938
POMA	POPAC	0.87	7.57	0.115	−0.940
VMA	HVA	1.69	14.67	0.115	−0.939
MA	PAC	5.57	47.80	0.117	−0.934

TABLE IV

EFFECT OF SUBSTITUTION (R) AT THE β-CARBON ATOM

S	P	k_S	k_P	α_{SP}	$\log \alpha_{SP}$
R = $CH(NH_2)COOH$	R = CH_2COOH				
DOPA	DOPAC	0.57	4.42	0.129	−0.890
TYR	POPAC	0.98	7.57	0.129	−0.890
PHE	PAC	3.85	47.80	0.081	−1.092
PSER	MA	1.04	5.57	0.187	−0.729
3MDOPA	HVA	1.86	14.67	0.127	−0.897
R = $CH(NH_2)COOH$	R = CH_2NH_2				
DOPA	DA	0.57	0.56	1.02	0.01
TYR	TA	0.98	0.96	1.02	0.01
3MDOPA	3MDA	1.88	1.86	1.01	0.00
PSER	PEOA	1.04	1.03	1.01	0.00
DOPS	NE	0.15	0.145	1.03	0.01
PHE	PEA	3.85	4.06	0.95	−0.02
R = CH_2NH-CH_3	R = CH_2NH_2				
E	NE	0.28	0.145	1.93	0.29
MET	NMET	0.93	0.48	1.94	0.29
R = $CH(CH_3)NH_2$	R = CH_2NH_2				
SYN	OCT	0.51	0.26	1.96	0.29

tion increases upon substituting a hydrogen, which is attached to the nitrogen or carbon, by a methyl group, as shown in the two lower sections of Table IV. Similarly an intercalating methylene group also increases the retention of a homologue with respect to that of the parent substance as demonstrated in Table V. Nevertheless, the log α value for the methylene group is only about half the log k increment found for aliphatic α-amino acids under similar chromatographic conditions [9].

The relative retention values, which express the effect of a given substituent, are surprisingly consistent. In most cases, however, the magnitude of the α-values is determined by the charge on the molecules, i.e., α_{SP} of a given substituent is not the same for neutral molecules as for those containing an ammonium group. Such an effect of ionization is expected in view of the different behavior of monopoles, dipoles and zwitterions [7].

In order to illustrate further the effect of a charged amino group on the retention, the data are arranged in a different way in Table VI. In compounds A the substituent R on the α-carbon atom is invariably a carboxylic group whereas in substances B the moiety R contains a primary amino group which is assumed to be fully protonated at the pH of the eluent. Thus, the relative retention values, α_{AB}, give an indication of the effect of replacing a fully ionized substituent by a carboxyl group which can be more or less dissociated under the conditions of the experiment. The pK_a values for the carboxylic groups of the compounds under investigation fall in the range 2—4.5 [10]. The acids having a hydroxyl group on the α-carbon (X_3 = OH) have generally a lower pK_a, therefore, a higher degree of dissociation than those in which X_3 = H at the eluent pH 2.1 The effect of the ionization of a carboxyl group on the retention of weak acids has extensively been investigated [7] and the findings have been corroborated in a study on the effect of pH on the retention of amino acids [9]. Full protonation of carboxylic groups having pK_a values below 3 requires an eluent pH significantly lower than 2 and it is not recommended to use such acidic eluents in commercially available liquid chromatographs. According to the theory [7] the increase in the relative retention should be greater when a largely undissociated carboxyl group is replaced by a substituent containing a protonated amino group than that occurs upon the replacement of a largely dissociated carboxyl group. Indeed the relative retention values, with two exceptions, are quite consistent when arranged in two groups depending on the substituent X_3, as seen in Table VI. Nevertheless, the relatively large effect observed with substances having a hydroxyl group on the α-carbon atom cannot be fully accounted for by the greater

TABLE V

EFFECT OF A 1-CH_2-GROUP ON THE RETENTION

Substituent	S	P	k_S	k_P	α_{SP}	log α_{SP}
—CH_2—	HVA	VA	14.67	9.13	1.61	0.21
	DOPAC	DOBA	4.42	2.66	1.66	0.22

dissociation of the adjacent carboxyl group. A more detailed analysis regarding the changes in the dipole moments upon introduction of such a substituent is required for a satisfactory theoretical interpretation [7, 8].

The relative retention values presented here can be used to estimate the retention of this type of compound. Thus, the tentative identification of certain peaks on the chromatogram obtained under similar conditions is facilitated. The sample population is not large enough to establish a statistically acceptable quantitative structure-retention relation. Nevertheless, the data appear to be consistent enough to express quantitatively the effect of replacing a hydrogen by certain substituents on the retention. Such substituent parameters which are given by the appropriate log k increments, are designated by τ and listed in Table VII. The meaning of these parameters is similar to that of the π-values introduced by Hansch [11] for use in quantitative structure—activity relationships (QSAR), in medicinal chemistry and related areas. The π-values are obtained from partition coefficients measured in octanol—water system as opposed to our τ-values which come from chromatographic measurements by using a bonded hydrocarbonaceous stationary phase. Nevertheless, both phenomena are subject to similar linear free-energy relationships which have wide currency in physico-chemical investigations [12]. Indeed, the log k values of certain amino acids have been found to be co-linear with the logarithm of the corresponding partition coefficients in octanol-water [9]. Unfortunately, literature data are not available to compare the τ values listed in Table VII with the corresponding π values. On the other hand, a general dis-

TABLE VI

EFFECT OF REPLACEMENT OF R CONTAINING AN IONIZED AMINO GROUP BY A CARBOXYLIC GROUP

	A	B	k_A	k_B	α_{AB}	$\log \alpha_{AB}$
R	COOH	$CH_2NH_3^+$				
X_3 = OH	POMA	OCT	0.87	0.26	3.35	0.53
	VMA	NMET	1.69	0.48	3.52	0.55
	DOMA	NE	0.51	0.145	3.52	0.55
X_3 = H	HVA	3MDA	14.8	1.86	7.96	0.90
	DOPAC	DA	4.42	0.56	7.89	0.90
	POPAC	TA	7.57	0.96	7.89	0.90
R	COOH	$CH(NH_3^+)COOH$				
X_3 = OH	DOMA	DOPS	0.51	0.15	3.40	0.53
	MA	PSER	5.57	1.04	5.36	0.73
X_3 = H	DOPAC	DOPA	4.42	0.57	7.75	0.89
	POPAC	TA	7.57	0.98	7.72	0.89
	HVA	3MDOPA	14.67	1.88	7.80	0.89
	PAC	PHE	47.8	3.85	12.42	1.09

TABLE VII

LIST OF THE LOG K INCREMENTS, τ, OBTAINED FOR THE EFFECT OF REPLACING A HYDROGEN BY A GIVEN SUBSTITUENT ON THE RELATIVE RETENTION.

Substituent	τ	Substituent	τ
4-OH	−0.80*	α-NH_3^+	−0.89
	0.60		
3-OH	−0.53***	β-NH_2^+-CH_3	0.29
	−0.24 §		
3-OCH_3	0.52	β-CH_3	0.29
α-OH	−0.94*		
	−0.58**		

*no ionized amino group in the molecule
**ionized amino group in the molecule
***X_1 = H
§ X_1 = OH

cussion of the use of chromatographic data in this particular field is given in the review by Tomlinson [13].

In using the data presented here a caveat is necessary, however. As has already been pointed out, the extent of dissociation of ionogenic groups in the molecules has a great effect on the magnitude of both the capacity factors and the relative retentions. Since the degree of dissociation depends on the pK_a values of the ionogenic groups and the pH of the eluent, the data presented here rigorously apply only at pH 2.1. When chromatographic analysis is carried out at higher pH, the ionization of the acidic and basic groups may change. The concomitant changes in retention, however, can be estimated on the basis of earlier treatment [7]. That the extent of the dissociation of ionogenic groups in the molecule has to be taken into account is also apparent from the two different τ-values obtained for the same substituents in charged and neutral species. It should be kept in mind, therefore, that the pH of the medium plays an important role in determining not only the capacity factors but also the τ-values for ionogenic substances. On the other hand, a variety of non-polar bonded stationary phases, which differ in hydrocarbon chain length, carbon loading and silica support, are commercially available. The properties of the stationary phases have an effect on the optimum eluent composition for such separations and the relative retention values likely to be different in another chromatographic system.

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Biomedical Applications

CHROMBIO. 135

RAPID, QUANTITATIVE HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHY OF PSEUDOURIDINE*

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SUMMARY

A rapid, precise, and accurate chromatographic method for the determination of pseudouridine (ψ) in urine by high-performance liquid chromatography (HPLC) has been developed. The ribonucleosides were first isolated with an affinity gel containing immobilized phenylboronic acid. The response for ψ was linear well above and below the range necessary to determine urinary ψ. Good precision was obtained for both matrix-dependent and matrix-independent samples. Supporting experimental data are presented on precision, recovery, chromatographic methods, sample cleanup and application to the analysis of urine samples from normal males and females, and patients with advanced colon cancer. In a comparison of 40 normals with 10 colon cancer patients, 9 of the 10 patients had a ψ:creatinine (Cr) ratio greater than $\bar{x} + 2\sigma$ for the normal population. This HPLC method is now being used extensively in our laboratory as a routine method for determination of ψ in urine from patients with various types of cancer and in chemotherapy response studies. Data are presented on the dynamics of ψ excretion by normal males and females. When the excretion of ψ was normalized with the excretion of creatinine, it was noted that samples collected at random have the same ψ:Cr ratio value as for the 24-h total collection, thus, allowing the use of random samples. The constancy of the ψ:Cr ratio implies that RNA turnover is constant and ψ excretion is independent of diet. Base values are presented for the ψ:Cr

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ratio for normal males 22.6, and females 26.8 (nmoles/μmole). Excellent agreement was found for the values obtained by this HPLC method, with published ion-exchange and gas—liquid chromatography data. This method offers a potential as a screening test for various types of cancer and contributes to biochemical research.

INTRODUCTION

Many RNA researchers are interested in studying the synthesis and regulatory activity of RNA in vivo. Pseudouridine (ψ), the only carbon—carbon ribofuranosyl nucleoside, is found only in RNA and at high concentration. Pseudouridine is not re-incorporated and the lack of catabolic breakdown [1, 2] of ψ should make the excretion rate of this nucleoside an ideal index of RNA activity. Above normal levels of ψ have been found in the urine of patients with various types of cancer [3—6] while recently Borek et al. [7] have reported an elevated tRNA turnover rate in tumor tissue. This suggests that the relationship between RNA metabolism and cancer merits further study.

Several chromatographic methods for the determination of ψ in urine have been developed. Randerath's group developed a thin-layer chromatographic (TLC) method [8] using chemical tritium labeling prior to chromatographic separation and quantitation by liquid scintillation counting of the spots scraped from the TLC plates. This method gives good sensitivity but the sample preparation is laborious and the low resolving power of TLC leads to some problems of incomplete separation.

The high resolution ion-exchange chromatographic (IEC) methods of Uziel [9] and Mrochek [6, 10] require long column regeneration time. Chang et al. [11] have developed a gas—liquid chromatographic (GLC) method for ψ but extensive charcoal cleanup and derivatization for volatility are required. Recently Gehrke and co-workers [12—14] reported on the chromatography and an elegant method for the determination of nucleosides in urine by reversed-phase HPLC with ultraviolet detection at 254 nm.

In this study, we modified our HPLC method for the rapid analysis of only ψ. The resulting chromatographic method is rapid, precise, accurate, and sensitive. The cleanup process was changed to make it more rugged and was also significantly shortened and simplified. The HPLC chromatography requires only 7.5 min per sample. In our laboratory this method is now being used for all routine ψ determinations.

EXPERIMENTAL

Apparatus

All chromatographic studies were conducted with Waters Assoc. (Milford, Mass., U.S.A.) equipment; M-6000A solvent delivery system, model U6K universal injector, and model 440 absorbance detector. The recorder used was a Fisher Recordall Series 5000. The column was a Waters Assoc. μBondapak C_{18}/Porasil (300 × 4 mm I.D.)

The temperature of the column was maintained at 24° using a constant temperature circulating bath, Haake Model FJ (Saddle Brook, N.J., U.S.A.),

connected to an aluminum column jacket. The jacket was composed of two aluminum blocks (24 × 7 × 2.2 cm) precisely grooved to accommodate two columns and a thermometer when bolted together. Each block had two holes (6.0 mm), drilled completely through the block lengthwise, and fitted with Swagelok fittings and copper tubing to allow the constant-temperature water to circulate along four sides of the columns before recycling through the bath. The aluminum column jacket blocks were specially designed and made in the University of Missouri, Science Instrument Shop.

Peak areas, retention times, and concentrations based on an external standard were calculated by a Hewlett-Packard 3352B Laboratory Data System (Hewlett-Packard, Avondale, Pa., U.S.A.). The system consists of a Hewlett-Packard 2100 computer with 16 K memory, 18652A analog to digital converters (A—D), ASR33 teletype, and a 2748B high speed photo reader. The columns used for the boronate gel were glass 5 × 150 mm (Fischer and Porter, Warminster, Pa., U.S.A.) modified by attachment of a 50-ml spherical reservoir to the top of the column. An Eppendorf Model 3200/30 microcentrifuge, Model 3300 rotary shaker, as well as various sizes of Eppendorf pipets (Brinkman, Westbury, N.Y., U.S.A.) were used in the sample cleanup procedure. A Micro Gram-Atic Balance (Mettler, Hightstown, N.Y., U.S.A.) was used to weigh milligram amounts of ψ for the calibration solutions.

Chemicals

The pseudouridine used in these investigations was obtained from Sigma, St. Louis, Mo., U.S.A. Other chemicals were purchased from the following sources. Ammonium acetate and formic acid A.C.S. certified grade (Fisher Scientific, St. Louis, Mo., U.S.A.), ammonium hydroxide, analytical reagent grade (Mallinckrodt, St. Louis, Mo., U.S.A.), ammonium dihydrogen phosphate (J.T. Baker, Phillipsburgh, N.J., U.S.A.). Methanol, distilled in glass (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.). All water used for the preparation of buffers and aqueous solutions was purified by a three-step process. The first step was reverse osmosis using an RO-Pure apparatus (DO640 Barnstead Co., Boston, Mass., U.S.A.). A nanopure D1794 four-cartridge water purification system was then used. A charcoal cartridge for adsorption of organics, two mixed bed ion-exchange cartridges for removal of cations and anions, and a filtration cartridge for removal of all particles larger than 0.22 μ were used. Finally, the nanopure water was distilled in a Corning all-glass still (Corning, Corning, N.Y., U.S.A.).

Buffers

A stock buffer concentrate was prepared as 2 l of a 2.0 *M* solution of $NH_4H_2PO_4$. This concentrate was then sterilized by filtering through a Millipore GS-22 filter (0.22 μ) and stored in glass at 4°. A 1-l volume of the working buffer was prepared daily by diluting a 5-ml aliquot of the buffer concentrate with ca. 200 ml of water, adding 10 ml of methanol, diluting the solution to 1 l with water and filtering through a Millipore GS-22 filter. Diluting the buffer concentrate prior to adding the methanol prevents the salt from precipitating out. Stored buffers were maintained in a cold room at 4° and discarded after five days.

Calibration standard solutions

A stock solution of ψ was prepared to give a concentration of about 1.00 m*M*/ml in distilled nanopure water. The working standard solution was a 10 μM solution. A 60-μl volume of this solution was used to calibrate the chromatography system.

Samples, collection, and storage

The urine samples were collected and maintained at ice temperature. Aliquot samples were frozen and stored at −70°. The normal male control urines were from laboratory personnel with an age span of 20—60 years. The cancer patients selected had advanced colon cancer and at the time of the urine collection the patients were not receiving anti-neoplastic drugs or other anti-tumor therapy. Urine samples from cancer patients were obtained through the courtesy of the National Cancer Institute Solid Tumor Service. Normal female volunteers from the local chapter of the American Cancer Society provided urine samples. Dr. James Hueser supervised the collection. No diet restrictions were imposed.

Phenylboronate affinity gel

An affinity gel with immobilized phenylboronic acid functionality was used for isolation of ribonucleosides prior to HPLC separation and quantitation of ψ [12]. Isolation is the most crucial step in the method. A detailed, stepwise procedure is described as follows:

CLEANUP OF URINE SAMPLES FOR CHROMATOGRAPHY OF ψ

Analytical method

Column preparation

(1) Place ca. 1 ml 0.25 *M* NH_4 Ac buffer (pH 8.8) in the column (Fischer and Porter No. 274—461, 150 × 5 mm, custom fitted with a 50-ml reservoir).

(2) Slurry the gel in its 0.1 *M* NaCl storage solution and transfer to the column with a Pasteur pipet (Fisher Scientific, No. 13-678-5B).

(3) Introduce the phenylboronate gel (200—400 mesh) below the surface of the buffer in the column. Care must be taken to prevent the gel from contacting the sides of the reservoir as it adheres to glass.

(4) Allow the column to begin draining and add more gel to a height of 40 mm (bed volume 0.8 ml).

(5) Rinse the gel with ca. 20 ml 0.25 *M* NH_4 Ac (pH 8.8) buffer. No pressure is used on the column. All solutions are allowed to drain by gravity flow. The flow-rate varies from column to column averaging about 10 ml/h for the 0.25 *M* NH_4 Ac buffer (pH 8.8) and about 20 ml/h for the 0.1 *M* HCOOH solution.

(6) Allow the buffer to drain to the top of the affinity gel bed then add 50 ml of 0.1 *M* HCOOH rinse. The gel expands and contracts depending on the pH and ionic strength of the solution with which it is equilibrated. Formic acid causes the gel to contract visibly but the bed volume is based on the initial volume of the gel in 0.25 *M* NH_4 Ac buffer (pH 8.8).

(7) Percolate ca. 10 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8) through the column to equilibrate it with this buffer. The gel column is now ready for sample loading when the buffer has drained to the top of the gel bed.

Sample cleanup

(8) The urine sample is thawed and shaken well to ensure sample homogeneity. Draw *exactly* a 0.50-ml aliquot with a 500-μl Eppendorf pipet, and place in a 1.5-ml Eppendorf microcentrifuge tube.

(9) Add 200 μl of 2.5 *M* NH_4 Ac buffer (pH 9.5) to the sample with a 200-μl Eppendorf pipet and mix the sample for 5 min on a vortex mixer (Eppendorf Model 3300 Rotary Shaker).

(10) Centrifuge the sample for 5 min at 12,000 *g* (Eppendorf Model 3200/30 microcentrifuge).

(11) Transfer the sample with a Pasteur pipet on to the column, being careful not to disturb the precipitate.

(12) Add 0.5 ml of the 0.25 *M* NH_4 Ac buffer (pH 8.8) to the sample tube and mix for 5 min on the vortex shaker.

(13) Centrifuge for 5 min at 12,000 *g*.

(14) Transfer the wash on to the column with the same Pasteur pipet.

(15) Follow the sample and wash through the column with 3 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8).

(16) Percolate an additional 3 ml of 0.25 *M* NH_4 Ac buffer (pH 8.8) through the column and after this wash has drained to the top of the gel bed, the column is ready for elution.

Elution of nucleosides

(17) Elute the nucleosides with 5 ml of 0.1 *M* HCOOH. Collect the eluate in a 10 ml volumetric flask.

(18) Bring the sample to volume with glass distilled nanopure water (see reagents section) and mix well by inversion, repeat mixing prior to HPLC analysis.

(19) After elution, strip the columns with ca. 20 ml of 0.1 *M* HCOOH and store in the same solution. Leave ca. 1 ml formic acid above the bed.

(20) To re-use the columns drain off the formic acid and repeat the process from step (7).

Reagents

(1) Ammonium acetate buffer, 0.25 *M* (38.54 g/2 l) with pH adjusted to 8.8 with concentrated ammonium hydroxide. The ammonium acetate used was A.C.S. certified grade from Fisher Scientific and the ammonium hydroxide was analytical reagent grade from Mallinckrodt.

(2) Ammonium acetate buffer, 2.5 *M* (385.4 g/2 l) with pH adjusted to 9.5 with concentrated ammonium hydroxide.

(3) Formic acid, 0.1 *M* (10.33 g concentrated formic acid/2 l). The formic acid used was A.C.S. certified grade from Fisher Scientific.

RESULTS AND DISCUSSION

Reversed-phase HPLC of ψ

A 60.0-μl aliquot of each cleaned sample (equivalent to 3 μl urine) was injected and chromatographed at 1 ml/min with 0.01 *M* $NH_4H_2PO_4$ buffer containg 1% (v/v) methanol on a 4 × 300 mm μBondapak C_{18} column (Waters Assoc.). The 1% methanol buffer gave good separation of the early eluting compounds in urine.

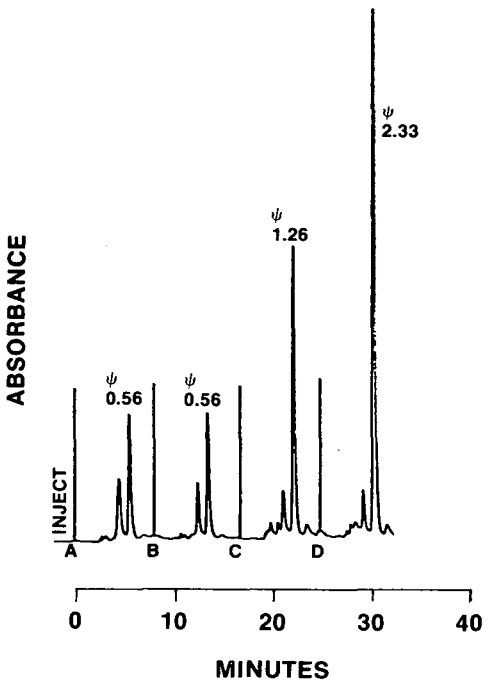

Fig. 1. Reversed-phase HPLC isocratic separation of ψ in urine. Sample: 60 μl of HCOOH eluate, ca. 3 μl urine; (A) 1a, (B) 1b, (C) 2, (D) 2 with 194.82 nmoles ψ /ml added; column: μBondapack C_{18}, 300 × 4 mm I.D.; buffer: 0.01 *M* $NH_4H_2PO_4$, 1% methanol, pH 5.0; flow-rate: 1.0 ml/min; detector: 245 nm, 0.05 a.u.f.s. temperature: 24°; units: nmoles.

Fig. 1 is a series of representative chromatograms. Chromatograms A and B are independently cleaned-up aliquots of urine sample one, which contained 0.56 nmoles of ψ in 3 μl of urine. Chromatogram C is for urine sample two (1.26 nmoles in 3 μl of urine). Chromatogram D represents sample two spiked prior to cleanup with ψ at a level of 194.82 nmoles in 1 ml of urine.

Precision of HPLC determination of ψ

The precision of the HPLC determination of ψ (Table I) was investigated using both standards and samples. Repeated injection of a ψ standard gave a relative standard deviation (R.S.D.) of 0.5% (Table I), and repeated injection of the same urine sample gave a matrix-independent R.S.D. of 1.1%. Then 20 different samples were analyzed with 10 being randomly duplicated. This gave a matrix-dependent R.S.D. of 1.1%. The study demonstrated that

TABLE I

PRECISION OF HPLC CHROMATOGRAPHY OF PSEUDOURIDINE

$n = 10$.

Experiment	$\bar{x}$ (nmole ψ /ml)	σ	R.S.D. (%)
Standard*	96	0.49*	0.51
Pooled control urine** matrix independent	159	1.8**	1.1
Different urines matrix dependent	234	2.6***	1.1

*One standard solution chromatographed ten times.
**Pooled urine passed through gel column then injected 10 times on HPLC column.
***Calculated by pairs using $\sigma = \sqrt{\frac{\Sigma(x_1 - x_2)^2}{2P}}$ where x_1 and x_2 are members of the pair, and P = number of pairs = 10.

the HPLC chromatography of ψ is not affected by the sample composition.

The rapidity of the chromatographic determination of ψ is facilitated by the high concentration and narrow peak width of ψ. The other ribonucleosides are at a concentration of at least one order of magnitude lower than ψ, and with the chromatography conditions for ψ (1% methanol) they are eluted as broad bands. This allows samples to be injected every 7.5 min without interference by the compounds still eluting from previous injections.

Linearity of the HPLC determination of ψ

The linear dynamic response range for ψ has been found between 1—50 nmoles. This wide linear range is more than sufficient for routine determination of urinary ψ.

pH Effect on the retention of ψ by the phenylboronate

Of all ribonucleosides ψ is the least strongly retained by the phenylboronic acid gel. A study on the effect of pH on the quantitative retention of ψ by the phenylboronic acid gel was made. Also, we wished to confirm that the gel column used had sufficient capacity to handle 1-ml aliquots of urine.

The pH of a pooled normal urine having a very high creatinine value (16.4 m*M*) was adjusted with 1.0 *M* NaOH and duplicate aliquots were withdrawn at each of the pH's 8.0, 8.5, 9.0, 9.5, 10.0. When 1.0-ml aliquots at pH 8.0 were loaded on the gel columns the values for ψ were 9.0% lower than those at pH 9.0 and greater. Aliquots of 0.50 ml showed no pH effect in the range between pH 8.0 and pH 10.0. The R.S.D. was 5.5% for the 1.0 ml loads and 1.6% for the 0.50 ml load.

The quantitative retention of ψ by the phenylboronic acid gel was dependent not only on the pH of the sample loaded on the column but also on the concentration of compounds in the urine other than the ribonucleosides. These experiments led to the decision to use a 0.5 ml sample and pH adjustment to 9.0 before placement on the 40 × 5 mm boronate column for cleanup.

TABLE II

NORMAL CONTROL VALUES AND DISTRIBUTION FOR PSEUDOURIDINE FROM RANDOM AND TOTAL 24 h COLLECTIONS

$\bar{x}:\bar{x}_{24}$ is a ratio for average value of each (random and total) collection to average value for 24 h total collection.

Time and type of collection	*Male*					*Female*				
	n	$\bar{x}$ (nmoles ψ /μmole Cr)	σ (nmoles ψ /μmole Cr)	RSD (%)	$\bar{x}:\bar{x}_{24}$	n	$\bar{x}$ (nmoles ψ /μmole Cr)	σ (nmoles ψ /μmole Cr)	RSD (%)	$\bar{x}:\bar{x}_{24}$
8 am	10	22.8	2.60	11.4	1.04	15	26.7	4.62	17.3	1.02
10 am	10	22.9	1.65	7.2	1.04	15	28.6	4.40	15.6	1.09
3 pm	10	22.5	2.98	13.2	1.02	15	25.9	3.70	14.4	0.98
24 h	27	22.0	1.68	7.6	1.00	28	26.3	4.9	19.0	1.00
Total*	57	22.4	2.16	9.4	1.02	73	26.7	4.50	16.9	1.02

*The total is the combination of all random and 24-h samples.

Recovery of ψ from gel column

Recovery of ψ for the total chromatography method was determined on a pooled normal urine with a very high creatinine concentration adjusted to pH 9.0 which was confirmed as the optimum pH. Aliquots of 1 ml were spiked with 50 μl of a standard solution containing 95.0 nmoles of ψ. The recoveries were unacceptable with an average of 77.3% on 9 different gel columns with an R.S.D. of 18.2%. The experiment was repeated with 0.50-ml aliquots of urine spiked at the same level. The average recovery increased to 95.4% with an R.S.D. of 5.8%, giving further confirmation of the capacity of the gel column.

The last 3 ml of wash from 1 ml urine loads on the gel column were collected and analyzed. Less than 1% of the ψ was found in this portion of the wash, implying that in the 1 ml loads the ψ was not all retained initially.

Previous capacity studies showed that the gel column quantitatively retained much greater amounts of nucleosides (ca. 30—40 μmoles) than present in 1 ml of urine. Evidently compounds other than ribonucleosides, in the urine exceeded the gel capacity, causing a loss of ψ.

Precision of method for urinary ψ

The precision for a pooled control urine sample taken through the cleanup and chromatographic process was then checked. Nine independent analyses gave an R.S.D. of 2.4%.

Dynamics of excretion

Normal males and females excrete 22.6 and 26.8 nmoles of ψ per μmole of creatinine, respectively (Table II). This reflects a 20% lower excretion of ψ and a 30% lower excretion of creatinine by females. The pseudouridine coefficient (μmoles ψ/kg per 24 h) for males is 4.1 and for females is 3.3; whereas the corresponding creatinine (Cr) coefficient for males is 185 and for females 129 (μmoles Cr/kg per 24 h). The larger RSD, % for the ψ : Cr ratio for female normals might reflect hormonal control of tRNA activity, but further study is required. Our study revealed that the dynamics of excretion of creatinine paralleled those of ψ for a normal population. When ψ was normalized with the excretion of creatinine, the ψ to creatinine ratio values for samples collected at random versus a total 24 h collection were the same (Table II). The constancy of excretion must stem from the constant turnover of RNAs independent of diet. This is of considerable importance in clinical applications where only a random sample can be easily obtained. Also, taking a random sample eliminates the inconvenience and lack of reliability of the 24-h collection. Excellent agreement was found between the normal values obtained by this HPLC method and those of Mrochek et al. [6] by cation-exchange, and Waalkes and co-workers [3, 4] using gas—liquid chromatography.

Excretion of ψ by colon cancer patients

A comparison was made for the ψ :Cr excretion ratio in 40 normals and 10 colon cancer patients and it was found that 9 of the 10 colon cancer patients had a ratio greater than $\bar{x} + 2\sigma$ for the normal population (see Figure 2).

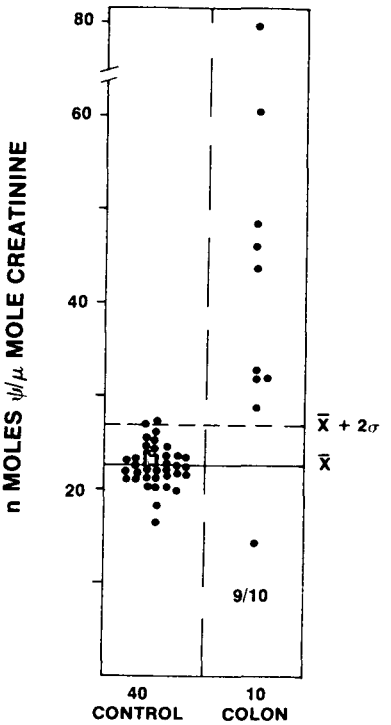

Fig. 2. Elevation of urinary pseudouridine excretion in colon cancer patients.

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Biomedical Applications

CHROMBIO. 132

QUANTITATIVE ANALYSIS OF 6,11-DIHYDRO-11-OXO-DIBENZ[*b*,*e*] OXEPIN-2-ACETIC ACID (ISOXEPAC) IN PLASMA AND URINE BY GAS—LIQUID CHROMATOGRAPHY

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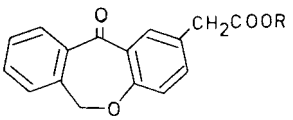
SUMMARY

A method is described for the quantitative analysis of 6,11-dihydro-11-oxo-dibenz[*b*,*e*]-oxepin-2-acetic acid (isoxepac) in plasma and urine. Isoxepac and internal standard are extracted from acidified plasma and urine, converted to the corresponding methyl esters and analysed by gas—liquid chromatography using a flame ionization detector. The method is accurate and precise over the range 0.1—30 μg/ml. The method has been applied to the analysis of plasma and urine from both healthy volunteers and patients receiving therapeutic oral doses of isoxepac.

INTRODUCTION

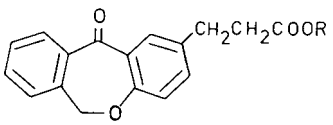
The compound 6,11-dihydro-11-oxo-dibenz[*b*,*e*]oxepin-2-acetic acid, isoxepac (also HP 549, I in Fig. 1), is currently being developed as a new anti-inflammatory agent which also possesses some analgesic activity [1, 2]. In order to obtain detailed pharmacokinetic information from clinical studies, it was necessary to develop an analytical method for isoxepac in plasma and urine. It was expected, from the known metabolism of isoxepac in animals [3], that peak plasma levels in man would be in the range 1—20 μg/ml following a single oral dose of isoxepac at the anticipated therapeutic level of 50—200 mg.

Initial investigations into the gas chromatographic properties of isoxepac established that satisfactory chromatography could be obtained after conversion of isoxepac to its methyl ester (II in Fig. 1), and that adequate sensitivity was available from flame ionization detection.



I R = H

II R = CH_3



III R = H

IV R = CH_3

Fig. 1. Structural formulae of isoxepac (I), P 74 1187 (III) and their methyl esters (II and IV, respectively).

MATERIALS AND METHODS

All solvents and reagents were of analytical grade and were used without further purification.

Preparation of diazomethane

Diazomethane in ether was prepared by a scaled-down version of the method described by de Boer and Backer [4]. As diazomethane is toxic, all work was performed in a fume cupboard. The explosion hazard associated with diazomethane was minimised by keeping the diazomethane at low concentrations and only using glassware fitted with Clearfit joints (Fisons, Loughborough, Great Britain).

The apparatus consisted of a two-necked, round-bottomed flask (100 ml) equipped with a PTFE-coated magnetic stirring bar. A dropping funnel (100 ml) was fitted into one neck of the flask, and a distillation head and condenser into the other. A solution of potassium hydroxide (0.6 g) in 95% ethanol (12.5 ml) was placed in the flask and heated to 65° with stirring. A solution containing N-methyl-N-nitrosotoluene-4-sulphonamide (BDH, Poole, Great Britain) dissolved in diethyl ether (2.2 g in 30 ml) was added slowly from the dropping funnel. Diazomethane and ether co-distilled from the reaction mixture. The resulting yellow distillate of ethereal diazomethane is usable for at least a week if kept refrigerated.

Standard solutions

Standard solutions of isoxepac and P 74 1186 (III in Fig. 1), the internal standard used in the method, were separately prepared by dissolving either compound in the minimum volume of 1 *M* Na_2CO_3 required for solution and diluting to a concentration of 0.1 mg/ml with distilled water.

Extraction and derivatisation

As solutions of isoxepac are light-sensitive, all operations must be carried out in subdued light. Dichloromethane (5 ml), 1 *M* HCl (0.5 ml) and 10 μg of the internal standard, P 74 1187 (0.1 ml of the 0.1 mg/ml standard solution) are

added to plasma (2 ml) in a screw-capped test tube (Sovirel; V.A. Howe, London, Great Britain). For urine analyses, 20 μg of internal standard (0.2 ml of the 0.1 mg/ml standard solution) is used, other quantities being unchanged. The plasma or urine is extracted for 10 min using an inversion mixer at 20 rpm (Heto Rotamix; V.A. Howe). After the layers have been separated by centrifugation at 2000 *g* for 5 min, the upper aqueous phase is aspirated and discarded. Any emulsions which may have formed are broken by mixing the contents of the test tube briefly with a vortex mixer, and centrifuging once more. The organic phase, after being transferred to a tapered test tube, is heated to 40° in a water bath and removed by a gentle stream of nitrogen. Ethereal diazomethane (0.5 ml) is added to the tube, mixed with the residue using a vortex mixer, and after 5 min removed by a gentle stream of nitrogen at room temperature. The residue is then re-dissolved in ethyl acetate (50 μl for plasma extracts; 100 μl for urine extracts) and aliquots (5 μl) are analysed by gas–liquid chromatography.

Gas–liquid chromatography

Analyses were performed on a Hewlett-Packard 5710A gas chromatograph equipped with a flame ionization detector. The glass column (1.8 m × 4 mm I.D., Hewlett-Packard configuration 5) was packed with 3% OV-11 on Chromosorb W HP (100–120 mesh). The oven temperature was 265°, and the injection port and detector were maintained at 300°. The carrier gas flow-rate was 60 ml/min of nitrogen. Under these conditions, isoxepac methyl ester and P 74 1187 methyl ester (IV in Fig. 1) eluted after 5.2 and 6.9 min, respectively. Examples of chromatograms obtained from plasma extracts are shown in Fig. 2.

Gas–liquid chromatography–mass spectrometry

An AEI MS 30-DS 50, mass spectrometer computer system coupled via a membrane separator to a Pye 104 gas chromatograph was used to obtain mass spectra. Chromatography was performed at 280° on a glass column (1.5 m × 4 mm I.D.) packed with 3% OV-17 on Chromosorb W HP (100–120 mesh) using helium, flowing at 45 ml/min, as the carrier gas. The mass spectrometer was operated at 45 eV, and 300 μA ionizing current; the ion source temperature was 250° and the separator temperature was 230°.

Liquid scintillation counting

Radioactive samples were counted on a Packard 2450 or 3255 liquid scintillation counter. Values (dpm) were calculated using an external standard channels ratio method. The scintillator used was NE 260 (Nuclear Enterprises, Edinburgh, Great Britain).

Quantification of Isoxepac levels

The concentration of isoxepac was determined from the peak height ratio of isoxepac methyl ester to internal standard methyl ester, and from a response factor obtained by analysing, in parallel with the unknown samples, blank plasma or urine to which had been added isoxepac (10 μg to plasma, 20 μg to urine) as well as internal standard.

During a series of routine measurements over a period of about one year, the

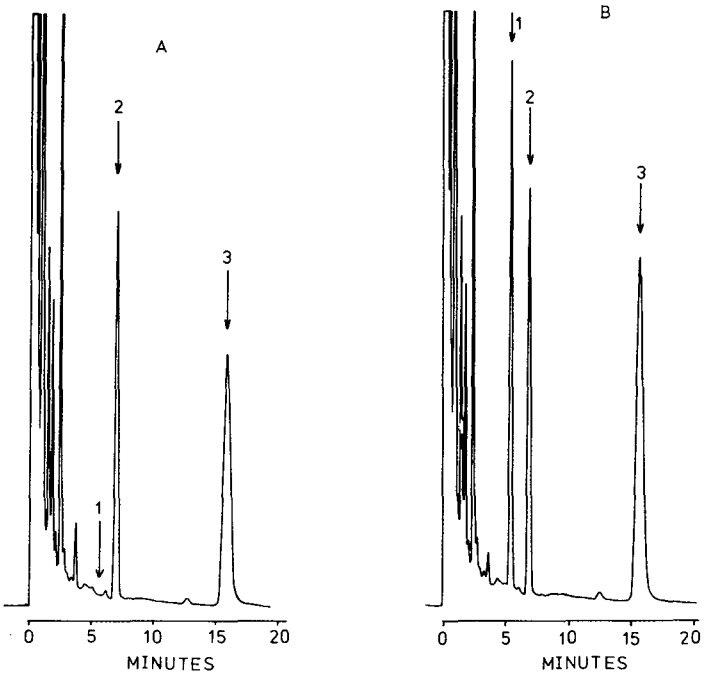


Fig. 2. Examples of chromatograms: A, control plasma to which had been added 5.21 μg/ml P 74 1187; B, control plasma to which had been added 5.25 μg/ml of isoxepac and 5.21 μg/ml of P 74 1187. The arrows 1, 2 and 3 indicate the respective retention times of isoxepac methyl ester, P 74 1187 methyl ester and cholesterol.

average value of the response factor was 1.24 (standard deviation, ± 0.06; 94 observations) for plasma and 1.31 (± 0.07; 48 observations) for urine.

RESULTS

Optimization of the extraction

The efficiency of extraction of isoxepac from plasma buffered to various pH values into dichloromethane, diethyl ether and diethyl ether—*n*-pentane (50:50, v/v) at pH <1 only, was determined using [^{14}C] isoxepac as follows. To plasma (2 ml) was added *ca.* 10 μg of [^{14}C]isoxepac (100 μl of a solution containing 600 dpm/μl), solvent (5 ml), and 1 *M* HCl (0.5 ml) or buffer (1.0 ml). After the plasma had been extracted and centrifuged (see Materials and methods), an aliquot (3.0 ml) of the organic phase was transferred to a counting vial and taken to dryness in a stream of nitrogen. Scintillator was added, and the amount of radioactivity in each vial was measured by liquid scintillation-counting.

The results (Table I) show that, although the extraction efficiency falls off rapidly with increasing pH, all of the isoxepac is recovered from plasma after acidification with 1 *M* HCl.

TABLE I

EFFICIENCY OF EXTRACTION OF [^{14}C]ISOXEPAC FROM BUFFERED PLASMA

Results are from duplicate determinations. The buffer was 0.1 *M* glycine—sodium chloride except for pH <1 which was 1 *M* HCl. The values quoted have not been corrected for any change in volume of the organic phase during extraction.

pH of added buffer	Recovery of [^{14}C]isoxepac (%)		
	Dichloromethane	Diethyl ether	Ether—pentane (50:50)
<1	99	106	97
	99	110	96
1.7	40	76	
	39	75	
2.0	24	59	
	24	60	
2.5	14	44	
	14	45	
3.0	8	29	
	8	29	
3.5	4	17	
	4	17	

Accuracy and precision

The accuracy and precision of the method were measured in the following experiment.Three standard solutions containing 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml of isoxepac were prepared. Aliquots of these solutions were then added to blank plasma samples (20 ml) such that the concentration of added isoxepac ranged from 0.03—31.2 μg/ml of plasma. The plasma samples were analysed on six occasions, and the results obtained are shown in Table II. Except for the two lowest concentrations, the precision is ± 3% or better and the results are accurate to within 5%. Although isoxepac could still be detected at the lowest concentration (0.03 μg/ml), interference from co-extracted material resulted in much poorer accuracy and precision.

Specificity

The specificity of the assay was established by combined gas—liquid chromatography—mass spectrometry of a plasma extract. The mass spectra of the peaks at the retention times of isoxepac methyl ester and P 74 1187 methyl ester were identical with the spectra from authentic standards.

TABLE II

DETERMINATION OF ISOXEPAC ADDED TO BLANK PLASMA

Each result is the mean of 6 measurements.

Isoxepac added (μg/ml)	Isoxepac found (μg/ml)	Standard deviation (μg/ml)	Percentage of theoretical
0.03	0.02	0.01	67 ± 50
0.10	0.09	0.01	90 ± 11
0.31	0.29	0.01	95 ± 3
1.01	0.98	0.02	97 ± 2
3.07	3.07	0.03	100 ± 1
10.2	9.96	0.04	97 ± 0.4
31.2	30.9	0.1	99 ± 0.3

DISCUSSION

Despite the hazards associated with its use, diazomethane was chosen for the esterification of isoxepac because its reaction with isoxepac is rapid, quantitative and free from by-products.

On-column methylation with a 0.2 *M* trimethylanilinium hydroxide solution in methanol (Methelute; Pierce and Warriner, Chester, Great Britain) was investigated, but was found to cause extensive decomposition of isoxepac.

During development of the method, solvents other than dichloromethane, and stationary phase other than OV-11, have been used. Extractions were carried out with diethyl ether and diethyl ether—*n*-pentane (50:50). As both of these solvents are less dense than plasma, it is easier and less time-consuming to remove them than it is for dichloromethane. However, it was found that they extracted more cholesterol and other lipophilic material from plasma and that the number of samples which emulsified on extraction was greater.

An advantage of using OV-17 rather than OV-11 as the stationary phase is that the retention time of cholesterol is about 4 min shorter on OV-17 under the conditions used. Its main disadvantage is that the background from co-extracted plasma constituents at the retention time of isoxepac methyl ester is greater. Thus, OV-11 can be used with greater sensitivity and accuracy, but with a possible drop in the through-put of samples; if a higher through-put of samples is needed, OV-17 can be used, but with some sacrifice of sensitivity.

Application of the method

The method has been in routine use for more than a year. It has been applied to the analysis of plasma and urine from healthy volunteers who had taken single, oral doses of 50, 100 and 200 mg isoxepac as well as to plasma and urine from patients suffering from rheumatoid arthritis who had taken 25, 50 or 150 mg isoxepac three times daily for 21 days.

In a typical experiment, a number of healthy volunteers were given a capsule

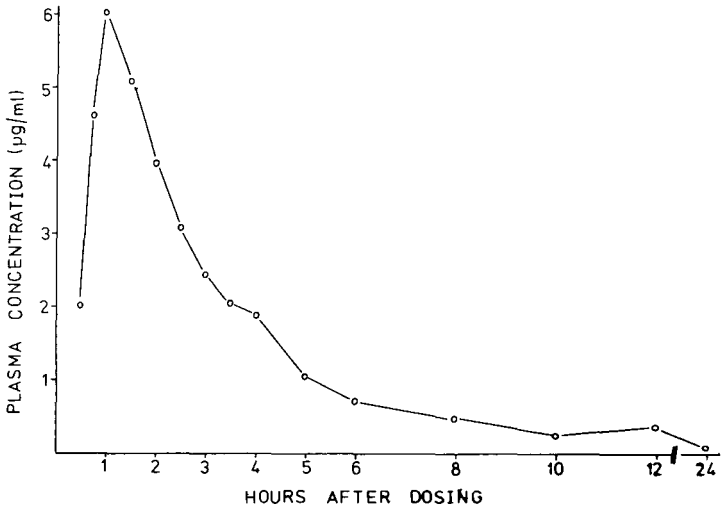


Fig. 3. Plasma level of isoxepac in a volunteer after a 50-mg oral dose of isoxepac.

TABLE III

URINE CONCENTRATIONS OF ISOXEPAC AFTER A SINGLE ORAL DOSE OF 50 mg ISOXEPAC

Total recovery of isoxepac, 6.01 mg.

Time after dosing (h)	Volume of urine collected (ml)	Conc. of isoxepac in urine (μg/ml)	Amount of isoxepac recovered (mg)
0—2	145	8.21	1.19
2—4	290	6.80	1.97
4—8	525	4.02	2.11
8—12	234	0.90	0.21
12—24	1500	0.25	0.38
24—36	848	0.08	0.07
36—48	670	0.12	0.08

containing 50 mg of isoxepac. Blood samples were withdrawn at various times during the next 24 h, and urine collected for various periods during the next 48 h. The average peak plasma level of six volunteers taking a 50 mg dose was 5.63 ± 1.03 μg/ml and the average recovery of isoxepac in urine was 7.3 ± 2.4 mg. Results from one of the volunteers are shown in Fig. 3 and Table III.

ACKNOWLEDGEMENT

The excellent technical assistance of Mrs. B.A. Bedford in the development and application of the method is gratefully acknowledged.

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CHROMBIO. 133

NEW ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF MEXILETINE PLASMA LEVELS IN MAN

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SUMMARY

A method for the determination of mexiletine in human plasma by gas—liquid chromatography with electron-capture detection is described. Plasma samples are extracted at pH 12 with dichloromethane after addition of the internal standard, the 2,4-methyl analogue of mexiletine. A derivative is obtained using heptafluorobutyric anhydride; according to gas chromatography—mass spectrometry it is a monoheptafluorobutyryl compound. The minimum detectable amount of mexiletine is 5 pg. Accurate determinations of human plasma levels were performed after oral or intravenous treatment.

INTRODUCTION

Mexiletine, [1-(1,6-dimethylphenoxy)-2-aminopropane; Kö 1173] is an anti-arrhythmic drug recently introduced in the management of ventricular arrhythmias [1—6].

As yet, only two methods for the quantification of unchanged mexiletine in biological fluids have been reported. The spectrofluorimetric method [7]

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is limited with respect to both specificity and sensitivity. In the gas—liquid chromatographic (GLC) method [7] the detection and quantification of the butyryl derivative of mexiletine is performed by a nitrogen-sensitive flame-ionization detector for which optimal operating conditions can be obtained and rapidly reproduced only with difficulty.

Besides, a GLC technique using heptafluorobutyric anhydride as derivatization reagent was recently described in a short note [8].

The aim of the present experimental study, therefore, was to develop a sensitive and specific GLC method using a linear and stable electron-capture detector (ECD) which should enable us to assess circulating plasma levels in patients after oral and intravenous administration.

EXPERIMENTAL

Reagents

All chemicals used were analytical grade. Heptafluorobutyric anhydride (Merck, Darmstadt, G.F.R.) was used as the derivatization reagent; to protect it from hydrolysis by moist air after opening the ampoule, it was stored in air-tight 3-ml vials (Reactivials, Pierce, Rockford Ill., U.S.A.). For the derivatization reaction the heptafluorobutyric anhydride (HFBA) was diluted (1:25, v/v) in ethyl acetate (J.T. Baker, Phillipsburg, N.J., U.S.A.) just before use. Hydrochloric acid (2 *N*) and sodium hydroxide (2 *N*) were prepared with glass-distilled water and stored in glass bottles. The extraction solvent was dichloromethane (Mallinckrodt, St. Louis, Mo., U.S.A.). Samples of mexiletine [1-(2,6-dimethylphenoxy)-2-aminopropane; Kö 1173] and of internal standard [1-(2,4-dimethylphenoxy)-2-aminopropane; Kö 768] were supplied by Boehringer (Paris, France).

GLC conditions

A Tracor 550 gas chromatograph equipped with a ^{63}Ni ECD was used. A glass column (400 cm × 4 mm I.D.) was packed with a mixed phase of phenylmethyl silicone fluids (2% OV-17 + 1% OV-275) coated on acid-washed, dimethylchlorosilane-treated, high-performance 80—100 mesh Chromosorb G (Johns Manville Products). This column was conditioned at 280° for 72 h (carrier gas nitrogen, 35 ml/min) after temperature programming (2°/min with low nitrogen carrier flow).

The operating conditions were: carrier gas (nitrogen) flow-rate 40 ml/min; scavenger gas (nitrogen) flow-rate 40 ml/min; column temperature 180°; injection port temperature 230° and detector temperature 285°. The ^{63}Ni ECD, which was not used with pulse current, gave a linear response for quantities ranging from 10—1200 pg of mexiletine heptafluorobutyryl (HFB) derivative.

Preparation of the derivative

Mexiletine (as free base) after extraction of its hydrochloride or of plasma was evaporated to a dry residue at 38° under a stream of dry nitrogen. A volume of 500 μl of ethyl acetate solution of HFBA (1:25, v/v) was added to this residue. The tubes were tightly stoppered and shaken and the reagent

solution was left to react for 30 min at 25°. The reaction mixture was then evaporated to a dry residue under a gentle stream of dry nitrogen.

Under the same conditions, the internal standard Kö 768 also reacts with HFBA.

Hexane (Mallinckrodt), 100 μl, was added to the residue; aliquots 90.5—1.5 μl) were injected into the gas chromatograph.

The completeness of the acylation was studied under various conditions: extended reaction times from 20 min—3 h, and the use of increasing amounts of HBFA solution. Increased concentrations of HFBA in these solutions gave identical recoveries, indicating quantitative derivatization conditions for mexiletine and internal standard.

Wentworth and Chen [9] have reviewed the effect of the detector temperature on the electron-capture detection (ECD). Accordingly, ECD temperatures ranging from 250° to 295° were tested for the HFB derivative of mexiletine; the best sensitivity was obtained at a temperature of 285°, which also reduced contamination of the cell detector.

GLC—mass spectrometry (MS) combination

The GLC—MS combination apparatus (Finnigan, Sunnyvale, Calif., U.S.A.) was operated at an accelerating voltage of 3.5 kV, an ionization voltage of 70 eV and a trap current of 65 μA. The column (180 cm × 4 mm I.D.) was packed with 2% OV-1 coated on the same support as already described. The apparatus was equipped with an accelerator voltage alternator for multiple-ion detection; the source was set to monitor in the electronic impact mode.

Mexiletine determination in patients

Protocol. A dose of 250 mg (3.5 mg/kg) of mexiletine hydrochloride was given intravenously (slow 10-min injection) to a 45-year-old patient. A 5-ml volume of heparinized blood was withdrawn 0, 0.5, 1, 2, 3, 4, 6, 9, 12, 18, 24 and 36 h after the dose was injected. After separating the plasma by centrifugation (3500 *g* for 15 min) 2-ml samples were taken for analysis according to the procedure described above.

Another patient was treated orally with 300 mg of mexiletine hydrochloride (5 mg/kg). Blood was withdrawn 0, 1, 2, 3, 4, 6, 9, 12, 18, 24 and 36 h after the dose was administered. Centrifuged plasma was treated following the aforementioned procedure.

Extraction procedure. A 100-μl volume of an aqueous solution (20 μg/ml, as a free base) of the internal standard (hydrochloride Kö 768) and 500 μl of 2 *N* NaOH (pH 12) were added to a 2-ml plasma sample in a glass-stoppered 30-ml centrifuge tube. The sample was shaken with 10 ml of dichloromethane for 15 min and then centrifuged at 3500 *g* for 15 min.

The aqueous phase was removed. The organic phase was mixed with anhydrous Na_2SO_4 (Merck) and then transferred to another tube and dried in a thermostatted bath at 40° under a gentle stream of nitrogen. Derivatization was performed on the dry residue according to the procedure described above.

Standard curve

Mexiletine standards were prepared by dissolving the appropriate amount of

mexiletine hydrochloride in 0.1 *N* HCl to obtain a stock solution of 20 μg/ml. Appropriate dilutions of this stock solution were then made to obtain the following concentrations: 0.20, 0.50, 0.75, 1.00, 1.25 and 1.50 μg/ml.

Control plasma samples (2 ml) were then spiked with these standard solutions and the internal standard (as described above) and were then extracted.

Peak areas of mexiletine and the internal standard were measured with an electronic integrator (Digital Readout CRS 204, Infotronics, Ireland) and their ratios were plotted as a function of mexiletine concentration.

RESULTS AND DISCUSSION

Preparation and structure of the heptafluorobutyryl derivative

Trifluoroacetic anhydride (TFAA) was selected as the acylating agent by Scott et al. [10] who used a GLC method for the isolation and quantification of two urinary metabolites of mexiletine with both the GLC—MS combination and nuclear magnetic resonance (NMR). Trifluoroacetyl derivatives can be obtained using a very large excess of TFAA and heating the reaction medium at 75° for 1 h. But this method, while perfectly convenient for thermal conductivity or flame-ionization detection, as well as for qualitative studies, is not suitable for assessing plasma concentration by GLC—ECD since the detector becomes polluted and there is a risk of its becoming saturated as it is difficult to achieve complete elimination of both the excess TFAA and the fluorinated degradation products of the reaction.

In this study, heptafluorobutylation provided a much higher selectivity and higher sensitivity and detector stability than trifluoroacetylation for determination in complex plasma extracts. HBFA was recently suggested by Willox et al. [8] as a derivatization agent. With their technique, however, the reaction takes place in a heated medium (90° for 30 min) and requires an excess of HFBA, whereas our method of obtaining the heptafluorobutyryl derivative involves a cold medium and less HFBA. Since operating conditions are not the same, it is impossible to know whether the derivatives obtained in each case have similar chemical structures. The conditions under which acylation of mexiletine (and its 2,4-methyl analogue) took place in this study resulted in a derivative containing one heptafluorobutyryl group on the amine moiety (see *m/e* 375 in Fig. 3). The chemical structure has been confirmed by GLC—MS. Fig. 1 shows the mass spectra of mexiletine (top) and its monoheptafluorobutyryl derivative (bottom). The mass spectrum of mexiletine (as free base) exhibits only two prominent peaks: *m/e* 58 (the C_3H_8N ion) which results from fission of the ether—methylene bond and *m/e* 44 (the C_2H_6N ion) which is the result of cleavage of the methylene—methine bond of the side chain. The molecular ion is at *m/e* 179 and the other peaks are at *m/e* 77, 91, 105, 107 and 121, corresponding to the possible fragmentation previously reported by Scott et al. [10] as shown in Fig. 2.

In the mass spectrum of the derivative, the molecular ion (*m/e* 375) is very small; it corresponds to the formation of the monoheptafluorobutyryl compound. In this spectrum the two most abundant ions were observed at *m/e* 254 and *m/e* 122 and correspond to the two ions formed by cleavage of the $-O-CH_2-$ bond. There is retention of the charge on the fragment

m/e 254 and a proton is transferred to the other part of the molecule (*m/e* 121) to form the ion at *m/e* 122.

Fig. 3 shows the possible structure of a few ions (*m/e* 121, 122, 163 and 254). Many of the other peaks correspond to well-known ions.

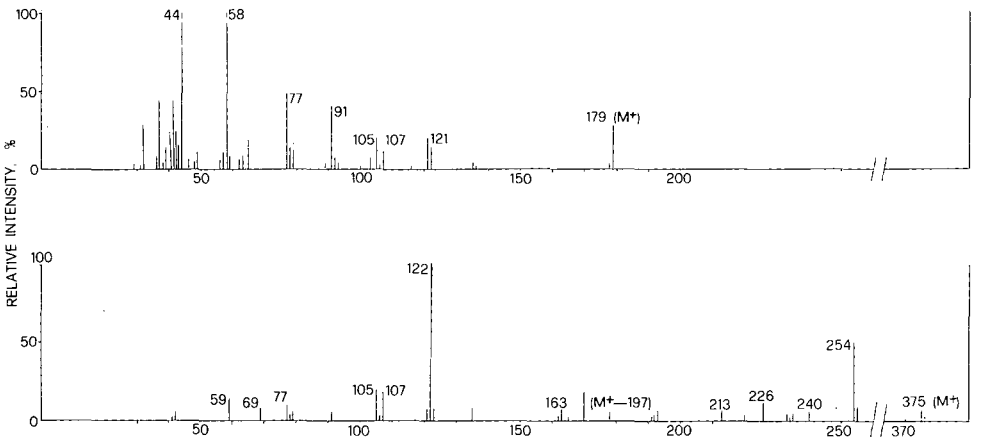


Fig. 1. Normalized mass spectra of mexiletine (top) and of its monoheptafluorobutyryl derivative (bottom).

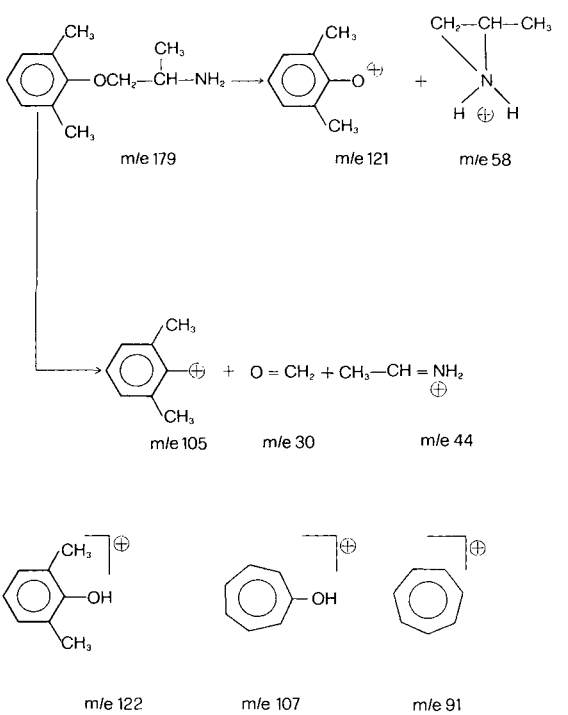


Fig. 2. Fragmentation pattern of mexiletine.

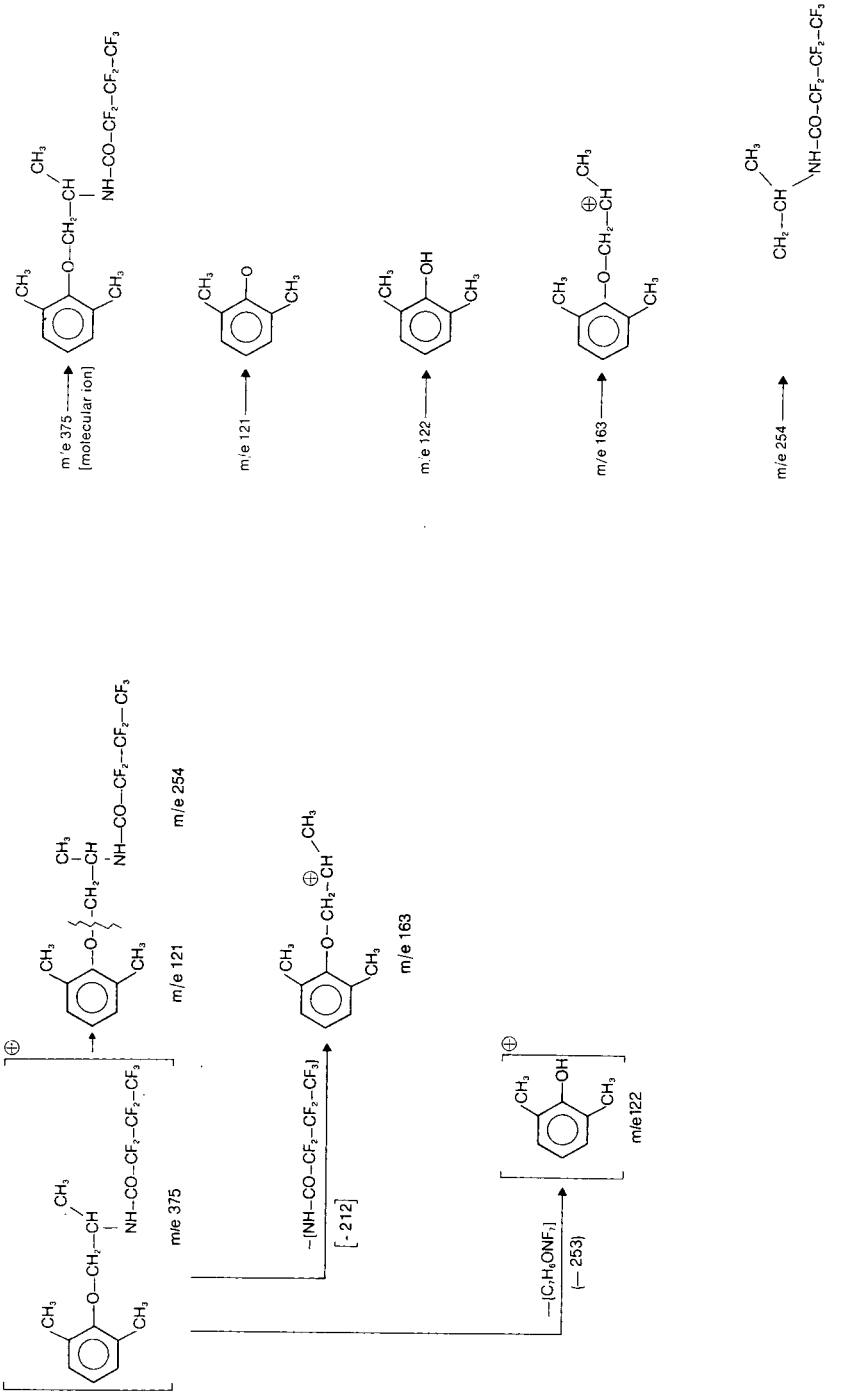


Fig. 3. Possible structure of a few ions resulting from fragmentation of the monoheptafluorobutyryl derivative of mexiletine.

Detector response and stability of the derivative

The monoheptafluorobutyryl derivative has excellent GLC properties and gives a very good response on the ^{63}Ni ECD.

The minimum detectable quantity being defined as the quantity that gives a signal three times that of the background noise level [11, 12], this for mexiletine corresponds to 5 pg under the GLC conditions used here.

This acylation reaction is quantitative and interference in the derivatization reaction by free acid in the anhydride reagent can be avoided by careful handling of HFBA as described in Analytical Procedure. The derivative is stable for at least 72 h at room temperature.

Chromatogram and retention times

With this method, intact mexiletine is measured and the drug is separated from its metabolites usually found in plasma and urine. No interference from normal plasma constituents or from drugs that would possibly be prescribed together with mexiletine (acetylsalicylic acid, α-methyldopa, clonidine, propranolol, pindolol, diazepam, chlordiazepoxide) has been found.

Fig. 4 shows a typical chromatogram obtained with the plasma extracts of

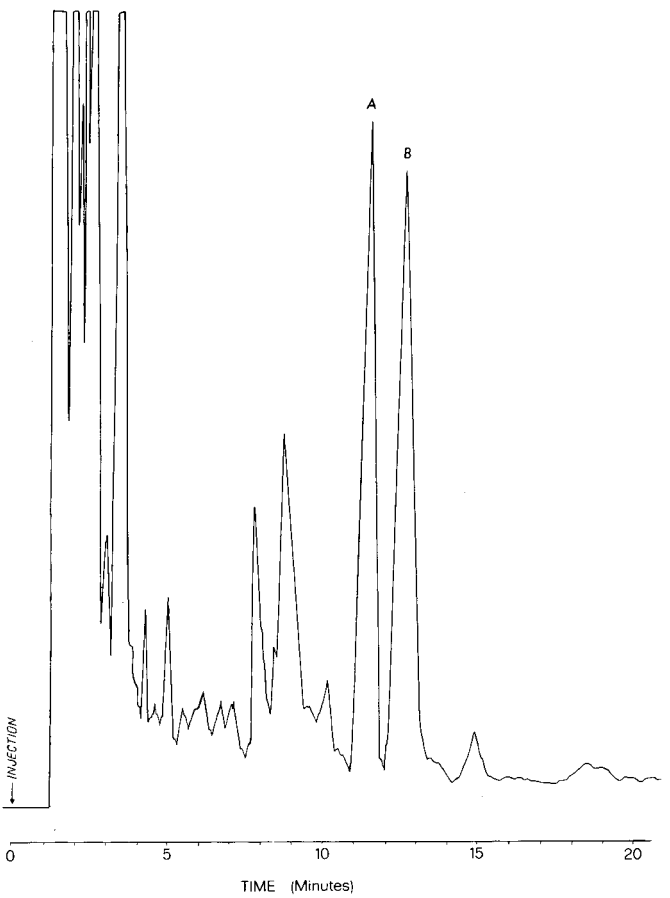


Fig. 4. Gas chromatogram of a calibration curve plasma extract. Retention times are: 11 min 40 sec for mexiletine (peak A) and 12 min 55 sec for internal standard (peak B).

the calibration curve. Retention times are 11 min 40 sec for mexiletine (peak A) and 12 min 55 sec for the internal standard (peak B).

Calibration curve, reproducibility and recovery

The calibration graph is obtained by plotting the ratios of the peak areas of the mono-HFB derivative of mexiletine to that of the internal standard against known amounts of mexiletine added to the plasma. Linearity is observed for concentrations from 0.2—1.0 μg/ml in plasma (r = 0.996; S.D. = 0.008). The precision of the method is adequate, as demonstrated by the recovery and reproducibility studies. The reproducibility of the method was determined in a 10-fold analysis of two plasma samples, one containing 1.0 μg/ml, the other 2.00 μg/ml, of mexiletine. The results are given in Table I. Recovery was assessed by adding known amounts of mexiletine to a pre-analysed plasma sample and calculating the concentration obtained as a percentage of the added concentration. This was carried out for two concentrations of added mexiletine, 0.75 and 1.25 μg/ml. Recoveries were 98.7 and 94.5% respectively.

TABLE I

REPRODUCIBILITY OF MEXILETINE ANALYSIS IN PLASMA

	Mexiletine added to plasma	
	1.00 μg/ml	2.00 μg/ml
	1.030	2.170
	1.020	2.154
	0.885	2.022
	1.011	1.981
	0.860	2.070
	1.030	2.050
	0.990	2.063
	1.075	1.730
	0.995	1.999
	1.085	1.770
mean	0.998	2.0009
S.D.	0.073	0.145
Coefficient of variation (%)	7.3	7.2

Determination of plasma levels of mexiletine

The method described, including the extraction procedure, permits analysis of 15—20 samples per day on a single-column instrument without automatic sampler.

Fig. 5 shows the decline of mexiletine plasma levels after i.v. administration to the 45-year-old patient. Pharmacokinetic data are calculated using a mono-

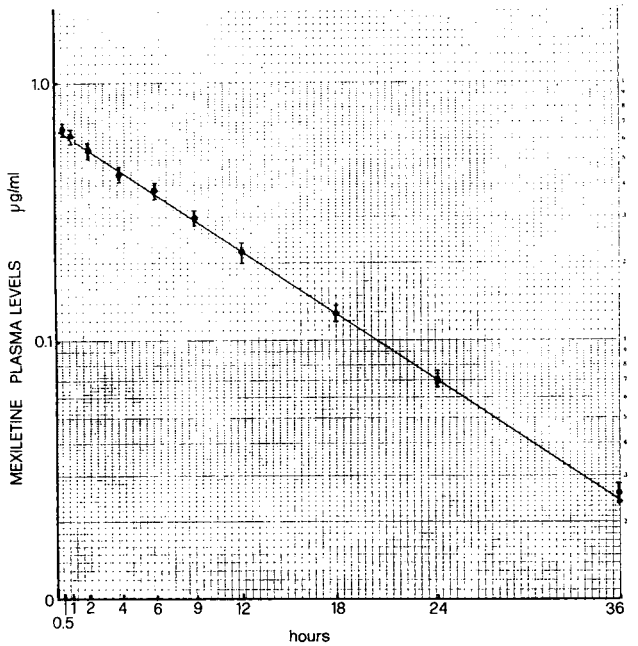


Fig. 5. Mexiletine plasma levels after a 250-mg i.v. dose (3.5 mg/kg) of mexiletine hydrochloride to a 45-year-old patient; each point represents the mean of three determinations (mean ± 2 S.D.).

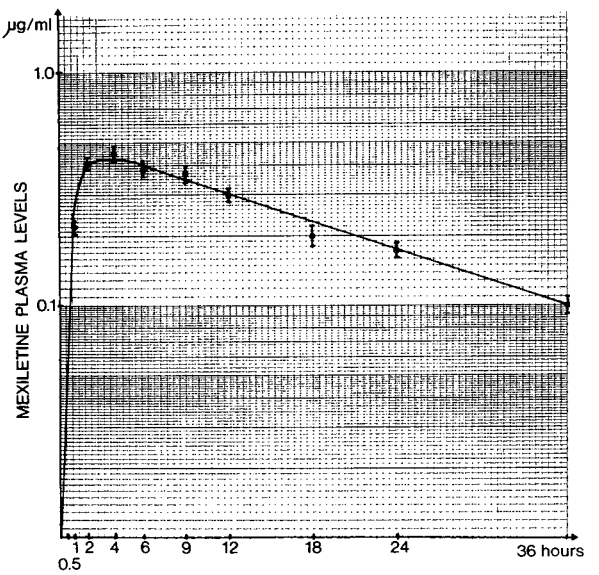


Fig. 6. Plasma climination curve after a 300 mg oral dose (4.0 mg/kg) of mexiletine hydrochloride. Each point represents the mean of three determinations (mean ± 2 S.D.).

compartmental open model because the loading dose was perfused over a period of 10 min. For this patient, the half-life is 7.9 h, fractional rate constant for elimination is $k_{el} = -0.087\ h^{-1}$ and the central volume of distribution is $V_c = 386$ l.

Fig. 6 shows the plasma elimination curve in an other patient to whom 300 mg of mexiletine hydrochloride were administered per os. In this patient, the calculated pharmacokinetic data are: half-life, 14.9 h; $k_{el} = -0.04649\ h^{-1}$.

Fig. 7 shows a GLC tracing for a plasma sample of this patient.

Urinary elimination of the metabolites (over the 48 h after drug administration) was studied and two metabolites that reacted with both TFAA and HFBA were investigated in particular. After reaction with TFAA their retention times expressed in Kovats indices were similar to those obtained by Scott et al. [10]: i.e. 1770 for compound a and 1837 for compound b. According to the GLC, MS and NMR studies carried out by Scott et al. [10] their structures could correspond to *p*-hydroxymexiletine (a), and to the free base with one of the methyl groups substituted by a $-CH_2OH$ group (b). These structures have also been confirmed by Beckett and Chidomere [13].

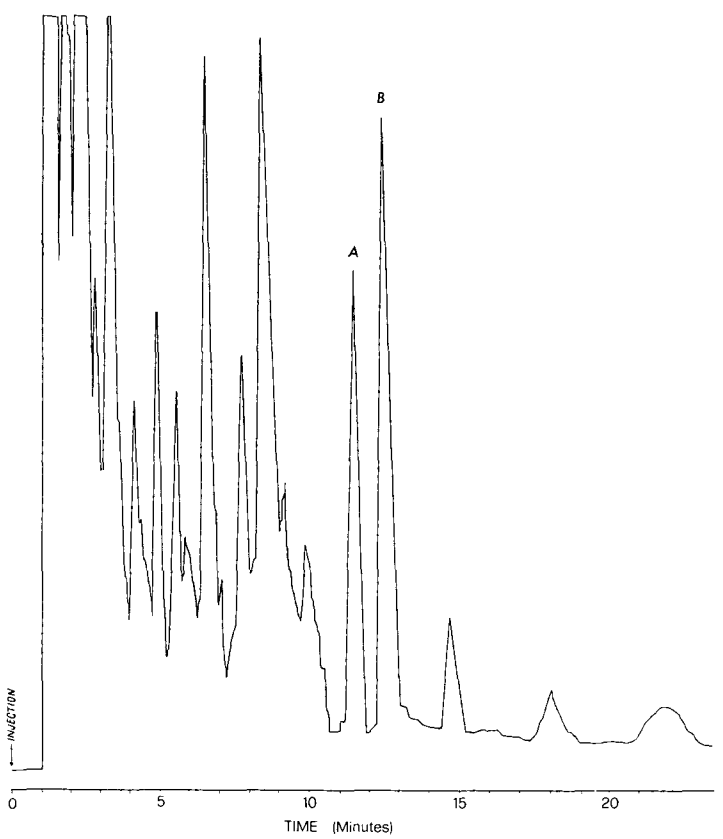
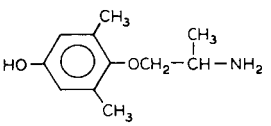
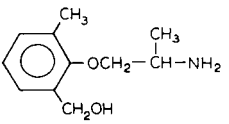


Fig. 7. Gas chromatograph of a plasma extract from a patient receiving a 300 mg oral dose of mexiletine hydrochloride. Peak A and peak B represent mexiletine and internal standard, respectively.


(a)


(b)

CONCLUSION

The present GLC method is simple, highly sensitive and specific because acylated mexiletine (as an unchanged drug) is separated from its metabolites and other drugs commonly used in the treatment of cardiac and vascular diseases. It can be used in single- as well as multiple-administration (particularly with low doses) pharmacokinetic studies.

ACKNOWLEDGEMENT

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CHROMBIO. 148

STUDIES ON THE METABOLISM OF 2,4′-ISOBUTYLPHENYLPROPIONIC ACID (IBUPROFEN) BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

DIALYSIS FLUID, A CONVENIENT MEDIUM FOR STUDIES ON DRUG METABOLISM

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SUMMARY

2,4′-Isobutylphenylpropionic acid (ibuprofen) has previously been demonstrated to yield four urinary metabolites, formed by ω1-, ω2- and ω3-hydroxylation and by a further oxidation of the primary alcohol of the ω1-hydroxylated metabolite to a carboxyl group. By synthesis and gas chromatography—mass spectrometry the suggested structure of the ω3-hydroxylated metabolite was verified in the present study. Moreover, a new metabolite, 2,4′-carboxyphenylpropionic acid, was demonstrated to be present in substantial amounts in dialysis fluid from a nephrectomized patient. In such patients ingested drugs cannot be excreted in the urine, but are metabolized to end products. Thus, dialysis fluid may be a convenient medium for studies on drug metabolism.

INTRODUCTION

The drug ibuprofen (2,4′-isobutylphenylpropionic acid) is a non-steroidal agent with anti-inflammatory, analgesic and antipyretic properties, which has gained acceptance in the treatment of rheumatoid arthritis and other rheumatic conditions. Using e.g. combined gas chromatography—mass spectrometry (GC—MS), previous workers [1, 2] have detected four metabolites, viz. the metabolites 1—4 shown in Fig. 1. Metabolites 2 and 4 have been synthesized [1], whereas metabolites 1 and 3 have been identified by interpretation of mass spectra of appropriate derivatives of the metabolites [2]. There was some uncertainty, however, with regard to the interpretation of the mass spectrum of

*To whom correspondence should be addressed at the Institute of Clinical Biochemistry.

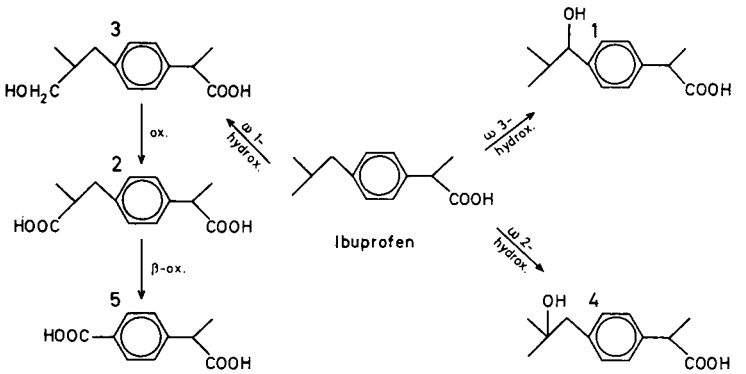

Fig. 1. Pathways of 2,4′-isobutylphenylpropionic acid (ibuprofen) metabolism and chemical structures of the demonstrated metabolites.

the postulated metabolite 1 [2].

The structure of metabolite 2, with the methyl branching in the longest side chain in the α-position to the carboxyl group, suggests that this compound may be further degraded by β-oxidation to yield 2,4′-carboxyphenylpropionic acid. This metabolite has not been demonstrated previously. A possible explanation might be that metabolite 2 is rapidly excreted in the urine and thus escapes a subsequent β-oxidation. In nephrectomized patients, however, drugs and their metabolites can not be excreted and may therefore be degraded to end products.

In the present study some possible metabolites of 2,4′-isobutylphenylpropionic acid were synthesized. Subsequently these metabolites were searched-for by combined GC—MS in dialysis fluid from a nephrectomized patient and in the urine from a healthy volunteer, both persons taking ibuprofen perorally. A new metabolite was identified (metabolite 5 in Fig. 1), the suggested structure of metabolite 1 [2] was verified, and it was found that dialysis fluid is a convenient medium for studies on drug metabolism. A preliminary report with some results from the present study has been presented previously [3].

MATERIALS AND METHODS

Chemicals

Ibuprofen was delivered by Boots (Nottingham, Great Britain). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, Ill., U.S.A.). N-Nitrosomethylurea for diazomethane production was delivered by K and K Labs. (Calif., U.S.A.). Stationary phases for gas chromatography (OV-17 and SE-30) and solid support (Gas-Chrom Q) were obtained from Applied Science Labs. (State College, Pa., U.S.A.). 2,4′-Carboxyphenylpropionic acid, 2,4′-(1-hydroxy-2-methylpropyl) phenylpropionic acid, 2,4′-(1-oxo-2-methylpropyl)phenylpropionic acid, and 3-hydroxy-2,4′-isobutylphenylpropionic acid were synthesized [4].

Dialysis fluid and urine samples

Dialysis fluid was obtained from a 24 year-old nephrectomized female under-

going hemodialysis regularly, twice a week. She suffered from rheumatoid arthritis and was daily given perorally two 200-mg tablets of ibuprofen. Special precautions were taken for collection of dialysis fluid. The flow of fresh dialysis fluid was diminished to obtain near-equilibrium passage of various metabolites across the dialysis membrane. The first 5 l of dialysis fluid were collected, divided into 40-ml portions and lyophilized. The samples were redissolved in 5 ml of water and subsequently treated exactly like the urine samples.

A 12-h urine sample was collected from a healthy, 34 year-old, male volunteer who had ingested three 200-mg tablets of ibuprofen. Five ml aliquots of the sample were used for the further studies.

Extraction of organic acids from dialysis fluid or urine samples was performed with diethyl ether (3 × 3 volumes) after adjusting the pH to 1 with 6 *M* HCl. The combined extracts were dried over anhydrous sodium sulphate.

Methylation was performed with diazomethane liberated from N-nitrosomethylurea. Ethylation was performed with ethanol–HCl. Trimethylsilylation was carried out in pyridine with BSTFA.

Gas chromatography and mass spectrometry

Two combined GC—MS instruments were used. One instrument consisted of a Varian 1440 gas chromatograph, a molecular separator of the glass frit type (kept at 230°) and a single-focusing mass spectrometer, type Varian CH 7 (Varian-MAT, Bremen, G.F.R.), operated with an ionisation energy of 70 eV. The gas chromatograph was equipped with a packed column (2 m × 1/4 in. O.D.) filled with 10% OV-17 on Gas-Chrom Q, 80—100 mesh. Helium was used as carrier gas (30 ml/min).

Multiple ion detection (selected ion monitoring, mass fragmentography) was carried out using a Varian 112 mass spectrometer fitted with a glass capillary column (SE-30, 25 m × 0.25 mm; LKB, Stockholm, Sweden) connected directly to the ion source.

Both GC—MS instruments were connected on-line to a computer system (Spectro System 100 MS; Varian-MAT). For the calibration of the mass spectrometers perfluorokerosene was used as reference substance.

High resolution mass spectrometric analyses were undertaken in an AEI MS 902 double-focusing instrument (70 eV ionizing energy, 100 μA ionization current). The samples were introduced by the heated direct-inlet probe. Perfluorotributhylamine was used as reference substance.

RESULTS

Fig. 2 shows gas chromatograms of the methylated diethyl ether extract of acidified dialysis fluid from a nephrectomized patient (a) and of urine from a healthy volunteer (b), both persons ingesting ibuprofen. In the urine sample large amounts of unmetabolized ibuprofen were found, together with only small amounts of metabolite 5. In the dialysis fluid, however, no trace of the unmetabolized drug could be detected, whereas substantial amounts of one of the metabolic end products of the drug, metabolite 5, were found. In addition to the drug metabolites several well-known metabolites normally occurring in the urine, e.g. hippuric acid, were observed in the chromatogram [5].

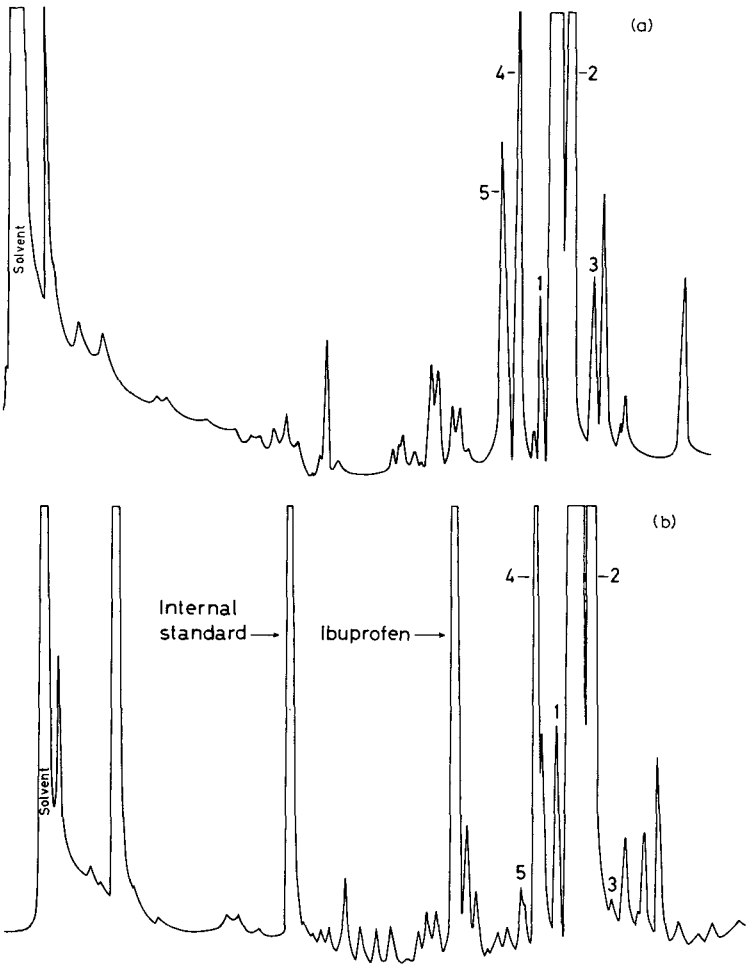

Fig. 2. Gas chromatograms of methylated diethyl ether extracts of acidified dialysis fluid from a nephrectomized patient (a) and urine from a healthy volunteer (b), both persons having ingested ibuprofen. GC was performed in a packed column (10% OV-17 on Gas-Chrom Q, 80—100 mesh), the temperature was programmed 80—300° (8°/min), and carrier gas was helium (30 ml/min). The GC peaks have been identified as the following compounds (as methyl esters): 1=2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid; 2=2,4′-(2-carboxypropyl)phenylpropionic acid; 3=2,4′-(2-hydroxymethylpropyl)phenylpropionic acid; 4=2,4′-(2-hydroxy-2-methylpropyl)phenylpropionic acid; 5=2,4′-carboxyphenylpropionic acid.

The metabolites giving rise to peaks 2 and 4 (metabolites 2 and 4 in Fig. 1) have been previously synthesized and identified as 2,4′-(2-carboxypropyl)-phenylpropionic acid and 2,4′-(2-hydroxy-2-methylpropyl)phenylpropionic acid [1]. The metabolites giving rise to peaks 1 and 3 (metabolites 1 and 3 in Fig. 1) have been detected and characterized by Brooks and Gilbert [2] using combined GC—MS. They identified the metabolites as 2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid and 2,4′-(2-hydroxymethylpropyl)phenylpropionic acid.

In the present work the four mentioned metabolites have been studied both as methyl esters and as methyl ester—trimethylsilyl (TMS) ethers, and the findings of Brooks and Gilbert [2] have been confirmed. With regard to the interpretation of the mass spectrum of the methyl ester—TMS ether of metabolite 1 they stated: "The abundant ion at *m/z* 133 was at first ascribed to an impurity retaining the unsubstituted isobutylphenyl moiety, but was still formed from a purified sample and remain unassigned pending further examination". The postulated metabolite 1, i.e. 2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid, was therefore synthesized [4]. Fig. 3 shows the mass spectrum of the

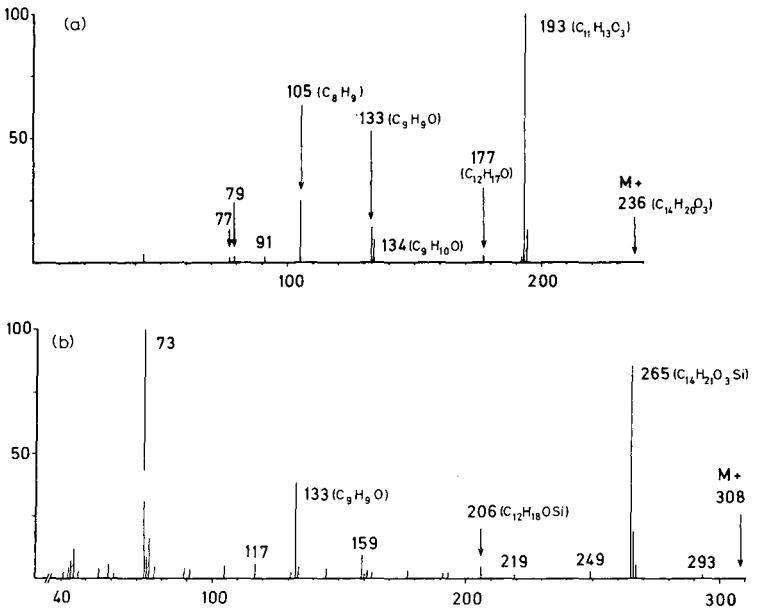

Fig. 3. Mass spectra of 2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid as methyl ester (a) and as methyl ester—TMS ether (b). The empirical formulae (as indicated in the figure) have been determined by high-resolution mass spectrometry.

methyl ester of this compound (a) and the mass spectrum of the methyl ester—TMS ether (b). The empirical formulae (as indicated in the figure) of the various fragments have been determined by high resolution mass spectrometry. A suggested scheme for the formation of the ion at *m/z* 133 in the mass spectrum from the methyl ester—TMS ether of 2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid is shown in Fig. 4.

The synthesized 2,4′-(1-hydroxy-2-methylpropyl)phenylpropionic acid had an identical mass spectrum as well as identical GC retention time with metabolite 1, both as methyl ester and as methyl ester—TMS ether.

Two other possible metabolites from 2,4′-isobutylphenylpropionic acid were synthesized, viz. 2,4′-(1-oxo-2-methylpropyl)phenylpropionic acid and 3-hydroxy-2,4′-isobutylphenylpropionic acid. The latter might be formed by an ordinary ω1-hydroxylation. Both of these compounds were searched for, first

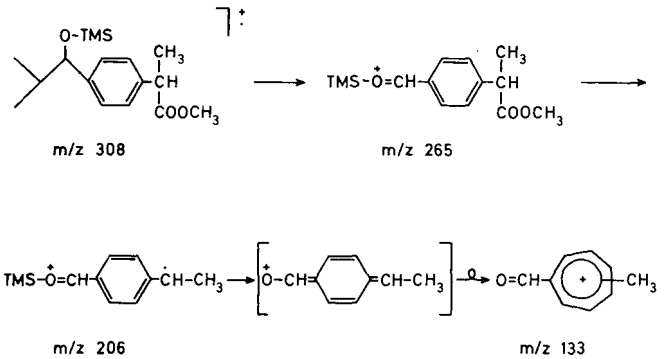

Fig. 4. Suggested fragmentation scheme for the formation of the ion at *m/z* 133 from 2,4'-(1-hydroxy-2-methylpropyl)phenylpropionic acid methyl ester—TMS ether.

by mass chromatography and later by multiple ion detection. However, not even traces of these compounds could be demonstrated in the samples (dialysis fluid or urine).

Peak 5 (Fig. 2) was identified as follows. The mass spectrum of this peak is shown in Fig. 5 (b). The molecular ion is at *m/z* 222; the fragment ions at *m/z* 191 and *m/z* 163 indicate a methyl ester. When the sample was treated with ethanol—HCl, the retention time of the peak was slightly increased and in the new mass spectrum (not shown) the molecular ion was found at *m/z* 250, thus showing the presence of two carboxylic groups. Ions at *m/z* 205 and *m/z* 177

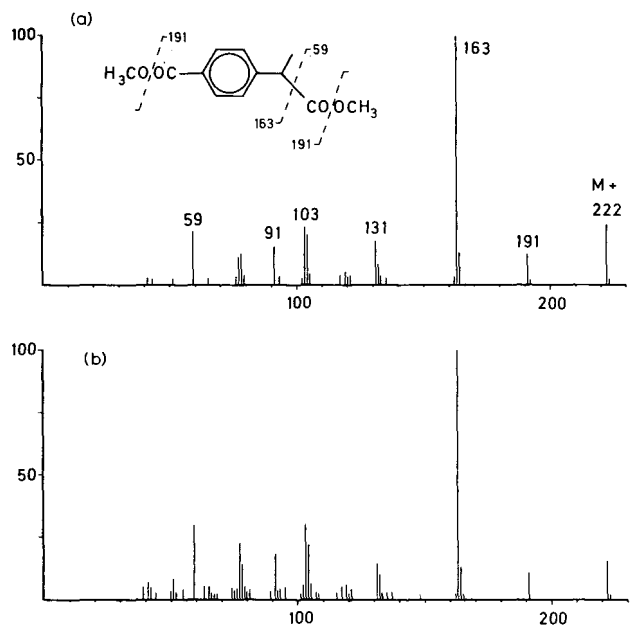

Fig. 5. Mass spectrum of authentic 2,4'-carboxyphenylpropionic acid dimethyl ester (a) and of the methylated ibuprofen metabolite (peak 5 in Fig. 2) detected in dialysis fluid from a nephrectomized patient (b).

demonstrated loss of an ethoxy and a carboethoxy group. The absence of hydroxyl groups was confirmed by the unchanged retention time and mass spectrum after trimethylsilylation of the dimethyl ester. The postulated structure of the new metabolite is shown in Fig. 1 (metabolite 5). This compound, viz. 2,4′-carboxyphenylpropionic acid, was synthesized [4], and the mass spectrum of the dimethyl ester is shown in Fig. 5 (a). This mass spectrum is identical to the mass spectrum of the methylated metabolite 5. Moreover, the two compounds had identical gas chromatographic retention times (0.50 relative to 2,4′-(2-carboxypropyl)phenylpropionic acid dimethyl ester in an OV-17 column, isothermally at 205°).

In the 2-methylpropionyl side chain of metabolite 2 (Fig. 1) the methyl branching is in the α-position to the carboxyl group. Thus, it is most likely that the new metabolite, 2,4′-carboxyphenylpropionic acid, is formed from metabolite 2 by an ordinary β-oxidation.

DISCUSSION

It has been shown by others [1, 2] and by us [3] that the metabolism of 2,4′-isobutylphenylpropionic acid takes place in the isobutyl side chain. Derivatives are formed by ω1-, ω2- and ω3-hydrolation and by a further oxidation of the ω1-hydroxylated metabolite. This is in accordance with the findings of Ruelius et al. [6] concerning the metabolism of the hypoglycemic agent 2-p-methoxybenzenesulphonamido-5-isobutyl-1,3,4-thiadiazole and our own findings concerning the metabolism of 4-isobutylphenylacetic acid [7].

Another possible site of attack for the ω-hydroxylation system would be at the 3-position in the propionic acid part of 2,4′-isobutylphenylpropionic acid. In the present study, however, not even traces of this postulated metabolite could be detected. Thus, it seems as if the neighbouring carboxylic group protects this methyl group from the ω1-hydroxylating enzymes.

The new metabolite, 2,4′-carboxyphenylpropionic acid (metabolite 5, Fig. 1), may be formed from 2,4′-(2-carboxypropyl)phenylpropionic acid (metabolite 2, Fig. 1) by β-oxidation. The reason why the new metabolite has not been previously detected may be that its precursor is rapidly excreted in the urine and thus for a large part escapes β-oxidation. In the nephrectomized patient in the present study the ingested drug could not be excreted in the urine, but was metabolized to end products. Such a product, 2,4′-Carboxyphenylpropionic acid, was found in substantial amounts in the dialysis fluid sample from the nephrectomized patient, whereas only small amounts were found in the urine sample from the healthy volunteer. Thus, we suggest that analyses of dialysis fluid from nephrectomized patients who for clinical reasons have to be given a certain drug, may yield new information on the metabolism of that particular drug.

ACKNOWLEDGEMENTS

The technical assistance of Miss Live Horn and Mr. Per Helland is gratefully acknowledged. Through the kind co-operation of the Chemistry Department

(Mr. G. Hvistendahl), University of Oslo, we had access to the high-resolution mass spectrometer.

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Biomedical Applications

CHROMBIO.123

A SIMPLIFIED ASSAY OF FUROSEMIDE IN PLASMA AND URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A simplified high-pressure liquid chromatographic method for determination of furosemide in plasma and urine has been developed using a fluorometric detector directly coupled to the column effluent. The method includes an ether extraction from acidified biologic samples. The mobile phase used for chromatography on a reversed-phase column (C_{18} hydrocarbon permanently bonded to silica particles) is sufficiently acidic to induce fluorescence of furosemide. The methylester of furosemide is employed as an internal standard. The sensitivity is 0.1 and 0.25 μg per ml plasma and urine, respectively. The applicability to pharmacokinetic studies of furosemide is shown.

INTRODUCTION

Furosemide is an extremely potent diuretic agent, the major use of which is in acute or chronic renal failure, congestive heart failure and liver cirrhosis [1, 2]. The earliest methods for analysis of furosemide were based on spectrophotometry [3] or spectrophotofluorometry [4] following extraction of serum and urine with organic solvents. These methods suffered from interference by the hydrolytic product, 4-chloro-5-sulfamoylanthranilic acid (CSA).

In order to improve the specificity, high-pressure liquid chromatographic (HPLC) methods have subsequently been developed [5—7] one of which employs fluorometry for quantification of the column effluent after acidification of individual fractions [7].

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The present paper describes an HPLC method utilizing a column packed with C_{18} hydrocarbon phase permanently bonded to very small silica particles (μBondapak). This type of chromatography obviates monitoring of salt concentrations and pH which is required when ion-exchange columns are used [5, 7]. Our method does not require acidification of individual fractions collected from the column effluent because the mobile phase is sufficiently acidic to render furosemide fluorescent. This allows continuous monitoring of the column effluent by fluorometry. Furthermore, the use of an internal standard improves accuracy of quantification.

MATERIALS AND METHODS

All analyses were performed utilizing a Waters Assoc. high-performance liquid chromatograph. A Model M6000 solvent delivery system, a Model U6K injector and a C_{18} μBondapak column (300 mm × 3.9 mm I.D.; particle size 10 μm) were used to perform the chromatographic separation. Furosemide and the internal standard, the methyl ester of furosemide, were measured with a fluorometer (Fluoro-Monitor, American Instr. Co.) employing a 70-μl Suprasil quartz flow cell, a Corning No.7-60 primary filter, a Kodak No.2A secondary filter and a General Electric No.F474·BL lamp. The mobile phase, methanol—water—glacial acetic acid (34:46:3), was delivered at a rate of 2.0 ml/min. The system was operated at room temperature.

Blood samples were collected in heparinized glass tubes and centrifuged to separate the plasma. Urine was collected in glass bottles without preservative. All samples were stored in the dark at −20°.

To 1 ml of plasma or urine were added 10—20 μg of the internal standard (IS) (4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid methyl ester, the methyl ester of furosemide), 0.1 ml of 5 *N* HCl, 0.5 ml distilled water and 10 ml of diethyl ether. The samples were shaken in 13-ml glass tubes with Teflon®-lined screw caps for 5 min and centrifuged for 5 min at approximately 800 *g*. 7—8 ml of the organic phase were transferred to a clean glass tube and the solvent evaporated to dryness with a gentle stream of dry nitrogen. The residue was dissolved in 0.5 ml of distilled water of which 40—100 μl were injected onto the column.

The compounds were quantified by comparing peak height ratios of furosemide and the internal standard from plasma or urine to peak height ratios of known concentrations of furosemide and the internal standard added to drug free of plasma or urine.

The internal standard was prepared by methylation of furosemide with ethereal diazomethane [8]. Diethyl ether (1 ml) saturated with diazomethane was added to approximately 5 mg of furosemide dissolved in 1.0 ml of methanol. This mixture was left at room temperature for 15 min. The solvent was evaporated with the aid of a gentle stream of dry nitrogen at room temperature. The residue was dissolved in 1.0 ml of methanol and the internal standard was isolated from the unreacted furosemide and byproducts by chromatography as described above. Identity of the methyl ester of furosemide was confirmed by mass spectrometry.

In urine samples of subjects treated with furosemide we discovered a peak

in addition to furosemide, the internal standard and CSA. Because the glucuronide had previously been described as a metabolite of furosemide [9], we incubated urine with a glucuronidase containing preparation (Gluculase®; Endo Lab., Garden City, N.Y., U.S.A.) at pH 5.5 and 37° for 24 h.

Furosemide was obtained from Hoechst-Roussel (Somerville, N.J., U.S.A.) and CSA from the U.S.P.C. (Rockville, Md., U.S.A.). Glass-distilled methanol was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other chemicals were ACS reagent grade obtained from commercial suppliers.

RESULTS AND DISCUSSION

Figs. 1A and 2A show chromatograms of blank plasma and urine, respectively, and demonstrate the lack of interfering compounds. Figs. 1B and 2B show the same biological samples spiked with the internal standard and with furosemide at concentrations of 0.25 and 0.5 μg furosemide per ml, respectively, demonstrating that these levels can be readily distinguished from the background.

The retention times of pure internal standard, furosemide and CSA were 12, 6 and 1.7 min, respectively. The retention times of these compounds were not altered when they were extracted from plasma. Thus, CSA will not interfere with furosemide analysis. Standard curves plotting peak height ratio of internal standard to furosemide on the y-axis and concentration of furosemide

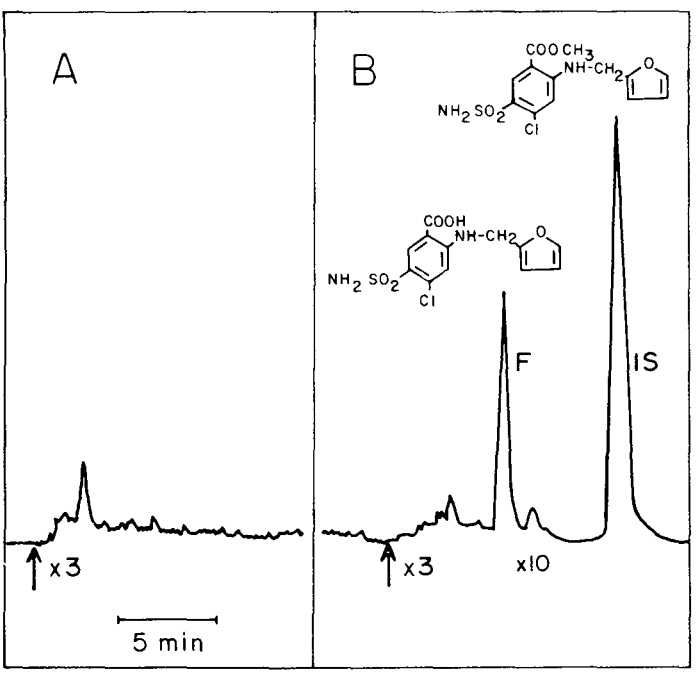

Fig. 1. Chromatograms of blank plasma (A) and the same plasma spiked (B) with furosemide (F) at a concentration of 0.25 μg/ml and the internal standard (IS). The arrows indicate time of injection. The sensitivity of the fluorometer is indicated by × 3 and × 10, × 3 being the more sensitive.

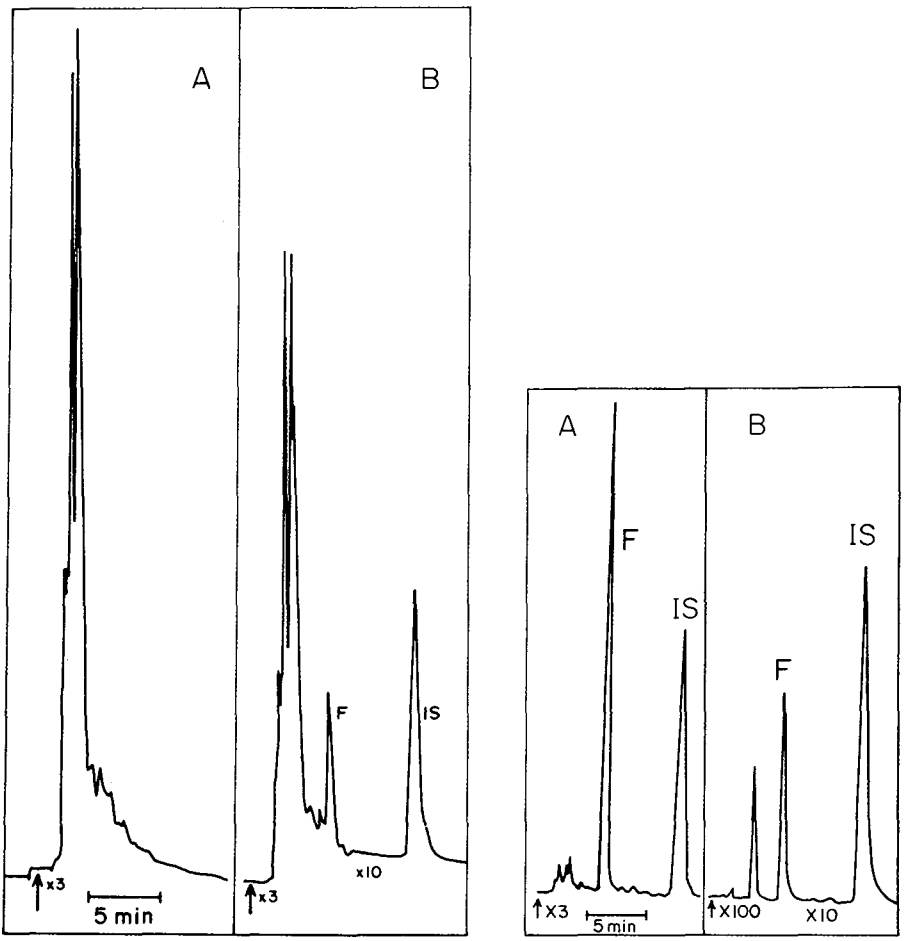

Fig. 2. Chromatograms of blank urine (A) and the same urine spiked (B) with furosemide (F) at a concentration of 0.5 μg/ml and the internal standard (IS). Symbols as in Fig. 1.

Fig. 3. Chromatograms of furosemide extracted from plasma (A) and urine (B) from a healthy male volunteer that received an intravenous dose of 80 mg furosemide. Concentrations: plasma, 4.1 μg/ml; urine, 15.2 μg/ml. See text for further explanation.

in samples of urine and plasma spiked with furosemide on the x-axis were linear, went through the origin and had a slope one. Identical curves were obtained for urine. The coefficient of variation for the analysis in urine (n = 5) was 5.3, 4.2 and 2.5% at levels of 0.5, 2.5 and 5.0 μg/ml, respectively. The coefficient of variation for analysis of furosemide in plasma was 9, 5.6 and 0.72% at levels of 0.2, 2 and 5 μg/ml, respectively.

Representative chromatograms of plasma and urine of a subject who had received 80 mg of furosemide intravenously are shown in Figs. 3A and B. No major peak other than furosemide and the internal standard could be observed in plasma. In urine an additional peak, clearly separated from furosemide and

CSA, with a retention time of approximately 3.5 min was observed. On incubation of this urine with glucuronidase this peak decreased while the furosemide peak increased (Figs. 4A and B). Thus, this peak may represent a conjugate, possibly the glucuronide of furosemide which has been suggested by Beerman et al. [9].

The applicability of this method to kinetic studies of furosemide is shown in Fig. 5 which depicts the plasma concentration profile of furosemide in a healthy adult volunteer after an intravenous dose of 80 mg furosemide.

The assay was checked for interference by drugs frequently used in patients who are treated with furosemide. For this, blank plasma was spiked with various drugs at concentrations indicated in Table I. Salicylic acid was the only drug tested that gave any interference. Its retention time was somewhat shorter than that of furosemide and hence it can be identified in this method. The level of salicylic acid added was about 20-fold higher than that found in the plasma of patients treated with high doses of aspirin [10].

Plasma of patients treated chronically with the drugs listed in Table II was analyzed before and after spiking with furosemide and the IS. There were no interfering peaks at the expected retention time of either furosemide or the internal standard before spiking and the recovery of the added furosemide was

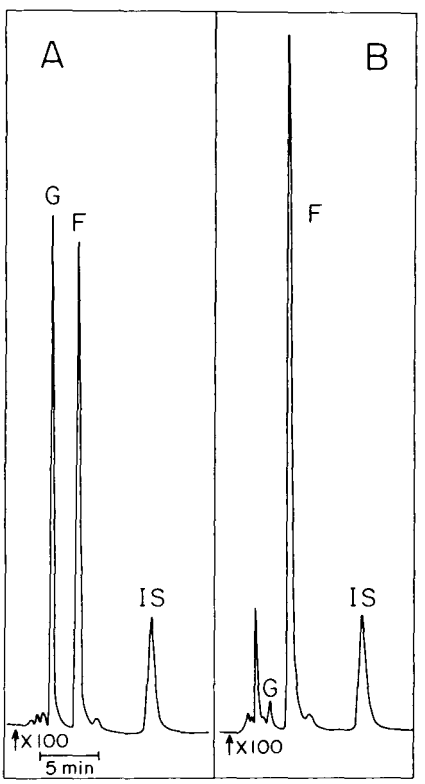

Fig. 4. Chromatograms of urine before (A) and after (B) incubation with Gluculase. IS= internal standard; F=furosemide; G=urinary metabolite of furosemide.

TABLE I

DRUGS ADDED IN VITRO TO PLASMAS CONTAINING FUROSEMIDE AND THE INTERNAL STANDARD AND TESTED FOR INTERFERENCE IN THE ANALYSIS OF FUROSEMIDE

—Indicates lack of interference.

Drug	Concentration (μg/ml)	Interference
Phenobarbital	50	—
Carbamazepine	50	—
Carbamazepine--10, 11-epoxide	12	—
Salicylic acid	1100	+
Guanethidine	0.08	—
α-Methyldopa	10	—
Hydralazine	40	—
Propranolol	0.5	—

quantitative as shown in Table II. Thus, neither the drugs nor their metabolites interfere at therapeutic dosage levels.

ACKNOWLEDGEMENTS

We are grateful to Dr. Grant R. Wilkinson for valuable discussion and to Dr. Brian Sweetman for the mass spectrometric confirmation of the structure of the internal standard.

TABLE II

MEASURED PLASMA CONCENTRATION OF FUROSEMIDE AFTER SPIKING WITH 0.5 μg/ml OF FUROSEMIDE

The plasma was obtained from patients treated chronically with therapeutic doses of the drugs listed below. Blanks of each sample were run prior to spiking; no interfering peaks were found in the blanks at the retention times of furosemide or the internal standard.

Drug	Furosemide recovered (μg/ml)
α-Methyldopa	0.52
Guanethidine	0.48
Hydralazine	0.46
Phenobarbital	0.48
Aspirin	0.52
Propranolol	0.46
Carbamazepine	0.46
Carbamazepine-10,11-epoxide	0.46

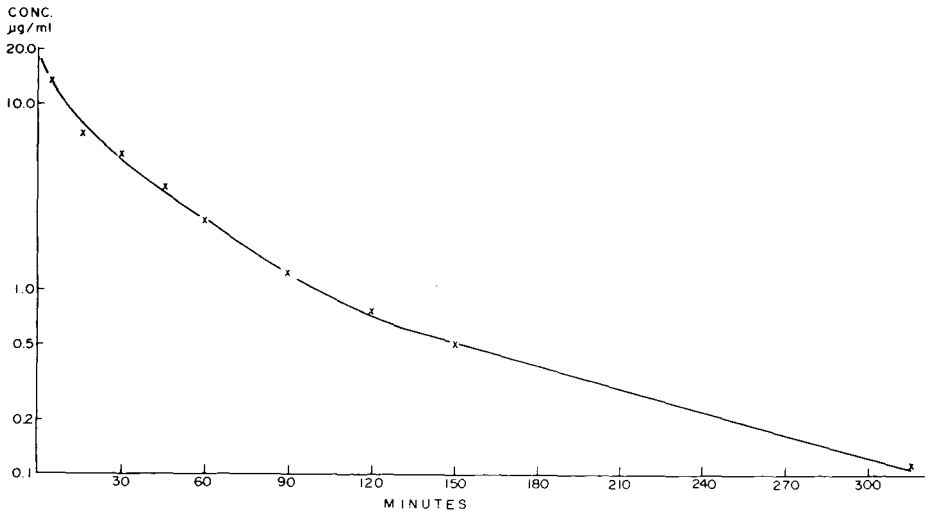

Fig. 5. Plasma concentration profile of furosemide (F) in a healthy adult male volunteer after an intravenous dose of 80 mg furosemide given at time 0.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS QUANTITATIVE ANALYSIS OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN PLASMA

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SUMMARY

A simple and rapid high-pressure liquid chromatographic procedure is reported for the simultaneous quantitative determination of propranolol and 4-hydroxypropranolol in plasma. Following an extraction the samples are chromatographed on a reversed-phase column and the components in the column effluent are detected by fluorescence monitoring. Using 1-ml plasma samples propranolol and 4-hydroxypropranolol concentrations at least as low as 1 ng/ml and 5 ng/ml, respectively, can be quantitated. The reproducibility of the method is satisfactory and no interference from endogenous plasma components or other drugs has been observed. A single plasma sample can be analyzed in approximately 20 min.

INTRODUCTION

Propranolol, a β-adrenergic blocking drug, has found wide application for the treatment of cardiac arrhythmia, sinus tachycardia, angina pectoris and hypertension [1], but its use has also been suggested for a number of other conditions, including dysfunctional labour [2], migraine [3], and anxiety [4]. Evidence has accumulated which suggests that the effectiveness of propranolol in the prophylaxis or treatment of certain conditions is related to the plasma concentration of the drug. Propranolol plasma levels in the range of 50—100 ng/ml at the end of a dosing interval are usually considered to be necessary for the suppression of ventricular ectopic beats [5] and inhibition of the tachycardia of strenuous exercise [6], while plasma levels of about 30 ng/ml and above are associated with attenuation of the symptoms of angina pectoris [7, 8]. However, it is possible that any such relationship between propranolol plasma level and therapeutic response is fortuitous in view of the fact that a metab-

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olite of propranolol, namely 4-hydroxypropranolol, has been reported to be approximately equipotent as the unchanged drug in producing β-blockade [9] and in addition the available data indicates that this metabolite is formed to a much greater extent following oral administration compared to intravenous administration of propranolol [10]. It would, therefore, appear to be desirable that an analytical procedure be available for the simultaneous quantitative determination of propranolol and its active metabolite in plasma. Such a method would permit re-evaluation of the question of whether or not relationships actually exist between propranolol plasma level and therapeutic response and an assessment of the role played by 4-hydroxypropranolol in a patient's response to propranolol therapy. Additionally, the analytical method may be valuable for the routine clinical monitoring of plasma concentrations of drug and active metabolite in patients undergoing therapy with propranolol.

Several methods have been reported for the determination of propranolol in plasma. The most widely used procedure is the fluorometric method of Black et al. [11] as modified by Shand et al. [12] which uses 4 ml of plasma and can be used for propranolol concentrations above about 5 ng/ml. This method may sometimes result in high background values which may be variable from day to day in the same individual [13]. Di Salle et al. [14] have described a gas chromatographic (GC) method for the determination of propranolol in plasma which involved a lengthy and laborious sequence of extractions followed by derivitization, GC and detection with an electron capture detector. Using plasma volumes of 0.5—2 ml it was possible to detect 1—5 ng/ml [13, 14]. Another GC method has been reported [15] in which the drug was extracted from 1 ml plasma following either a single or a back extraction procedure, derivatization and GC separation of the di-trifluoroacetyl derivatives of the drug and internal standard (oxprenolol) with subsequent electron capture detection. The total analysis time for each sample using the single extraction method (which could be used for propranolol levels greater than 5 ng/ml) was 45 min, while for the analysis of lower concentrations, when it was necessary to use the back extraction work-up technique, the assay turn around time was 70 min per sample.

All of the procedures discussed above suffer from the disadvantage that they only quantitate the unchanged drug. More recently the description of a quantitative method for the simultaneous determination of propranolol and its active metabolite, 4-hydroxypropranolol, has appeared in the literature [16]. In that method 1 ml of plasma, with sodium hydrogen sulphite, internal standard and buffer added, was extracted with 10 ml of ethyl acetate for 10 min followed by centrifugation for 5 min. After transfer to another tube the ethyl acetate layer was evaporated to dryness at 60° under a stream of nitrogen. At that stage trifluoroacetylation was carried out using a 15 min reaction time. The derivatized compounds were analyzed using the technique of selective ion monitoring by injection of an aliquot of the reaction mixture into a gas chromatograph—mass spectrometer. The minimum detectable concentration of propranolol and 4-hydroxypropranolol in plasma was 1 ng/ml and 5 ng/ml, respectively.

The purpose of this paper is to describe a rapid, sensitive and specific high-

pressure liquid chromatographic (HPLC) assay for the simultaneous determination of propranolol and 4-hydroxypropranolol concentrations in plasma.

EXPERIMENTAL

Reagents and standards

Propranolol hydrochloride, 4-methylpropranolol hydrochloride and 4-hydroxypropranolol hydrochloride were kindly supplied by Ayerst Labs. (New York, N.Y., U.S.A.). Standard solutions of the salts of propranolol and 4-hydroxypropranolol in methanol were prepared for spiking of blank plasma with known amounts of the two compounds; these solutions were stored at −20° when not in use. Fresh methanolic solutions were prepared periodically. An aqueous solution of 4-methylpropranolol hydrochloride which contained 150 ng/ml was prepared and stored at 4°.

Glass-distilled methanol, ethyl acetate and acetonitrile were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Phosphoric acid, sulphuric acid, sodium bicarbonate, sodium carbonate, and sodium hydrogen sulphite were ACS reagent grade and were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). An 0.06% solution of phosphoric acid in distilled water was prepared (final pH 2.6) and passed through a membrane filter for subsequent use in the preparation of the HPLC mobile phase. An aqueous solution with a pH of 2.2 was prepared by dilution of sulphuric acid with distilled water. Finally, a carbonate buffer (pH 9.5) was prepared by making a solution in distilled water which contained both sodium bicarbonate (0.5 *N*) and sodium carbonate (0.5 *N*).

Other drugs which were tested for potential interference of the assay procedure had, in most cases, been donated by pharmaceutical manufacturing companies.

HPLC instrumentation and conditions

The HPLC system consisted of a model M-6000A pump for delivery of mobile phase, a model U6K injection loop and a 30-cm μBondapak alkyl phenyl column (particle size 10 μm) obtained from Waters Assoc. (Milford, Mass., U.S.A.) together with a model FS 970 fluorescence detector of Schoeffel (Westwood, N.J., U.S.A.). The output from the detector was connected to a 10-mV potentiometric 10-in. recorder (Linear Instr. Corp., Irvine, Calif., U.S.A.). Chromatography was carried out at ambient temperature.

The mobile phase for the chromatography was prepared by mixing 27 parts of acetonitrile with 73 parts of 0.06% phosphoric acid solution, and this passed through the HPLC system at a rate of 2 ml/min (approximate operating pressure was 1700 p.s.i.g.). The fluorescence detector was operated with the wavelength of excitation set at 205 nm and an emission filter (KV 340) was used to select the fluorescence emission for detection. The recorder chart speed was 8 in/h.

Plasma sample preparation

Aliquots of plasma (1 ml) were pipetted into 13 × 100 mm screw-capped

culture tubes which contained 20 mg of sodium hydrogen sulphite. After the addition of internal standard solution (0.1 ml of 150 ng/ml 4-methylpropranolol hydrochloride solution), 1 ml of carbonate buffer (pH 9.5) and 3 ml of ethyl acetate, each tube was vortexed for 1 min to promote mixing of the immiscible phases. Following centrifugation at 800 *g* for about 2 or 3 min most of the ethyl acetate layer was transferred by Pasteur pipette to a 12-ml tube which had a tapered base and contained 0.1 ml of a dilute sulphuric acid solution (pH 2.2). Each tube was closed with a screw cap and vortex mixed for 1 min followed by brief centrifugation as described above. A 50-μl aliquot of the lower aqueous phase was injected into the HPLC.

Standard curves were prepared by spiking blank pooled human plasma with propranolol hydrochloride and 4-hydroxypropranolol hydrochloride such that the concentrations of each compound in plasma were 1, 2, 5, 10, 25, 50, 100, and 150 ng/ml, followed by extraction and chromatography as described above. The peak height ratios of propranolol and 4-hydroxypropranolol to 4-methylpropranolol were plotted against the concentration of propranolol and 4-hydroxypropranolol, respectively, in order to provide standard curves. A programmable calculator (Hewlett Packard model 97) was used to fit equations to the data.

Reproducibility studies

Reproducibility studies were performed at two concentrations for both compounds by doing six replicate analyses of plasma samples which had been spiked such that the concentrations were 10 ng/ml for one series and 50 ng/ml for the second series.

Drug interference studies

A number of other drugs and drug metabolites were tested for potential interference of the assay by injecting aliquots of stock solutions of the compounds being tested into the HPLC.

RESULTS AND DISCUSSION

A number of HPLC columns and mobile phases were tested during preliminary studies, but the μBondapak alkyl phenyl column with a mobile phase of acetonitrile—0.06% phosphoric acid (27:73) was the only system which afforded suitable resolution of the unchanged drug, metabolite and internal standard from each other and endogenous components from plasma. Chromatograms resulting from the analysis of blank human plasma together with similarly treated plasma which had been spiked with propranol and 4-hydroxypropranolol (10 ng/ml of hydrochloride salt of each in plasma) are shown in Fig. 1. The compounds eluted from the reversed-phase column as symmetrical peaks with retention times of 3.9, 7.9, and 11.6 min for 4-hydroxypropranolol, propranolol and 4-methylpropranolol, respectively. No interference in blank plasma was observed at the retention times of the three compounds of interest, although a very small endogenous peak eluted after the 4-hydroxypropranolol peak. The presence of this small peak does not appear to jeopardize the analysis of the metabolite since it eluted on the tail of the 4-hydroxypropranolol

peak. Analysis of other batches of blank plasma resulted in chromatograms which were qualitatively and quantitatively similar to that which is illustrated in Fig. 1.

The standard curve for propranolol in plasma was linear ($y = 0.0549x + 0.0026$, $r = 0.9994$) over the range of plasma concentrations from 1 to 150 ng/ml (based on the hydrochloride salt). Those lower and upper limits of the standard curve correspond to 0.875 and 131 ng of propranolol base per ml of plasma, respectively. The mean response factor (peak height ratio divided by propranolol concentration) for the eight point standard curve was 0.0555; the individual response factor values fell within a narrow range from 0.0505 to 0.0580 which indicates good linearity over the concentration range studied. The signal to noise ratio at the 1 ng/ml concentration was approximately 10 to 1, which suggests that quantitation and/or detection of considerably lower concentrations would be possible.

The standard curve for 4-hydroxypropranolol hydrochloride in plasma was constructed over the concentration range of 5—150 ng/ml (4.41—132 ng of the base per ml). This standard curve had a small inflection at about 40—50 ng/ml and was apparently linear above that value. This phenomenon was reproducible in that other standard curves prepared for the metabolite behaved in the same manner. The exact reason for such a behavior is unknown. A power equation provided a good fit ($y = 0.0130x^{1.0987}$, $r = 0.9992$) to the experimental data which enabled plasma concentrations to be determined from peak height ratio information by use of the equation.

The precision of the method was determined by six replicate assays at two concentrations for both the unchanged drug and metabolite. At the 10 ng/ml level the coefficient of variation for 4-hydroxypropranolol and propranolol was 15.4% and 9.6%, respectively, while at the 50 ng/ml level the corresponding values were 8.8% and 6.0%, respectively. These reproducibility data com-

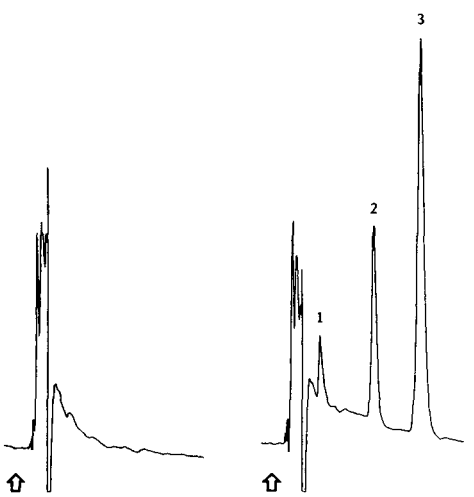

Fig. 1. Chromatograms of extracts of blank human plasma (left) and plasma spiked with 10 ng/ml of propranolol and 4-hydroxypropranolol (right). Peaks 1, 2 and 3 are 4-hydroxypropranolol, propranolol and 4-methylpropranolol, respectively. The arrow marks the point of injection.

pare favorably with those reported for the simultaneous mass fragmentographic assay for propranolol and its metabolite [16].

The metabolite, 4-hydroxypropranolol, is stable in plasma for at least one week if frozen [16]. However, in agreement with Walle et al. [16], it was found to be necessary that sodium hydrogen sulphite be added to the plasma prior to the first extraction in order to limit the extent of decomposition of the metabolite at the elevated pH values employed in that step, because stability decreases with increasing pH. In the absence of sodium hydrogen sulphite erratic results sometimes occurred, particularly at low concentrations of 4-hydroxypropranolol. Methanolic stock solutions of the metabolite stored at −20° appear to be stable for at least several days as assessed by quantitatively comparing chromatograms from freshly prepared solutions with those of older solutions. Additionally, 4-hydroxypropranolol appears to be adequately stable in the dilute sulphuric acid solution (pH 2.2) which was used for the back extraction and injection into the HPLC because no systematic trends toward decreasing peak height ratios were observed during the reproducibility studies in which all samples (six for each concentration) were extracted at the same time but injected over a period of hours.

The extraction work-up procedures in which the plasma is extracted with organic solvent followed by back extraction into a small volume of aqueous acid are relatively rapid and simple, and they serve several important functions. Firstly, the extraction sequence used provides a degree of sample clean-up because acidic and neutral compounds (endogenous and other drugs) would be separated from the compounds of interest. Secondly, the procedures used isolate propranolol, its metabolite and internal standard in a solvent which is suitable for direct injection into the HPLC, since aqueous sulphuric acid is miscible with the HPLC mobile phase. Finally, adequate concentration of the sample occurs by the back extraction into a small volume of acid so that time consuming evaporation steps are not necessary.

It should be pointed out that volumes of plasma smaller than 1 ml could be analyzed at the expense of assay sensitivity. For example, the analysis of 200-μl aliquots of plasma would permit quantitation of propranolol and 4-hydroxypropranolol levels of at least 5 and 25 ng/ml, respectively, which may be adequate for routine clinical monitoring of plasma levels.

A number of other basic drugs and drug metabolites were tested for potential interference of the assay but none of the compounds tested were found to interfere with the analysis of propranolol and its metabolite. The compounds investigated in this experiment were the other cardioactive agents and their metabolites, namely lignocaine, monoethylglycinexylidide, glycinexylidide, procainamide, N-acetylprocainamide, and quinidine. The other compounds which were also shown not to impair the usefulness of the assay were chlordiazepoxide, chlorpromazine, fluphenazine, perphenazine, prochlorperazine, trifluoperazine, and trimeprazine. In addition, chromatograms resulting from the analysis of various batches of blank plasma and plasma obtained from patients on other drug therapy showed no interfering peaks.

The method has been used for analysis of plasma samples collected from patients receiving propranolol. Fig. 2 shows chromatograms resulting from the extraction and chromatography of plasma samples obtained from patients

Fig. 2. Chromatograms of extracts of plasma obtained from two patients who had received propranolol intravenously. Peaks 1 and 2 are propranolol and 4-methylpropranolol, respectively. The arrow marks the point of injection. Concentration of propranolol in sample A and B is 101.0 ng/ml and 35.5 ng/ml, respectively.

who had received propranolol by the intravenous route. In these cases the 4-hydroxypropranolol peak is absent because that metabolite is not formed to any significant extent following intravenous administration of propranolol [10].

CONCLUSIONS

The HPLC method described permits the rapid simultaneous determination of plasma concentrations of propranolol and its active metabolite, 4-hydroxypropranolol. The preparation of plasma samples prior to chromatography is relatively simple and no evaporation or derivatization steps are necessary. The total turn around time for the analysis of a single sample is approximately 20 min but this would be shortened considerably when many samples are extracted simultaneously. The sensitivity and precision of the method are good and no interference is observed from plasma samples or from a number of other commonly used drugs. It is concluded that the analytical method described is suitable for routine clinical monitoring of plasma levels in patients or for use in research studies in pharmacokinetics.

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Biomedical Applications

CHROMBIO. 136

THE ESTIMATION OF QUINIDINE IN HUMAN PLASMA BY ION PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, accurate method for determination of quinidine in plasma has been developed using ion-pair extraction and high-performance liquid chromatography. The method, which is capable of distinguishing between quinidine and dihydroquinidine, involves acidification of plasma with perchloric acid, extraction with methyl isobutyl ketone and chromatography of the carbonate-washed extract on a silica gel column with a mobile phase of methylene chloride—hexane—methanol—perchloric acid (60:35:5.5:0.1) followed by fluorometric detection. The procedure is sensitive to below 50 ng/ml (coefficient of variation 6.6%) and compares favourably with a standard spectrofluorometric method when tested with plasma from volunteer subjects.

INTRODUCTION

The cardiac depressant quinidine is used widely in the treatment of certain cardiac arrhythmias. Due to the narrow range between its effective and toxic concentrations (3—5 μg/ml) [1] there is a need for a rapid, accurate and sensitive procedure for monitoring plasma levels of this drug, clinically.

Quinidine is presented as different salts in a variety of dose forms which generally contain 3—10% dihydroquinidine [2, 3]. In humans both of these substances undergo biotransformation [4] with the major urinary metabolites being either 2′-quinidinone, and 3-hydroxyquinidine or the dihydro analogues.

The fluorometric method of Cramér and Isaksson [5] used for over a decade for the assay of quinidine in plasma was found to be non-specific by several workers [6—8]. Armand and Badinand [8] modified the extraction procedure to remove most of the interfering metabolites. However, neither these procedures nor the more recent gas chromatographic procedures of Valentine

et al. [9] and Midha and Charette [10] differentiated quinidine from dihydroquinidine.

An additional problem with the use of direct spectrofluorometric procedures in the clinical situation occurs because cardiac patients may be exposed to several drugs which may interfere in assays. Recently such an interference has been described with triameterene [11, 12] which has extraction and fluorescence characteristics similar to those of quinidine.

The present paper describes the resolution of these problems by application of recent developments in ion-pair extraction and chromatography [13] together with the separatory power of high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

Quinidine sulphate was obtained from Burroughs Wellcome, (La Salle, Canada); its dihydroquinidine content was 9.3%. 2'-Quinidinone and 3-hydroxyquinidine were the generous gift of Dr. F.I. Carroll (Research Triangle Institute). Dated plasma, obtained from the Canadian Red Cross, was used for blanks and calibration curves.

The solvents used for chromatography were from Burdick and Jackson (Muskegon, Mich., U.S.A.). All other chemicals were of reagent grade, purchased locally.

Chromatography

Equipment. The chromatographic apparatus used consisted of a Rheodyne Model 7120 injector, Waters Model 6000A pump and Schoeffel Model FS 970 fluorescence detector.

Conditions. The column (250 × 3 mm I.D.) was packed by a balanced-density slurry method using silica gel (LiChrosorb Si-60, 5 μm, E. Merck, Darmstadt, G.F.R.). The mobile phase dichloromethane—hexane—methanol—70% perchloric acid (60:35:5.5:0.1) was pumped at a flow-rate of 3 ml/min, producing a back pressure of 3500 p.s.i.g. (Flow-rates of 2 ml/min, used in some experiments, gave equivalent results). The excitation wavelength of the detector was set at 325 nm, and the emission monitored using a sharp-cut filter at 420 nm. The gain of the detector was adjusted to give a peak-to-peak noise of 0.5% full scale (f.s.) at the sensitivity of 0.05 μA f.s. and the time constant setting at minimum. Monitoring at 0.02 or 0.01 μA f.s. was done with the time constant set at nominal 2 sec.

Solutions

Dilutions of perchloric acid (1 *M* and 3 *M*) were prepared from the 70% reagent (approx. 13 *M*) with fluorescence grade water. Methyl isobutyl ketone (MIBK) saturated with an equal volume of 1 *M* perchloric acid was prepared shortly before use.

Standards

Quinidine sulphate was dissolved in water at the concentration of 1 mg/ml.

Appropriate dilutions of this solution with blank plasma were made to give the desired concentrations.

Treatment of the human volunteers

Four healthy male volunteers, who abstained from food and water overnight, were administered 200 mg quinidine sulphate (commercial formulations). Venous blood was withdrawn into heparinized evacuated containers (Vacutainers; Becton-Dickinson, Toronto, Canada) just prior to the dose and at appropriate intervals during the next 30 h. The blood samples were immediately centrifuged and the separated plasma kept at −18° until analysis.

Procedures

To 0.5 ml plasma, in a 10 ml round-bottomed glass tube fitted with a PTFE-lined screw cap, 0.25 ml 3 *M* perchloric acid was added. After mixing briefly (Vortex), 1 ml of MIBK (perchloric acid saturated) was added, the mixture was shaken vigorously (Evapo-Mix, Buchler Instruments, U.S.A.) for 5 min and centrifuged for 5 min.

The organic layer was transferred to another tube and the aqueous phase (including the precipitate) re-extracted as above with 1 ml MIBK. The organic phase was combined and washed by shaking briefly (Vortex) with 0.3 ml saturated aqueous potassium carbonate solution and centrifuged. Exactly 100 μl of the washed extract was used for chromatography.

The fluorometric procedure of Armand and Badinand [8] was used with only minor modifications [14].

Quantitative analyses

The amount of quinidine in plasma samples was estimated by comparing the peak height obtained for the sample to a calibration curve, constructed daily, using spiked blank plasma. This curve took into account the actual quinidine sulfate content (90.7% of the weighed amount) of the standard material used.

Statistical evaluations

Correlation coefficient (r^2) and regression were determined by the linear least squares method ($y = b_0 + b_1 x$).

RESULTS AND DISCUSSION

Extraction

Quinidine, a basic substance, can be extracted into organic solvents either at alkaline pH [5] or in the presence of an ion-pair. The former method is somewhat delicate as alkaline solutions of plasma proteins have a tendency to emulsify when shaken vigorously with organic solvents. Furthermore, quinidine-free base is non-fluorescent and requires back-extraction into sulfuric acid for quantitation.

Ion-pair extraction, on the other hand can be accomplished with relative ease, provided an appropriate counter-ion and extraction solvent are used.

For the following reasons the counter-ion of choice in these experiments

was perchloric acid: being a strong acid it remains dissociated in water in the absence of buffers, giving rise to perchlorate ions; it prevents emulsification by virtue of its low pH; it forms a strongly fluorescent organic-soluble ion-pair with quinidine, with some solubility even in non-polar solvents such as hexane (personal observations).

Table I shows the distribution coefficients for quinidine perchlorate between plasma containing 1 *M* perchloric acid and various solvents. Of the solvents tested MIBK appeared to be the most efficient. More polar ketones were not investigated because of the mutual solubility of the organic and aqueous phases.

TABLE I

DISTRIBUTION COEFFICIENT OF QUINIDINE PERCHLORATE BETWEEN PLASMA AND VARIOUS SOLVENTS

Solvents used: Methyl isobutyl ketone (MIBK); ethyl *n*-butyl ketone (ENBK); di-*n*-propyl-ketone (DPK) and benzyl alcohol—toluene (4:1) (BzOH—Tol).

Quinidine (μg/ml plasma)	Solvent	V_{org}/V_{aq}	C_{org}/C_{aq}
1	MIBK	1.33	1.93
1	ENBK	1.33	0.59
1	DPK	1.33	0.45
4	BzOH—Tol	0.25	0.43

The concentration of perchloric acid did not influence the extraction efficiency, provided it was kept above 0.5 *M*. Proper equilibration of the phases required several minutes of vigorous, vortex-type mixing; extractions using a tumbling mixer (Fisher RotoRack) required at least 20—30 min. In neither case was emulsification a problem and the phases were easily separated by centrifuging them briefly. A final wash of the extract with aqueous saturated potassium carbonate was required to remove dissolved perchloric acid which interfered with the chromatography. In earlier experiments dibasic potassium phosphate saturated with MIBK was used. However the saturated carbonate, by also removing most of the dissolved water, gave longer column life.

The recovery of quinidine with the final procedure was 94.2% (coefficient of variation (C.V.) 5.2%). A single extraction gave efficiencies of approx. 70%. While this could reduce the accuracy of the method, in clinical monitoring the resultant saving in time may warrant such a compromise.

Chromatography

In order to avoid the back-extraction of quinidine into an aqueous system, forward-phase chromatography was chosen. Of the mobile phases tried, methylene chloride—hexane (60:35) appeared optimal for separating dihydroquinidine from quinidine, while methanol was used to adjust the retention time. Small amounts of perchloric acid were included to induce fluorescence. The amount of perchloric acid was not critical for fluorescence, but had some influence on the retention times.

Fig. 1 shows chromatograms of plasma extracts. Fig. 1A was obtained by extracting blank plasma to which 1 μg quinidine sulphate standard per ml was added. Dihydroquinidine (peak 1, Fig. 1A; k' = 4.0) was estimated to be 9.3% by comparing peak areas. It is well separated from quinidine (peak 2, k' = 5.0). Fig. 1B represents the chromatogram of a plasma extract of a volunteer 30 h after ingestion of 200 mg quinidine sulphate. In addition to dihydroquinidine and quinidine, a third peak (k' = 7.7) is apparent; the identity of this peak has not been established. Fig. 1C, from a blank plasma, shows no interfering peaks with retention times similar to quinidine and dihydroquinidine.

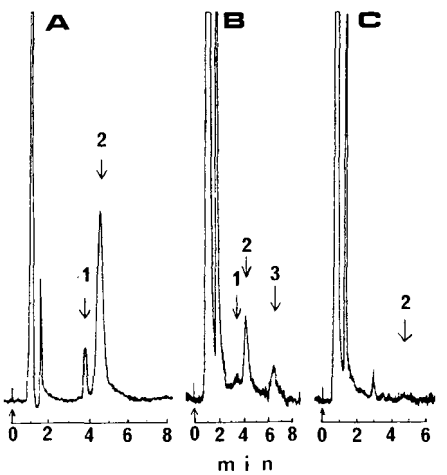

Fig. 1. Chromatographs of plasma extracts with and without quinidine. A, blank plasma spiked with 1 μg quinidine sulphate (containing 9.3% dihydroquinidine) per ml; B, plasma of a volunteer 30 h after the ingestion of 200 mg quinidine sulphate (containing dihydroquinidine) (estimated concentration: 79.3 ng/ml); C, blank plasma. Conditions: Flow-rate: A and C, 2 ml/min; B, 3 ml/min. Sensitivity, A, 50 nA full scale; B and C, 10 nA full scale. Peaks: 1 = dihydroquinidine; 2 = quinidine; 3 = unidentified.

To test for possible interferences, triamterene (k' = 10.3), 3-hydroxyquinidine (k' = 12.1) and 2'-quinidinone (k' = 5.0) have been chromatographed using the same system. Of these only 2'-quinidinone had a retention time identical to quinidine. Since, however, this metabolite contributed little to the measured in vivo plasma concentrations of quinidine (see below), no special attempts were made at this time to remove this interference.

Calibration curve

To blank plasma appropriate amounts of quinidine sulphate standard were added. These spiked standards were carried through the procedure and the peak heights obtained plotted against the concentrations. Table II summarizes the characteristics of the calibration curve. For concentrations of 0.05—2 μg/ml the C.V. values ranged from 3.75—6.94%. Good linearity was evidenced by the steady values for the peak height to concentration ratios. The correlation coefficient (r^2) for the 24 individual determinations was 0.998 and

TABLE II

CALIBRATION CURVE

Correlation: n = 24, r^2 = 0.998; Regression: slope = 0.0584 inches · μA f.s., intercept = 0.0008 inches · μA f.s. · μg^{-1} · ml.

Concentration (μg/ml plasma)	n	Mean peak height (inches · μA. f.s.)	ht*/C	S.D.	C.V. (%)
0.05	6	0.0030	0.060	0.0002	6.63
0.20	6	0.0132	0.066	0.0009	6.94
0.50	6	0.0301	0.060	0.0018	5.86
2.00	6	0.1174	0.059	0.0044	3.75

*Ratio of peak height to concentration.

the regression showed a negligible intercept (0.0008 inches · μA f.s.) compared to the slope (0.0584 inches · μA f.s. · μg^{-1} · ml).

Plasma concentrations in vivo

The validity of the method was tested by comparison of plasma profiles obtained by the described procedure with those obtained using a fluorometric procedure [8, 14] after administration of 200 mg of quinidine sulphate to four volunteers. Fig. 2 shows the profile for one volunteer from 0—30 h as

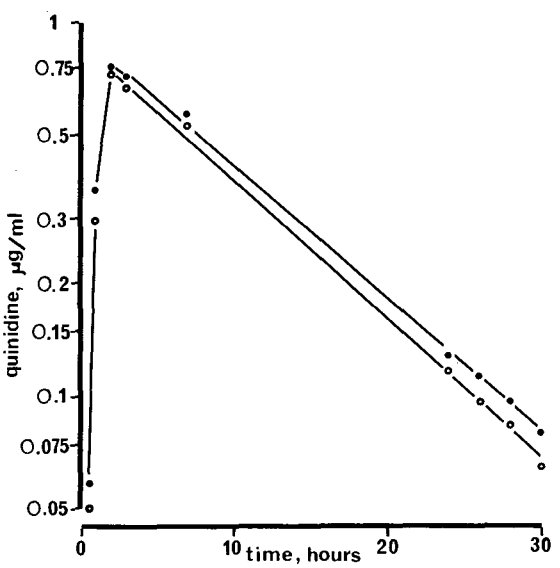

Fig. 2. Plasma profile of quinidine in a volunteer, estimated by fluorometry (•) and HPLC (○).

measured by both methods. Consistently lower values were found by HPLC, as expected, since dihydroquinidine was not measured. Similar results were obtained with the other three volunteers.

All plasma concentrations for the volunteers were 2—10% less by HPLC compared to fluorometry: mean for 1—7 h, 6.4% lower; the 24—30 h values, all in the 60—100 ng/ml range, were 15% lower.

The correlation between the chromatographic (ordinate) and fluorometric (abscissa) procedures was done on the data obtained on 37 random plasma samples, containing 50—1100 ng quinidine/ml. Least-square linear regression gave an intercept that was not significantly different from 0 (95% confidence interval: −9.44 ± 11.23). The regression line forced through the origin (r^2 = 0.999) had a slope of 0.95 ± 0.01 (95% confidence interval), significantly lower than unity, as expected.

Table III shows a comparison of the areas under the plasma concentration—time curve (AUC) for the four volunteers. All AUC values obtained by HPLC are 6—7% lower compared to fluorometry. The AUC for the means is 6.7% lower.

TABLE III

COMPARISON BETWEEN HPLC AND FLUOROMETRIC PROCEDURES FOR THE ESTIMATION OF AUC* FOR QUINIDINE

Volunteer No.	AUC ($\mu g \cdot h \cdot ml^{-1}$)		
	HPLC	Fluorometric	Diff. (%)**
1	10.03	10.77	−6.1
2	10.81	11.52	−6.2
3	9.86	10.54	−6.5
4	9.12	9.83	−7.3
Means***	9.85	10.56	−6.7

*Area under the plasma concentration—time curve, calculated by the trapezoidal rule.
**HPLC relative to fluorometry.
***AUC calculated from the mean plasma concentrations.

Since the values obtained by HPLC are within a few percent of the values obtained by fluorescence corrected for dihydroquinidine it would appear that 2'-quinidinone contributes little to the fluorescence of the quinidine peak. Whether this is because of a lower quantum efficiency at the wavelengths used, a relatively smaller importance in plasma as compared to urine or a combination of both cannot be established from the present study.

In conclusion, the present method can be used for the simultaneous assay of quinidine and dihydroquinidine in plasma. Its sensitivity makes it applicable to single-dose bioavailability studies in humans, while its speed would make it useful in clinical and toxicological monitoring of patients.

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DETERMINATION OF AMITRIPTYLINE AND NORTRIPTYLINE IN HUMAN PLASMA BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

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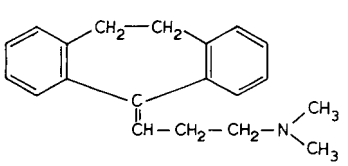
SUMMARY

A thin-layer chromatographic method for simultaneous determination of amitriptyline (AT) and nortriptyline (NT) in human plasma is described. Both substances are extracted from biological material by means of a single extraction. The extract is evaporated until dry and the residue quantitatively applied to a silica gel thin-layer plate. AT and NT are separated from interfering plasma components by chromatography. The spots are visualized by nitration, reduction and coupling with N-(1-naphtyl)ethylenediamine on the plate. The intensity of the azo-dyes formed can be measured densitometrically. Using 1 ml of plasma, the sensitivity limit was 0.5 ng/ml for both substances. About 10—15 plasma samples can be analysed per day. The method is applicable to pharmacokinetic studies after a single oral dose of 25 mg AT as hydrochloride in man.

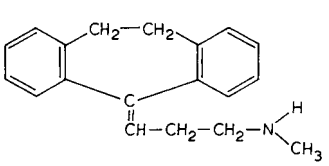
INTRODUCTION

In recent years several methods have been published for the determination of amitriptyline (AT) and nortriptyline (NT) in human plasma. Jørgensen [1] and Bailey and Jatlow [2] used a gas chromatographic procedure with a nitrogen detector. The sensitivity of this procedure was about 5 ng AT and NT/ml of serum. Wallace et al. [3] determined AT, after oxidation to a polyaromatic carbonyl derivative, by gas—liquid chromatography with an electron capture

AMITRIPTYLINE



NORTRIPTYLINE



detector. In this case no differentiation between AT and NT was possible. The sensitivity of the method was about 2 ng/ml of serum. A similar procedure is described by Hartvig et al. [4]. Prior to oxidation they separated the extracted AT and NT by column chromatography. A quantitative thin-layer chromatography (TLC) method for the determination of AT in human plasma has also been developed by Faber et al. [5], with a sensitivity of about 10 ng/ml of plasma.

All these methods have sensitivity limits which do not allow pharmacokinetic studies to be carried out after a normal single oral dose of 25 mg AT-hydrochloride in which case concentrations of as little as 0.5—1 ng AT per ml of plasma should be measurable.

We therefore tried to develop a sufficiently sensitive TLC method using direct densitometric measurement. This procedure was similar to the method published recently [6] for the determination of chlorpheniramine and codeine. By changing the reaction conditions and the reagents the necessary sensitivity for AT and NT was achieved. The procedure is described in this paper.

METHOD

AT and NT are extracted from alkalized plasma with pentane—isobutanol. The extract is concentrated and applied to a silica gel thin-layer plate. After separation, AT and NT are nitrated with nitric acid, reduced with sodium dithionite to the corresponding aromatic amines which are diazotized and coupled with N-(1-naphthyl)ethylenediamine. The intensities of the azo-dyes are directly proportional to the concentrations of AT and NT and can be measured densitometrically on the TLC plate.

Evaluation of the test conditions

Extraction

At the nanogram level the extraction of AT and NT from plasma is critical. To reduce the extraction of interfering plasma constituents it is advantageous to use apolar organic solvents. But it was observed that in apolar solvents the substances to be determined were partially adsorbed at the glass surfaces. Even siliconisation of the surfaces did not eliminate this effect. However, by adding 1% of isobutanol to pentane, the adsorption of AT and NT was practically eliminated.

The extraction yield of AT and NT from an aqueous solution is independent of the quantity of NaOH added. This quantity is however critical for extraction of these substances from plasma. A minimum concentration of NaOH is necessary to compensate for the buffer capacity of the plasma. We expected that the quantities of NaOH could be varied arbitrarily above this minimum level. We found however, that an optimal concentration exists above which the extraction yield diminishes considerably. We cannot explain this phenomenon. The highest recoveries from plasma (more than 90%) were found after addition of 0.2 ml of 2.5% NaOH to 1 ml of plasma.

Thin-layer chromatography

From a large number of solvents examined, the following systems were found to be the most suitable (vapour saturation of the jar): chloroform—ethanol—acetic acid (5:1:1.5), R_F values: AT ca. 0.3, NT ca. 0.45; chloroform—methanol (4:1), R_F values: AT ca. 0.35, NT ca. 0.1.

Detection

Principle of spraying. The best results were obtained by spraying the reagents. The layer should be completely and homogeneously wet, but without drops. For spraying, we used the commercial glass sprayers without modification.

Nitration. AT and NT can be nitrated at high temperature on silica gel thin-layer plates using nitric acid. The best results were obtained with a mixture of nitric acid (65%)—methanol (1:1). Two factors are critical for nitration on thin-layer plates. The temperature of 125—130° has to be reached in a short time and should be homogeneous over the whole plate. We achieved this by placing the thin-layer plate on a preheated aluminium block with a high heat capacity in a drying oven. A more complex problem was ventilation of the drying oven. We found that an intensive air-circulation and draining off of the nitric-acid vapours gave the highest nitration yield.

The structures of the nitration products were not identified, since this is of secondary importance for our project aim. The optimal nitration conditions were: temperature 125—130°; intensive air circulation and sucking-off the nitric acid vapours with a waterjet pump; reaction time 15 min.

Reduction. The reduction of aromatic nitro compounds to the corresponding amino compounds is possible using several reducing reagents. Titanium(III) chloride and thin(II) chloride have been used [6, 7]. However, for the reduction of the nitroderivatives of AT and NT sodium dithionite gave advantageous results. The reduction with this reagent on thin-layer plates depends mainly on the pH of the solution used. The best results were obtained at pH 6.5 using a 0.5 *M* sodium-phosphate buffer as solvent for sodium dithionite. The optimum reaction temperature was 80—85°. Air circulation had to be omitted since it reduced the reduction yield. The reaction period had to be limited to 8 min, longer periods led to lower recoveries.

The amino-compounds of AT and NT showed an intensive fluorescence under long-wave UV light (366 nm). Due to bad reproducibility, however, these fluorescences were found to be unsuitable for quantitative measurement.

Diazotizing and coupling. The procedure according to the Bratton—Marshall reaction was used with a 2% solution of sodium nitrite in 1 *M* HCl and a 1% solution of N-(1-naphthyl)ethylenediamine dihydrochloride in methanol. After spraying the plate with sodium nitrite it was necessary to completely dry the silica gel layer with a stream of cold air. Neglection of this step led to a reduction in sensitivity of the method.

Densitometry on the thin-layer plate

The intensity of the AT and NT spots can be measured with the commercial chromatogram spectrophotometers. We used the Zeiss chromatogram-spectralphotometer model PMQ II.

EXPERIMENTAL

Reagents

The following reagents were used: Sodium hydroxide (2.5%) in distilled water; equal parts of analytical-grade methanol and analytical-grade 65% nitric acid are mixed carefully and cooled thereafter; 4% sodium dithionite ($Na_2S_2O_4$) in 0.5 *M* sodium phosphate buffer pH 6.5 (this solution remains stable for about 1 h); 2% sodium nitrite in 1 *M* hydrochloric acid, prepared immediately before use; 1% N-(1-naphthyl)ethylenediamine dihydrochloride in analytical-grade methanol. This solution can be used for a few days; analytical-grade solvents: acetic acid, chloroform, ethanol, isobutanol, methanol, *n*-pentane.

Standards

Solution I: dissolve 10.0 mg of AT hydrochloride in 10 ml of methanol.

Solution II: dissolve 10.0 mg of NT hydrochloride in 10 ml of methanol.

Solution III: mix 1 ml of solution I and 0.25 ml of solution II and dilute with methanol to 10 ml.

Solution IV: 0.5 ml of solution III are diluted with chloroform to 100 ml. This solution contains 5 ng of AT hydrochloride and 1.25 ng of NT hydrochloride in 10 µl.

Solution V: 1 ml of solution III is diluted with distilled water to 100 ml.

Plasma standard I: 0.5 ml of solution V are diluted with a blank plasma to 25 ml (20 ng AT/5 ng NT/ml). Starting with this plasma standard the following plasma standards are prepared by dilution: plasma standard II; 10 ng AT/2.5 ng NT/ml, plasma standard III; 5 ng AT/1.25 NT ng/ml and plasma standard IV; 2.5 ng AT/0.625 ng NT/ml.

Materials for TLC

The following materials are used: Merck (Darmstadt, G.F.R.) silica gel 60, F_{254} 0.25 mm precoated thin-layer plates, 20 × 20 cm; 10 µl automatic capillary-dosage pipettes from Desaga (Heidelberg, G.F.R.). Chromatographic tanks for 20 × 20 cm plates; Zeiss chromatogram-spectral photometer PMQ II.

Procedure

Extraction. Mix well 1 ml of plasma and 0.2 ml of 2.5% sodium hydroxide in a 15 ml centrifuge tube. Add 5 ml of pentane—isobutanol (100:1) and shake for 5 min on a reciprocating shaker. Centrifuge the samples for 5 min at 700 *g*. Transfer 4.5 ml of the organic phase into a 10—15-ml glass tube. Evaporate the extracts in a shaking thermostat at a temperature of 55—60°. Process 1 ml of each of the plasma standards I—IV along with the samples.

Application to the TLC-plates. The cold extraction-residues are dissolved in 30 µl of chloroform. Apply these solutions to a silica gel TLC plate (20 × 20 cm) heated to about 60°, with 10 µl pipettes. The distance from the bottom and side edges should be 1.5 cm and between the spots 1.2 cm. Rinse the tubes carefully with 30 µl of chloroform and apply the rinsings to the plate. For localisation of AT and NT after separation place 5 µl of solution III on the boundary points.

Chromatography. The chromatograms are developed in a jar, lined with filter-paper to achieve vapour saturation. The tank is preconditioned with the solvent, either chloroform—ethanol—acetic acid (5:1:1.5) or chloroform—methanol (4:1), about 10 min before use. The migration distance of the solvent from the bottom edge is 10 cm. Dry the plate and mark the zones of AT and NT under short-wave UV light (254 nm).

Detection. Spray the plate with the nitration-mixture until the layer is uniformly wet. After the liquid has completely penetrated into the silica gel, place the TLC plate in a drying oven with a temperature of 125—130°. To achieve a high nitration yield, the plate is located on a preheated aluminium block, intensive air circulation is maintained and the acid vapours are sucked off by a powerful waterjet pump. After 15 min cool the plate and spray with the dithionite reagent. The reduction is performed in a drying oven at a temperature of 80—85° for 8 min without ventilation. After cooling, AT and NT show under long-wave UV light a pale-yellow fluorescence. Spray the plate with a fresh nitrite solution, dry for 10 min with a stream of cold air (hair drier) and spray with the N-(1-naphthyl)ethylenediamine dihydrochloride reagent. After 1 min dry with air at a temperature of about 50—60° (hair drier). As already mentioned, all spraying must be performed very carefully.

Scanning. The operating conditions for the Zeiss chromatogram-spectral photometer PMQ II are as follows: tungsten lamp 550 nm; entrance diaphragm 3.5 mm; slit width 0.5 mm; ordinate extended 4 times; damping 1; scanning speed 10 cm/min; paper speed 10 cm/min.

Calculation. Up to 20 ng of AT or NT hydrochloride per spot, the peak heights are directly proportional to the quantity chromatographed. Therefore, the peak heights of the standards are divided by the corresponding plasma concentrations resulting in the height of 1 ng/ml of plasma. The mean value of all the plasma standards applied to the plate is compared with the height of the signal of the samples to be analysed.

$$\frac{H_S}{H_{PS}} \text{ ng/ml} = \text{concentration of substances in plasma (ng/ml)}$$

where H_S = height of the signals of the samples
H_{PS} = mean height of the plasma standards corresponding to 1 ng/ml in plasma

RESULTS

Linearity.

As already mentioned, a linear relationship between the heights of the peaks and the amounts on the thin-layer plate was observed up to 20 ng of AT and NT hydrochloride. For levels higher than about 20 ng/ml the plasma has to be diluted in such a manner that 1 ml of the solution contains not more than 20 ng.

Sensitivity limit

The minimum concentration of AT and NT hydrochloride that was determined in plasma was 0.5 ng/ml.

Recovery

Up to 20 ng AT or NT/ml plasma, the overall recovery of the procedure was more than 90%, independent of the concentration.

Specificity of the method

The extraction, chromatographic separation and derivatization guarantee a high degree of specificity. We analysed a great deal of blank plasma but no interference by the plasma was found.

Reproducibility of the method

The relative standard deviation of the whole procedure was ± 8% for concentrations down to 2 ng/ml. For smaller concentrations the value rose by a factor of 2—3.

Practicability

The procedure allows a well-trained technician to analyse 10—15 unknown plasma samples per day.

Application of the procedure

To check the applicability of the method, plasma samples were analysed. Four patients received a single oral dose of 25 mg of AT as hydrochloride in solution. Plasma samples were collected up to 48 h after medication. The levels found are summarized in Table I.

TABLE I

PLASMA LEVELS (ng/ml) OF AMITRIPTYLINE (AT) AND NORTRIPTYLINE (NT) CALCULATED AS HYDROCHLORIDES AFTER A SINGLE ORAL ADMINISTRATION OF 25 mg AT AS HYDROCHLORIDE IN SOLUTION

Time after medication (h)	Patient I		Patient II		Patient III		Patient IV	
	AT	NT	AT	NT	AT	NT	AT	NT
0.5	0.7	0.6	0.8	0.3	1.2	0.9	0.6	0.5
1	1.1	0.9	0.9	0.4	2.6	1.0	0.9	0.5
2	5.2	1.2	2.5	0.4	6.5	1.8	2.4	0.8
4	3.0	1.2	2.4	1.5	4.1	1.7	3.2	0.8
6	3.1	1.4	3.8	1.8	5.6	1.6	4.7	0.8
8	3.0	1.6	3.3	1.1	5.7	1.6	3.4	0.9
10	3.7	1.2	3.2	0.7	5.5	1.8	2.9	0.8
24	2.8	1.1	2.4	1.0	4.2	1.6	2.5	0.8
34	0.9	0.9	1.4	0.8	2.7	1.1	2.1	0.5
48	0.7	0.7	1.4	1.7	1.8	1.2	1.4	0.5

DISCUSSION

On comparison of the results from Jørgensen [1] with our data, the levels of AT are seen to be somewhat lower than expected. But the differences are explained by the different test set-up and the variations between individuals. The concentrations of NT are rather low after a single oral dose of AT and the sensitivity of most published methods is not sufficient to determine these quantities. However, the described method is sensitive enough, and highly specific for AT and the metabolite NT. The procedure is simple and not very time-consuming, but requires some experience. The sensitivity of the procedure is sufficient for pharmacokinetic studies after a single dose of 25 mg AT as hydrochloride. The determination of nanogram amounts of drugs in biological materials is always susceptible to contamination. In general, repeated sophisticated cleansing of the glass-ware and reagents is necessary. It is therefore very surprising that in order to determine AT and NT in nanogram quantities no similar problems were encountered. The normal analytical grade solvents, normal distilled water and the conventional reagents were used. The only drawback of the procedure is the necessity of scanner use for thin-layer plates. However, we agree with other authors who use this technique that such an instrumentation will become standard in the near future, because of the great number of potential applications, available for direct spectrodensitometry or measurement after derivatization, to assays in clinical laboratories. The same procedure can probably be applied to other tricyclic substances of similar structure.

ACKNOWLEDGEMENT

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Note

Rapid determination of urinary oxalic acid by gas—liquid chromatography without extraction

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The existing methods for the gas—liquid chromatographic (GLC) analysis of urinary oxalic acid have until now necessitated separation of oxalic acid prior to derivatization [1]. We have previously developed a method [2] using an ethanol—diethyl ether mixture for extraction, and now describe a new procedure which avoids this time-consuming step and the potential hazard of using diethylether.

EXPERIMENTAL

Reagents

Hydrochloric acid and absolute ethanol (R.P. Normapure) were from Prolabo (Paris, France) and sulphuric acid (1.84 density, Suprapure) was from Merck (Darmstadt, G.F.R.). A stock solution of 140 mg oxalic acid $\cdot 2\,H_2O$ (analytical grade, Prolabo) in 100 ml distilled water was prepared. This corresponds to a 1000 μg/ml solution of anhydrous oxalic acid.

Apparatus

A rotating vacuum evaporator (Büchi Rotavapor), Jouan centrifuge, 3-ml graduated pyrex test-tubes (65 mm, ϕ 10 mm) and ground-glass stoppered pyrex test-tubes (90 mm, ϕ 10 mm) were used.

GLC Specifications

A Hewlett-Packard Model 7610 gas—liquid chromatograph was equipped with a flame ionization detector and a temperature programming device. A glass column (1.50 m × 1/8 in. I.D.) was packed with 15% DEGS on Chromo-

sorb W AW (80—100 mesh) as the stationary phase. Injector temperature was 230°, and the detector temperature 200°. The oven temperature programme was 10 min at 110°, increased by 10°/min to 180°, then kept constant for 10 min.

Procedure

When necessary the volume of the 24-h urine sample is made up to 2 l in order to improve the recovery of the dissolved dry residue (see below). The urine sample is then acidified with 1% HCL and a 30-ml aliquot is evaporated to dryness under reduced pressure at 65° with a rotating vacuum evaporator. The dry residue is dissolved in 1 ml ethanol and the evaporating vessel washed twice with 1 ml ethanol. The three extracts are transferred to a graduated test-tube and the volume adjusted to 3 ml with ethanol. Sulphuric acid (0.1 ml) is added to catalyse the ethylation. The test-tube is centrifuged at 300 *g* and the supernatant immediately transferred to a ground-glass stoppered test-tube and placed in an incubator at 37° overnight.

Two aliquots of 1000 μg (1 ml) and 2000 μg (2 ml) of the stock solution of oxalic acid undergo the same procedure and are used as standards. Three microlitres of each sample are injected into the GLC column for analysis.

RESULTS AND DISCUSSION

Table I compares the data obtained by GLC of urine samples with and without extraction. The results of the two methods are in excellent agreement. The Student's t-test has been performed on paired series. For normal subjects $t = 0.15$; from the Table t is 1.298 for $\alpha = 0.10$ and degree of freedom, d.f. = 66. For subjects with lithiasis $t = 0.10$; from the Table t is 1.301 for $\alpha = 0.10$ and d.f. = 52. Moreover, Fig. 1 shows identical chromatographic profiles for two samples of the same urine, one corresponding to an ethanol—diethyl ether extract, the other to the direct evaporation product.

Recovery of [^{14}C]oxalic acid is 70% with the extraction technique and 90%

TABLE I

GLC OF URINE SAMPLES WITH AND WITHOUT EXTRACTION

	No. of subjects	Oxalic acid (mg/24 h)		Range for both methods (mg/24 h)
		Extraction method	Direct method	
Normal subjects	34	27.14 ± 8.5*	27.44 ± 8.54	10—45
Subjects with lithiasis	27	45.59 ± 20.67	45.04 ± 20.61	10—105

*Mean ± 1σ.

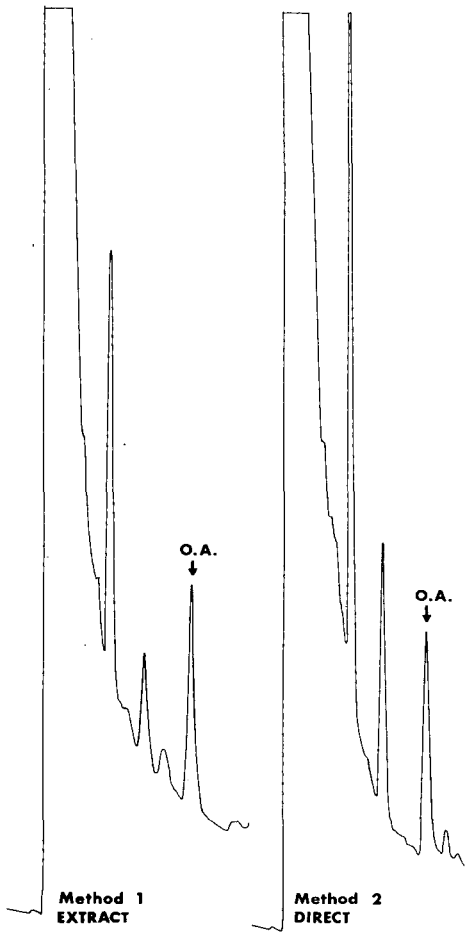

Fig. 1. Oxalic acid (O.A.) obtained from a sample analysed by each method. Injection at 110° on a 15% DEGS column. Retention time, 8 min.

using the direct method. The ethylation rate for both methods, compared with the standard ethyl oxalate, is near 99%.

In order to test the specificity of the direct method we checked the possible interference from another compound at the locus of the peak characteristic of ethyl oxalate. Thirty ml of urine were concentrated to 2 ml and oxalic acid was specifically extracted with 2 ml tributyl phosphate [3]. The remaining aqueous phase underwent total evaporation and ethylation and after injection of the product no peak corresponding to ethyl oxalate could be detected within the limits of sensitivity of the chromatographic technique.

CONCLUSION

The direct determination of oxalic acid on a urine sample by GLC without prior extraction gives results identical to those obtained with the extraction method. Moreover, it has several technical advantages. The extraction rate prob-

lem no longer occurs neither does the usual loss of oxalic acid during the distillation of the ethanol—diethyl ether mixture. The technique is time- and solvent-saving, a factor which has to be taken into account for routine clinical determinations. Safety for routine applications is increased by avoidance of the use of diethyl ether. Finally, a point which should be emphasized: repeated injections of a non-purified urine extract does not shorten the life of the chromatographic column.

ACKNOWLEDGEMENT

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Note

Determination of glutamine and glutamic acid in biological fluids by gas chromatography

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Over the past ten years, refined methods for gas chromatographic (GC) analysis of amino acids have been developed. An excellent recent review [1] describes the present status of GC methodology. The advantages of GC include rapid analysis, sensitivity, low cost, versatility and the ability to combine GC with mass spectrometry for identification of unknown substances. Disadvantages include a need to remove interfering substances from biological fluids and the necessity of derivatizing amino acids prior to chromatography. In most derivatization schemes it has been assumed that glutamine (Gln) and asparagine (Asn) are converted to the same derivatives as glutamic acid (Glu) and aspartic acid (Asp), respectively, so that one determines only Gln + Glu and Asn + Asp. This is of no consequence for protein hydrolysates, since the amide forms are converted to the dicarboxylic acids during hydrolysis. For determination of free amino acids in biological fluids, however, the ability to make a separate determination of Gln and Glu is highly desirable. Gln plays a significant role as a nitrogen carrier and is often elevated in disease states characterized by hyperammonemia, such as hepatic encephalopathy, Reye's syndrome, and inborn errors of metabolism involving the urea cycle.

Using GC methods developed by Gehrke and co-workers [2—8], we have been analyzing amino acids in biological fluids and have noted the constant presence of an unidentified peak in both normal and abnormal specimens of plasma and urine. By high resolution mass spectrometry we have identified this peak as a derivative of pyroglutamic acid which forms from Gln under

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the direct esterification procedure of Roach and Gehrke [4]. A similar situation occurs with Asn. The purpose of this report is to present two points: (1) Using the widely accepted direct esterification method for making N-trifluoroacetyl (N-TFA) *n*-butyl esters, calculation of Glu + Gln or Asp + Asn in samples containing significant amounts of the amide forms will be too low unless these additional derivative peaks are recognized. (2) We present a practical method whereby the additional peak can be used to quantitate Gln and Glu separately; a similar analysis could be performed for Asn and Asp, but in clinical situations this is less likely to be important.

EXPERIMENTAL

Materials

Pure amino acids for standards were obtained from Mann Labs., New York, N.Y., U.S.A. The internal standard used was tranexamic acid (Aldrich, Milwaukee, Wisc., U.S.A.). Pyroglutamic acid was obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Ion-exchange cleanup of biological fluids prior to analysis was accomplished using Amberlite CG-120 and CG-400, both 100—200 mesh (Mallinckrodt, St. Louis, Mo., U.S.A.), as the cation- and anion-exchange resins, respectively. Derivatization reagents were *n*-butanol 3.0 *N* in HCl or 1.25 *N* in HCl, methanol 1.25 *N* in HCl (all from Regis, Morton Grove, Ill., U.S.A.), methylene chloride (Fisher "Spectranalyzed", Fisher Scientific, Pittsburgh, Pa., U.S.A.), and trifluoroacetic anhydride (TFAA, Pierce, Rockford, Ill., U.S.A.).

The gas chromatograph was a Hewlett-Packard F&M 402 dual column instrument (Palo Alto, Calif., U.S.A.) equipped with flame detection, effluent splitter, and temperature programming. An Infotronics CRS-208 digital integrator (Columbia Scientific, Austin, Texas, U.S.A.) was used for peak area determination. Column packings of "EGA" (0.65% w/w of ethylene glycol adipate on 80—100 mesh acid-washed Chromosorb W) and "mixed OV" (2% w/w OV-17, 1% w/w OV-210 on 100—120 mesh Supelcoport) were obtained already prepared (according to methods of Gehrke et al. [6] from Analytical Biochemistry Labs., Columbia, Mo., U.S.A.). Reaction tubes were heavy-walled 5 ml tubes with aluminum screw caps and PTFE seals (Regis, Morton Grove, Ill., U.S.A.). Oil baths at 100°±2° and 150°±2° were aluminum pans heated by hot plates equipped with magnetic stirring to ensure uniform temperature of the baths. A special rack [9] permitted flow of dry nitrogen into the reaction tubes as desired. Peaks for mass spectrometric analysis were collected in glass capillary tubes (O.D. 1.6 mm) which were used as the source in a high-resolution MS 902 mass spectrometer (AEI, Manchester, Great Britain).

Methods

The volume of plasma routinely analyzed was 0.25 ml, and of urine, an amount equivalent to 250 μg creatinine. Internal standard (25 μl of a 40 mg/dl solution of tranexamic acid) was added at the beginning of the analysis. Plasma was deproteinized with 1.0 ml of 1% picric acid. Ion-exchange cleanup of the samples followed the methods described by Zumwalt et al. [5] except that the

volumes were scaled down. Pasteur pipets with glass wool plugs were used as the ion-exchange columns, approximately 300 mg resin was employed, wash volumes were 5 × 1 ml distilled water, and elution volumes were 2 ml. Routine derivatization followed the direct esterification method of Roach and Gehrke [4], with amounts of reagents appropriate for 100 μg total amino acids. Experiments employing the transesterification method of derivative preparation followed the procedures described by Lamkin and Gehrke [2]. GC with temperature programming was as described by Gehrke et al. [6].

For experiments analyzing synthetic mixtures of Gln and Glu, no ion-exchange cleanup was necessary, the internal standard was phenylalanine, and 0.25 ml sample was used.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of N-TFA *n*-butyl ester derivatives of

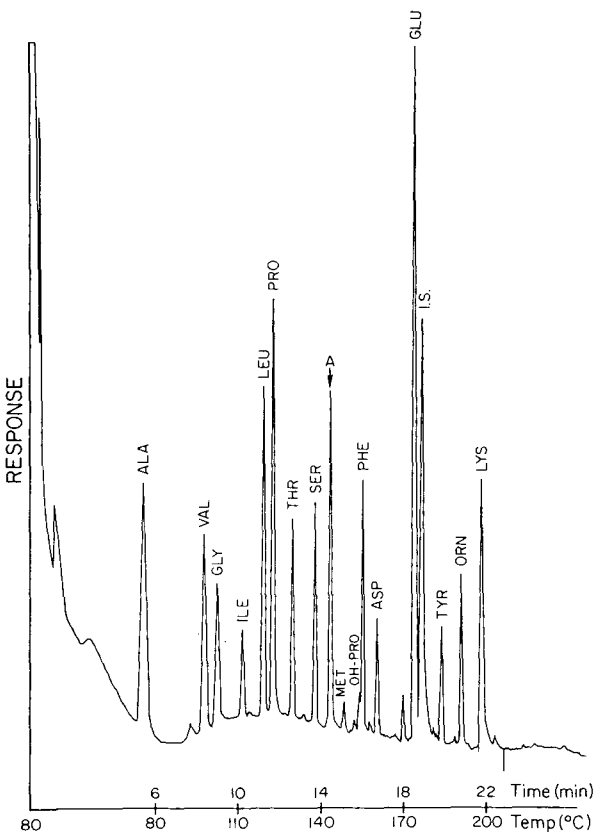

Fig. 1. Typical gas chromatogram of N-TFA *n*-butyl esters of amino acids in normal human plasma, cleaned prior to derivatization by cation- and anion-exchange. Conditions: Hewlett-Packard F&M 402 GC; column, 0.65% w/w EGA on 80—100 mesh acid-washed Chromosorb W, 6 ft. glass, I.D. 2 mm; nitrogen flow 60 ml/min; initial temperature 80° with 6 min delay, then 7.5°/min. Sample was 0.25 ml of fresh plasma, final volume of derivative, 80 μl, and 2 μl, was injected. Internal standard (I.S.) is tranexamic acid; peak labelled A is seen in all normal plasma and urine.

normal human plasma amino acids analyzed by GC. Of particular interest in Fig. 1 is the peak labelled A which appears between serine and methionine on the EGA column. Using an effluent splitter, this was collected in a glass capillary tube for mass spectrometry. To check recovery, one such collected sample was redissolved in CH_2Cl_2 and injected back on to the column; to our surprise, the reinjected sample appeared at a higher retention temperature (between Asp and Glu on EGA), indicating that some chemical change had occurred. When the material was recovered from the capillary tube with CH_2Cl_2—TFAA (3:1), however, the peak appeared at its original location, suggesting that this substance easily lost its trifluoroacetyl group on collection. Other amino acid derivatives did not exhibit this behavior.

Another sample of peak A was collected and analyzed by high resolution mass spectrometry. The mass of the principal ion was 185.1045±0.0020; by computer search the only tenable empirical formula was $C_9H_{15}NO_3$. The largest intensity fragment had mass 84.0444, empirical formula C_4H_6NO; this corresponds to loss of $-COOC_4H_9$. Thus, the underivatized compound was of the form (C_4H_6NO) COOH, and it was concluded that peak A represented the unstable trifluoroacetylated butyl ester.

A likely candidate for the underivatized compound was pyroglutamic acid. The nitrogen in this compound, having amide character, would be expected to form a less stable trifluoroacetyl derivative than would the R-NH_2 of amino acids.

But free pyroglutamic acid in plasma or urine could not account for this peak, since lacking a basic group, pyroglutamic acid would be lost during cation-exchange cleanup. Rather, the substance represented by peak A must

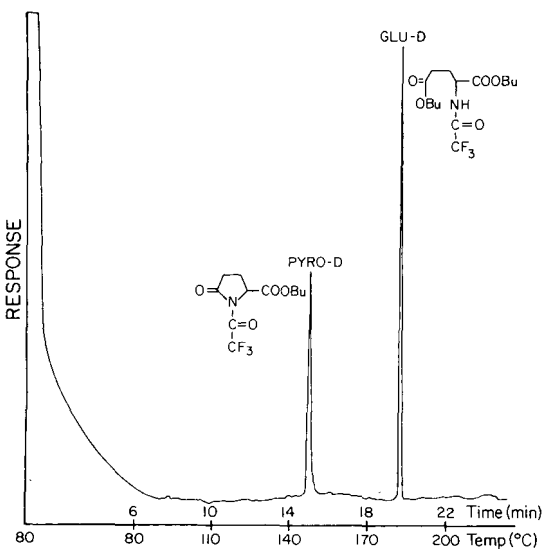

Fig. 2. Gas chromatogram on EGA of pure Gln derivatized by the direct esterification method. GC conditions are given in the legend to Fig. 1. The higher temperature peak is the dibutyl ester, pure Glu gives *only* this peak. The lower temperature peak has exactly the same properties as peak A in normal plasma (Fig. 1), and its structure was deduced by mass spectrometry.

be formed during derivatization. The origin of peak A became clear when pure crystalline Gln was derivatized. The result (Fig. 2) showed that two peaks were present, the first of which had a retention time identical to the unknown peak A, and had the same recovery properties. Derivatization of pure Glu gave only the second peak; derivatization of pure pyroglutamic acid gave a similar result to Gln except that peak A was relatively more intense.

The conclusion from these findings is that Gln (but not Glu) cyclicizes partially to *n*-butyl pyroglutamate during the esterification reaction; the N-TFA *n*-butyl ester of pyroglutamic acid is then formed during acylation, but hydrolyzes easily (with loss of CF_3COOH) so that the mass spectrum obtained is of the non-acetylated compound.

That Gln can cyclicize to pyroglutamic acid when heated has been known for years [10]. The probable reaction scheme is shown in Fig. 3.

Derivatization of pure Asn shows that it too yielded an extra peak (between methionine and phenylalanine on EGA) which presumably represented pyro-aspartic acid; however, it was proportionally less intense, accounting for about 15% of the total detector response.

How it is that this additional glutamine derivative has been previously overlooked? A major reason is that much of the analytical effort has been devoted to protein hydrolysates, in which Gln and Asn have already been converted to the corresponding dicarboxylic acids. Furthermore, commercially available amino acid standards usually do not contain the amide forms. Zumwalt et al. [5] did a detailed study of GC analysis of amino acids in biological substances, but their work employed the older transesterification procedure for forming the *n*-butyl esters [2]. Following this procedure (which is more time-consuming and involves forming first the methyl esters and then conversion to the butyl form), we found that Gln and Asn were 100% converted to the dibutyl esters.

The presence of two derivative peaks (PYRO-D and GLU-D in Fig. 2) for Gln might be viewed as a nuisance. However, one can use the extra peak to quantitate Gln and Glu separately.

A quantity f, is defined by $f = \dfrac{\text{PYRO-D area in Gln std.}}{\text{GLU-D area in Gln std.}}$ (1)

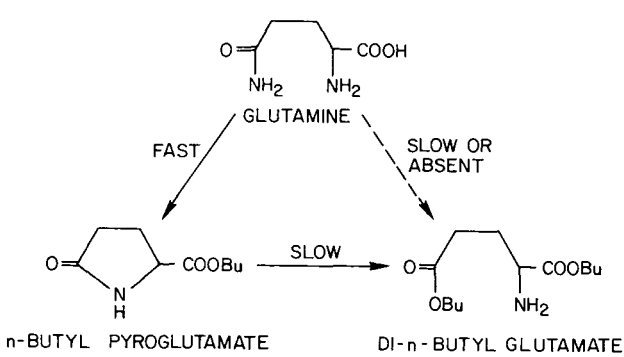

Fig. 3. Proposed reaction scheme for glutamine in acidic *n*-butanol at 100°.

(The basic premise of the calculation is that f is the same for glutamine in the standard and the unknowns.)

The PYRO-D peak is used to quantitate Gln:

$$\text{Amount of Gln in sample} = \frac{\text{PYRO-D area}_{\text{sample}}}{\text{PYRO-D area}_{\text{Gln std.}}} \times \frac{\text{I.S. area}_{\text{Gln std.}}}{\text{I.S. area}_{\text{sample}}} \times \begin{pmatrix}\text{Amount}\\ \text{of Gln in}\\ \text{Gln std.}\end{pmatrix} \quad (2)$$

where I.S. = internal standard.

When the contribution of Gln to the GLU-D peak is subtracted out, the remainder of the GLU-D peak area is used to quantitate Glu:

$$\begin{pmatrix}\text{Amount}\\ \text{of Glu in}\\ \text{sample}\end{pmatrix} = \frac{\left(\text{GLU-D area}_{\text{sample}} - \frac{\text{PYRO-D area}_{\text{sample}}}{f}\right)}{\text{GLU-D area}_{\text{Glu std.}}} \times \frac{\text{I.S. area}_{\text{Glu std.}}}{\text{I.S. area}_{\text{sample}}} \times \begin{pmatrix}\text{Amount}\\ \text{of Glu in}\\ \text{Glu std.}\end{pmatrix} \quad (3)$$

We investigated the effects of bath temperature, time of esterification, concentration of HCl in the n-butanol, and concentration of Gln on f in equation (1). These results are depicted in Fig. 4. The sensitivity of f to temperature, time, and [HCl] is apparent and makes it necessary to run a Gln standard with each batch of samples. However, f was constant to within 5% over a wide

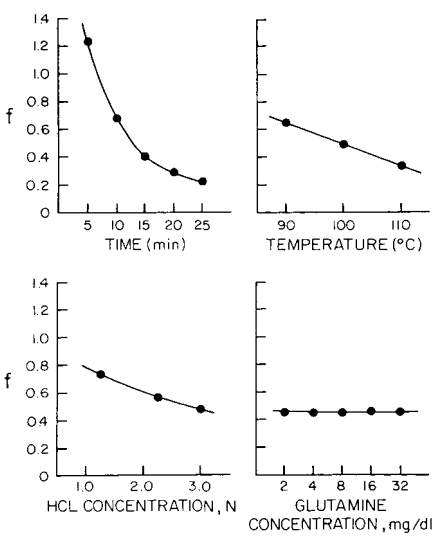

Fig. 4. Effect of esterification time, temperature, concentration of HCl, and concentration of Gln on f, the ratio of peak areas of the pyroglutamate derivative and the N-TFA dibutyl glutamic acid ester. Higher temperature, longer reaction time, and more HCl favor formation of the latter. When these factors are held constant, however, variations in concentration of glutamine do not affect f.

range of Gln concentrations when all samples were esterified simultaneously.

Mixtures of Gln and Glu of known composition were analyzed to determine the accuracy of these methods. The results, shown in Table I, show errors of 6% or less in separate determination of Glu and Gln. The last concentration used (Glu 2.0 mg/dl, Gln 8.0 mg/dl) represents approximately the levels found in plasma and urine.

If only Gln + Glu is of interest, it is possible to multiply the area of the PYRO-D peak by (RMR of GLU-D)/(RMR of PYRO-D), where RMR = relative molar response, and add this value to the GLU-D peak area to compute Glu + Gln by comparison with a standard containing only Glu. We found the value of (RMR of GLU-D)/(RMR of PYRO-D) to be 1.67 ± 0.10. This should be independent of f, so long as no other non-volatile derivatives of Gln are formed. Using this method, the values for Glu + Gln shown in the last column of Table I were computed; errors are 5% or less.

A similar analysis could be performed for Asn and Asp. However, in biological fluids Asn is present in low concentrations and f for Asn is low (about 0.2) so that the pyroaspartic peak is difficult to separate from background. In other situations where larger amounts of Asn are present this method would be applicable.

In conclusion, this paper demonstrates that an extra derivative is found from Gln and Asn using the direct esterification method [4]. Ignoring this derivative will lead to underestimation of Glu + Gln in biological fluids, but taking advantages of its presence enables one to quantitate Glu and Gln separately. We have found this to be a considerable advantage in the study of patients with metabolic disorders, particularly those associated with hyperammonemia.

TABLE I

ANALYSIS OF MIXTURES OF Gln AND Glu OF KNOWN COMPOSITION.

Sample	Concentration of amino acid (mg/dl)				
	Added		Found*		
	Glu	Gln	Glu**	Gln**	Glu + Gln***
Glu std.	4.00	0	4.00	0	4.00
Gln std.	0	8.00	0	8.00	8.01
Mixture 1	4.00	2.00	4.13	1.95	6.09
Mixture 2	4.00	4.00	4.11	3.92	8.04
Mixture 3	4.00	8.00	4.01	8.26	12.29
Mixture 4	2.00	8.00	1.93	8.47	10.40

*Each value represents the average of three determinations.
**Calculated according to eqns. 1—3.
***Calculated by multiplying PYRO-D peak by 1.67 and adding this to the GLU-D peak.

ACKNOWLEDGEMENTS

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Note

Gas chromatographic determination of volatile sulfur compounds in the expired alveolar air in hepatopathic subjects

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Characteristic halitosis of patients in hepatic coma has been described as being of diagnostic value by many clinicians, since early times. In previous literature, methyl mercaptan was isolated from the urine of patients in hepatic coma and exhibiting *fetor hepaticus* following acute massive necrosis of the liver. It was speculated that the breath odor was caused by a mixture of mercaptans and dimethyl sulfide [1, 2]. Subsequently, the concentration of methyl mercaptan was reported to be significantly elevated in the breath of patients with hepatic cirrhosis. A correlation between the breath odor and the dimethyl sulfide concentration in the breath of cirrhotics was also demonstrated after oral administration of methionine [3].

In recent gas chromatographic analysis, the flame photometric detector has been extensively used for the selective detection of sulfur and phosphorous compounds. By the application of this apparatus, trace amounts of volatile sulfur compounds have been detected in the field of environmental hygiene and toxicology, especially in analyses of atmospheric air pollutants and pesticides.

This paper presents our gas chromatographic procedure for the analysis of human expired alveolar air and the application of this technique in the determination of the overnight fasting level of volatile sulfur compounds, mainly methyl mercaptan and dimethyl sulfide, in the expired adveolar air in patients with diseases of the liver.

EXPERIMENTAL

Apparatus

A gas chromatograph (Model GC-5AP$_5$TFF$_p$, Shimadzu, Kyoto, Japan)

equipped with a flame photometric detector (FPD) and with a flame ionization detector (FID) monitor, was used for analysis. The cryogenic vapour preconcentration device and subsequent heat-desorption transfer system (flash sampler) are schematically illustrated (Fig. 1). The sample tube, column, connecting glass tubing and gas-tight syringe were all treated with 0.05 *N* phosphoric acid. The contact of metal surfaces by the sample was avoided, except for the needles of the connector and the injection syringe.

The glass column (3 m × 3 mm I.D.) was packed with 10% polyphenyl ether (5 rings) OS-124 on Shimalite TPA 60—80 mesh. Column temperature was initially isothermal at 40° for 5 min, then increased to 90° at the rate of 10°/min with a hold at 90°. For routine examination, the procedure takes about 30 min from the gas sampling to the end of cooling process. The FPD with a 394 mμ filter was operated at 750 V. Detector temperature was 140°. The gas flows were nitrogen as a carrier 50 ml/min, hydrogen 50 ml/min, and air 50 ml/min.

Reagents

Nitrogen-balanced standard hydrogen sulfide (H_2S) gas (13.8 ppm) was prepared by Nihon Sanso (Tokyo, Japan). Standard benzene solutions of methyl mercaptan (MM) (1μg/μl) and dimethyl sulfide (DMS) (0.1 μg/μl) were obtained from Wako (Osaka, Japan). Special grade ethyl mercaptan (EM) and dimethyl disulfide (DMDS) were also obtained from Wako. Each of these solutions was further diluted and adjusted to a suitable concentration for daily calibration.

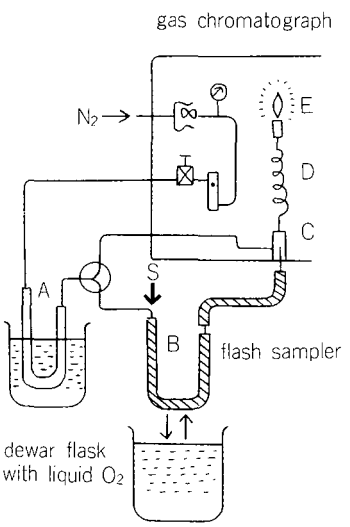

Fig. 1. Schematic diagram of cold-trap preconcentration and analytical system. A = Freeze-out trap for carrier gas (N_2) packed with molecular sieve 5A, 30—60 mesh, stainless steel; B = Sample tube packed with 1, 2, 3, tris (cyano ethoxy) propane (TCEP) 25% on Shimalite, 60—80 mesh, 31 cm × 4 mm I.D., glass, C = Injection port; D = Column; E = FPD with FID monitor; S = Sample injection by gas-tight syringe.

Separation of volatile sulfur compounds

The retention times in minutes are as follows; H_2S 1.67, MM 3.18, EM 4.18, DMS 4.68, and DMDS 15.17, respectively. Fig. 2 shows the typical chromatograms obtained from a 75-year-old male patient with hepatic cirrhosis and secondary diabetes.

Calibration of volatile sulfur compounds

Between 0.2 and 50 ng of these compounds were injected into U-shaped sample tubes using Hamiltone gas-tight syringes of 10 ml capacity (Hamiltone Whittier, Calif., U.S.A.) or SGE microsyringes of 5 μl capacity (Scientific Glass, North Melbourne, Australia). The line produced by plotting the logarithm of dose vs. the logarithm of peak height was used for calibration.

Procedure

Routinely, 100 ml of expired air was collected in the pharyngeal region with a gas-tight syringe of 100 ml capacity (TOP Surgical Manufacturing Co., Tokyo, Japan) towards the end of a prolonged uninterrupted expiration subsequent to 20-sec breathholding. In such samples, the composition of the alveolar air is in equilibrium with the air dissolved in alveolar capillary blood, and the alveolar carbon dioxide tension is estimated as equivalent to the carbon dioxide tension in oxygenated mixed venous blood. The specimen was immediately injected into the sample tube (↓S: Fig. 1).

Reproducibility of the quantitation of volatile compounds in the expired alveolar air

Table I shows duplicate analyses in five subjects. In order to obtain reliable

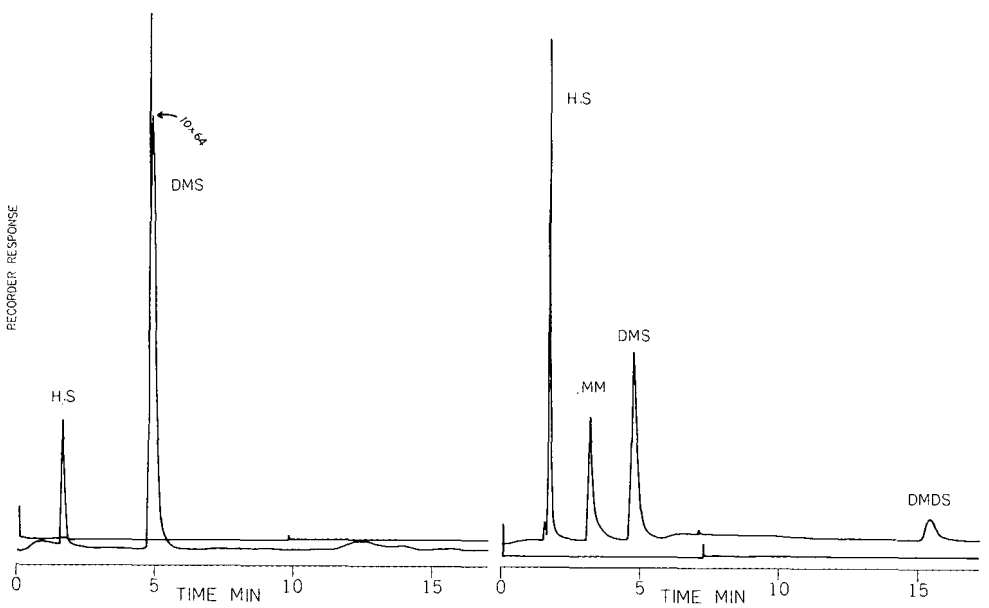

Fig. 2. Gas chromatogram obtained from a 75-year-old male with hepatic cirrhosis and secondary diabetes. Left: expired alveolar air; right: urinary headspace gas; sample size: 20 ml; detector sensitivity and range: FPD = 10 × 32, FID = 10^3 × 256.

and reproducible data in such an experiment, we must emphasize the importance of the sampling procedure for the expired alveolar air as described above. In other experiments on the gas chromatographic quantitation of acetone or ethanol, duplication has been clearly demonstrated [4, 5].

Subjects

Concentrations of MM and DMS in the expired alveolar air were determined after 12-h fasting in 97 subjects; 53 normal controls and 44 patients with diseases of the liver (13 acute hepatitis; 11 chronic hepatitis; and 20 cirrhosis of liver).

RESULTS AND DISCUSSION

Since DMS concentration was sufficiently detectable in 100 ml samples of expired alveolar air, for the requirements of the analyses, we repeated our estimation every 30 min, routinely determining both DMS and MM. By means of the t-test, the concentration of DMS was shown to be significantly elevated in cirrhotics (Table II).

Previously, sulfur compounds were collected from 60—80 l of tidal air as mercuric salts and analyzed by gas chromatography and flame ionization detection [3]. Their fasting average values were: in 7 normal subjects, MM 0.8 and EM 5.9; in 6 compensated cirrhotics, MM 3.0 and EM 4.7; and in 12 severely decompensated cirrhotics, MM 4.4 and EM 11.5 ng/l. But, since several kinds of volatile sulfur compounds have been detected from the oral cavity [6—8], the contamination by mouth air of the expired alveolar had to be carefully excluded.

Because of improvements in expired alveolar air sampling, use of cold-trap preconcentration procedure, utilization of all-glass and PTFE tubing, and the selectivity of FPD with FID monitor, the time required for sampling, pretreat-

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF VOLATILE SULFUR COMPOUNDS IN EXPIRED ALVEOLAR AIR (ng/dl)

N = normal subject; LC = patient with liver cirrhosis

Patient	MM	DMS
M.H. (N)	—	0.45
	—	0.46
H.K. (N)	2.25	0.58
	2.20	0.64
A.K. (LC)	—	3.9
	—	3.9
S.I. (LC)	3.18	15.6
	4.80	14.7
T.M. (LC)	1.10	0.73
	1.15	0.73

TABLE II

FASTING LEVELS OF MM AND DMS IN THE EXPIRED ALVEOLAR AIR (MEAN ± SE, ng/dl)

Experimental group	MM	DMS
53 Normal (control)	0.71 ± 0.21	1.54 ± 0.09
13 Acute hepatitis	0.48 ± 0.27	1.48 ± 0.50
11 Chronic hepatitis	0.23 ± 0.23	2.30 ± 0.86
20 Liver cirrhosis	0.94 ± 0.33	4.05 ± 1.06*

*vs. normal control, $t = 2.3611$, $P < 0.05$.

ment and gas chromatographic analysis has been markedly shortened, and the concentrations of MM obtained by us were about ten times higher than previous workers had obtained [3].

Volatile sulfur compounds constitute the *fetor hepaticus* and they seem to play an important role in the induction of hepatic encephalopathy, as do ammonia, short chain fatty acids, etc. [9]. Methionine toxicity in liver cirrhosis has been studied in relation to mercaptans and dimethyl sulfide [3, 10—12]. The physiological significance of these compounds in normal and pathological states must be further studied.

As the quantitative analyses of trace amounts of volatile sulfur compounds in blood are still complicated and uncommon [13, 14], it is important to take full advantage of the expired alveolar air analysis in the field of clinical biochemistry.

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Note

Separation of neurohypophyseal proteins by reversed-phase high-pressure liquid chromatography

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The class of neurohypophyseal proteins collectively called the "neurophysins" has recently occupied the attention of many investigators [1]. Separation of these molecules from various pituitary preparations using open column liquid chromatography has been investigated and standard gel electrophoretic methods used as analytical techniques [2].

The physical properties of these molecules are, briefly: they form aggregating systems in aqueous solutions, are all of monomer molecular weight $\sim$ 10,000 D and contain an unusually high proportion of disulfide bonds. However, the property which makes them of special interest to high-pressure liquid chromatography (HPLC) is their rather high solubility in aqueous methanol [3].

The theory of HPLC as applied to columns consisting of microparticulate packing coated with non-polar stationary phases has received much attention. The form of liquid—liquid chromatography using these columns and sometimes called "reversed-phase" is commonly thought to be most appropriate for separation of nonionogenic compounds [4]. On the other hand, it has also been shown recently that separation of polar compounds can be achieved using a non-polar stationary phase and elution with aqueous solutions containing no organic component [5]. To a practical biochemist involved in chromatographic separation of *proteins* the theoretical prediction of which columns and eluents to use in a particular case is therefore by no means clear. Indeed, it is commonly thought that protein separations require ion-exchange/sieving type packings. Be that as it may, it seems intuitively clear that proteins such as the neurophysins might exhibit a competition between solubility in a non-polar stationary phase and a partially organic mobile phase and thus be separable on that basis. The present work shows that this is true, and leads to a convenient analytical separation (which can presumably be scaled up to preparative level using a

larger column) which takes only a fraction of the time needed for a polyacrylamide gel electrophoresis (PAGE) determination, is quantitative without staining or labelling, and is at least several times more sensitive in detection. The column packing used is one commonly employed for separation of low-molecular-weight peptides.

EXPERIMENTAL

The neurophysins used in these experiments were prepared from fresh frozen bovine posterior pituitary glands in a manner previously described [2] and which is in accord with the results of other workers insofar as their purity as determined by analytical PAGE [1]. The HPLC experiments reported here were performed using a Micromeritics Model 7000B chromatograph equipped with a Model 785 variable wavelength UV detector. Solvent methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and water was glass distilled from the deionized laboratory water supply. J.T. Baker (Phillipsburgh, N.J., U.S.A.) reagent grade sodium acetate was used for the buffer preparation. The HPLC column was packed in this laboratory using a Micromeritics Model 705B slurry packer. The column dimensions were 25 × 4.6 mm I.D. and the packing was E.M. Labs. (Elmsford, N.Y., U.S.A.) designation "RP-18". Using standard tests the column was found to be about 90% as efficient (expressed as number of theoretical plates and height equivalent of theoretical plates) as similar commercial columns available from several manufacturers. The standards were low-molecular-weight compounds.

Chromatographic conditions were: sample injection as 2 mg/ml solutions in phosphate buffer, pH 5 (our standard conditions for neurophysin solutions), detection in a 10-mm path length cell (volume = 10 μl) at 215 nm, elution with a mixture of 0.01 *M* acetate buffer (pH 5.7) and methanol. It was found that the ratio of buffer to methanol was very critical (see data below), and with detection at this wavelength the most practical way of obtaining stability was to mix a large proportion of the buffer—methanol mixture of approximately correct ratio with small increments of pure methanol in the chromatograph's solvent blending system to bring about the final composition.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms which result from injection of neurophysins I and II along with scans of analytical PAGE gels of the same components. To be noted in the chromatograms is the strong solvent front due to the phosphate buffer (taken to be the void volume, V_0). In the neurophysin I chromatogram the peak immediately following this front is real and accounts for 26% of the total UV-absorbing area. Injection amounts for neurophysins I and II differ in order to make the figure clearer for publication since peak widths vary. The PAGE scans show what would ordinarily be considered homogeneous proteins of each kind. Using gel isoelectric focussing (experiments not shown) it was shown that neurophysin I presents itself as two bands while neurophysin II remains homogeneous even in that method of

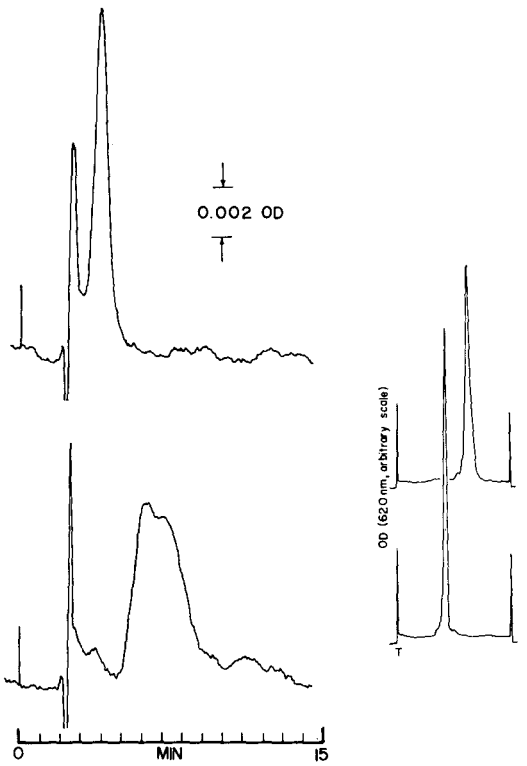

Fig. 1. Main figure: HPLC chromatograms, flow-rate 1 ml/min, detection at 215 nm, eluent composition; 48.4% 0.01 *M* acetate buffer (pH 5.7), 51.6% methanol. Upper: neurophysin I, 2 μg injected as phosphate buffer (pH 5) solution. Lower: neurophysin II, 6 μg injected as phosphate buffer (pH 5) solution. Inset figure: PAGE scans, 7.5% acrylamide gels, Tris—glycine buffer (pH 7.9), stained with amido black. "T" indicates top of gel. Upper: neurophysin I, 50-μg load. Lower: neurophysin II, 50-μg load.

separation. Table I gives the HPLC relative elution volume values (elution volume, V_e, for each component/V_o as defined above) for the major peaks obtained as a function of eluent composition.

It is clear from the above that the neurophysins can be easily separated from each other. It should be noted that the intrinsic detector sensitivity (0.005 a.u.f.s.) is about four times greater than that used in these experiments. This makes the minimum detectable amount of neurophysin about 0.3—1.0 μg. Aside from the tremendous decrease in time per determination over PAGE one of the advantages of this method is that detected signal is much more easily related to amounts of protein than by staining. The wavelength of 215 nm was chosen because it is the isosbestic point at which random and helical peptide bond absorptions, from the $\pi \rightarrow \pi^*$ transition, are equal [6]. Thus, at this wavelength UV absorption is approximately independent of protein composition and the residue molar adsorptivity (neglecting contributions from tails of aromatic residue near UV-bands) is 10^3 l·mole^{-1} ·cm^{-1} ·residue^{-1} [6]. Using this value and the number of residues in the neurophysins (approximately 100 [7]) the observed integrated absorbances account very closely for the

TABLE I

V_e/V_0 VALUES FOR ELUTION OF THE MAJOR COMPONENTS OF NEUROPHYSINS I AND II (NPI AND NPII) VS. ELUENT COMPOSITION

A: 0.01 *M* acetate buffer (pH = 5.7); B: methanol

Composition	V_e/V_0			
	NPI_1	NPI_2	$NPII_1$	$NPII_2$
48.4% A 51.6% B	1.11	1.69	2.70	3.12
49.5% A 50.5% B	1.18	2.33	4.17	5.00
50.5% A 49.5% B	1.61	4.76	8.33	10.0

total protein injected. Finally, the chromatograms (not shown) near the limit where the proteins are almost totally retained by the column show dramatically that PAGE derived, "chromatographically pure", protein is by no means pure as far as HPLC is concerned, without recourse to two-dimensional electrophoretic experiments.

The advantages of this HPLC system over currently used methods are obvious, and it seems hard to believe that this is the only protein system to which this reversed-phase method can be applied. It must be emphasized that success with proteins using UV detection at high sensitivity can only be hoped for with a detector tuned very closely to the steep shoulder of the peptide bond absorption band which has its maximum at 190 nm, but which is obscured at that wavelength by solvent absorption.

ACKNOWLEDGEMENTS

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Note

Chromatography of glycosylated hydroxylysines and hydroxylysine on Dowex 50 resins of different degrees of cross-linking

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Determination of the excretion rates of hydroxylysine (Hyl) and its glycosylated derivatives glucosylgalactosylhydroxylysine (GlcGalHyl) and galactosylhydroxylysine (GalHyl) has been shown to be useful in assessing the activity of diseases which affect collagen metabolism [1-3]. We have developed a method for measurement of the urinary excretion of GlcGalHyl, GalHyl and Hyl which is based upon separation of these compounds by chromatography on Dowex 50W-X8, followed by determination of the Hyl present in that portion of the column eluate which contains each compound [4]. The separation of the individual glycosylated hydroxylysines and Hyl is excellent; however, all urine samples obtained from normal individuals and from patients with a variety of diseases contain chromogenic material which is eluted from the chromatographic column prior to the elution of GlcGalHyl. This material exhibits chromatographic heterogeneity and is designated Fraction I (Fr I). The separation of this material from GlcGalHyl on Dowex 50W-X8 is adequate but the estimation of GlcGalHyl present in pooled eluates from calibrated chromatographic columns [4] would be more reliable if the separation of Fr I from GlcGalHyl could be improved without sacrifice of the separation of the other compounds of interest. This paper describes a modified chromatographic method which achieves this goal.

EXPERIMENTAL

Standard solutions

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Hydroxylysine (mixed DL and DL-allo) was purchased from Sigma (St. Louis, Mo., U.S.A.). GlcGalHyl and GalHyl were prepared from bovine lens capsule (Pel-Freez Biologicals, Rogers, Ariz., U.S.A.) collagen by the method of Spiro [5]. A 24-h urine sample obtained from a patient with Paget's disease was used as a reference urine sample to compare the different Dowex 50W resins. Aliquots of this urine sample were stored at −20° until chromatographed.

Reagents for column chromatography

Dowex 50W resins having different degrees of cross-linking were purchased from either Sigma (8% cross-linked) or Bio-Rad Labs., Richmond, Calif., U.S.A. (2% and 4% cross-linked). All resins were 200—400 mesh and were washed according to the procedure of Moore and Stein [6]. The washed resins were equilibrated with 0.1 *M* citrate buffer, pH 3.42 [6]. The eluting buffer contained 69 g citric acid monohydrate, 96 g sodium citrate dihydrate and 8 g disodium ethylenediamine tetraacetate in a final volume of 8 l. The pH of buffer was adjusted to 5.00 with 10 *M* NaOH (approximately 4.4 ml/l of buffer). This buffer is 0.08 *M* in citrate and contains 172 mequiv. Na/l. Following completion of each chromatogram the resin beds were washed with 0.2 *M* NaOH and then re-equilibrated with the 0.1 *M* citrate buffer, pH 3.42.

Chromatographic procedure

The following 24 × 1 cm ion-exchange columns were prepared: Dowex 50W-X8, Dowex 50W-X4, Dowex 50W-X2 and Dowex 50W-X8: Dowex 50W-X4 combined 1:1 (wet vol./wet vol.). The columns containing the combination of Dowex 50W-X8 and Dowex 50W-X4, designated combined-resin columns, were prepared either by layering the 4% cross-linked resin over the 8% cross-linked resin or by blending equal volumes (wet) of the two resins and then pouring the slurry into the chromatography column. Either 1 ml of a mixture containing GlcGalHyl, GalHyl and Hyl or a 1-ml aliquot of the reference urine, pH 2—3, was washed into the resin bed with the 0.08 *M* citrate buffer, pH 5.0. The chromatogram was developed at a flow-rate of 8 ml/h which was controlled by a syringe pump (Model 975 Infusion Pump, Harvard Apparatus Co., Dover, Mass., U.S.A.) fitted with adapters to hold four 60-ml plastic disposable syringes. The column effluents were collected in 1- or 2-ml fractions. Chromatography was carried out at room temperature. The eluate fractions were analyzed for the presence of Hyl and glycosylated hydroxylysines as described below.

Spectrophotometric analysis

Prior to spectrophotometric analysis, the glycosylated hydroxylysines and substances present in Fr I were hydrolyzed in order to release free hydroxylysine and Fr I chromogen(s) respectively. One ml of the column eluate fractions was placed in 100 × 13 mm culture tubes fitted with PTFE-lined screw-caps, and 1.0 ml of 4 *M* HCl was added to give a final concentration of 2 *M* HCl. The tubes were closed and hydrolysis was performed for 4 h at 105°. Following hydrolysis the solution was neutralized by adding 0.22—0.23 ml of 10 *M* NaOH to each ml of the hydrolysate solution. The volume then was adjusted to 5.0 ml

with 1.2 *M* Na_2HPO_4, 0.3 *M* citric acid buffer, pH 7.0 [7] and, if necessary, the final pH was adjusted to between 6.8 and 7.0 by the addition of either 2 *M* NaOH or 1 *M* HCl. Analysis for Hyl was then carried out as described by Blumenkrantz and Prockop [7]. Column eluate fractions which contained free Hyl did not require hydrolysis.

RESULTS

The elution positions of GlcGalHyl, GalHyl and Hyl standards were determined on each column. Table I summarizes the volumes of column eluate which separate Fr I, the glycosylated hydroxylysines and Hyl when 1-ml aliquots of the reference urine sample were chromatographed on Dowex 50W resins of varying cross-linking. The separation of GlcGalHyl from Fr I was improved by use of either a 4% or 2% cross-linked resin. However, there was a decrease in the separation of GlcGalHyl from GalHyl and of GalHyl from Hyl on the 4% cross-linked resin and a marked diminution in these two separations on the 2% cross-linked resin. The elution volume of the chromogenic material present in Fr I was not changed and the use of resins with fewer cross-linkages did not result in further fractionation of the substances present in this material. Analysis of the results obtained with the 8%, 4% and 2% cross-linked resins suggested that improved separation of GlcGalHyl from Fr I might be achieved by use of a 6% cross-linked resin without a significant decrease in the separation of the other compounds.

To our knowledge Dowex 50W-X6 resin is not commercially available, therefore chromatographic columns were packed with the 1:1 combination (wet vol./wet vol.) of Dowex 50W-X8 and Dowex 50W-X4. Fig. 1 shows the elution pattern obtained when 1 ml of the reference urine was chromatographed on a layered combined-resin column. There was no significant difference between the separations obtained on the layered combined-resin

TABLE I

SEPARATION OF FRACTION I, GLYCOSYLATED HYDROXYLYSINES AND HYDROXYLYSINE ON DOWEX 50W, 200—400 MESH RESINS WITH VARIOUS PERCENTAGES OF CROSS-LINKING

Cross-Linking (%)	Volume of eluting buffer between fractions (ml)*			Elution Volume of Hyl**
	Fr I—GlcGalHyl	GlcGalHyl—GalHyl	GalHyl—Hyl	
8	8	22	46	180
4	38	14	22	178
2	48	4	6	138
Combined-resin	26	20	34	176

*The results are expressed as the number of millimeters between the completion of the elution of each compound and the beginning of the elution of the following compound and are the average of two determinations.

**Volume of buffer required to complete the elution of Hyl.

columns and the blended combined-resin columns. Combined-resin columns were used over a period of months and no significant change in elution pattern of the substances present in the reference urine sample was observed during this period. Use of the combined-resin chromatographic columns resulted in improved separation of Fr I from GlcGalHyl, while excellent separation of GlcGalHyl from GalHyl and of GalHyl from Hyl was retained. The volumes of combined-resin column eluate which separate Fr I, the glycosylated hydroxylysines and Hyl are shown in Table I.

Although there was some variation, the volumes of eluate containing Fr I, the glycosylated hydroxylysines and Hyl were not significantly different during chromatography on resins of different degrees of cross-linking as compared to their elution volumes on Dowex 50W-X8. Urine samples which contain the amounts of glycosylated hydroxylysines and Hyl excreted by normal adults [1, 4] can be analyzed on calibrated chromatographic columns by pooling the eluate volumes previously demonstrated to contain GlcGalHyl, GalHyl and Hyl and by analyzing 1-ml aliquots (GlcGalHyl and GalHyl) or 2-ml aliquots (Hyl) of the pools [4].

Spectrophotometric analysis of those portions of the column eluate which contained Fr I and the glycosylated hydroxylysines without prior acid hydrolysis resulted in the detection of only trace amounts of chromogenic material. Acid hydrolysis of the column eluate fractions which contained Hyl did not affect the results of the spectrophotometric analyses of these fractions.

DISCUSSION

Separation of glycosylated hydroxylysines and Hyl on Dowex 50W-X8 is satisfactory; however, when the column eluates which contained GlcGalHyl, GalHyl and Hyl were pooled separately [4] and then analyzed, unexpectedly high values for GlcGalHyl occasionally were obtained. In these instances, further testing revealed that a portion of the Fr I chromogenic material had been included in the GlcGalHyl pool. Moore and Stein [8] demonstrated the effect of resin cross-linking on the chromatographic behavior of amino acids and polypeptides and noted that improved separations sometimes could be achieved by blending resins of different degrees of cross-linking. In order to

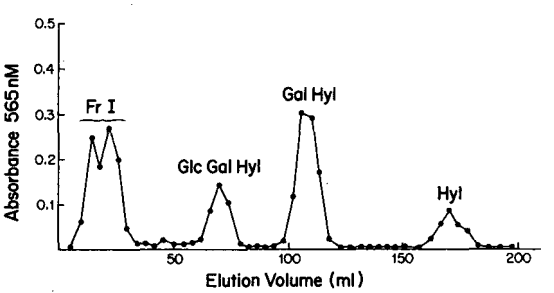

Fig. 1. The elution pattern of Fr I chromogens, GlcGalHyl, GalHyl and Hyl obtained when the reference urine was chromatographed on a layered, combined-Dowex 50W-resin column. Resin bed volume was 19.1 ml.

improve the separation of Fr I from GlcGalHyl without compromising the separation of the other Hyl-containing substances, we investigated the use of resins of different degrees of cross-linking. Among the types tested, this objective was best attained by use of a resin bed consisting of a 1:1 combination of 8% cross-linked Dowex 50W and 4% cross-linked Dowex 50W. Use of a combined-resin column has eliminated the problem of admixture of Fr I chromogen with the GlcGalHyl-containing portion of the column eluate.

The composition of the buffer was also a factor in the results obtained. Use of a 0.1 *M* citrate buffer, pH 5.0, which contained 182 mequiv. Na/l [4] to develop the combined-resin column resulted in good separation of Fr I from GlcGalHyl but the separation of GlcGalHyl from GalHyl was not as satisfactory as with the buffer described in this report. Less than optimal separation of all Hyl-containing compounds precludes the pooling of fractions collected from calibrated columns and measurement of the Hyl content of aliquots of the pools. If individual column eluate fractions are analyzed for Hyl, the original buffer does yield satisfactory separations and has the advantage of a shorter total running time since Hyl is eluted at 135—145 ml rather than at approximately 175 ml.

These investigations described in this report do not provide an indication of the nature of the substances present in Fr I. Six percent of the chromogen present in the Fr I component of the reference urine sample employed in this investigation is accounted for by the presence of peptide-bound hydroxylysine while the remainder of the chromogenic material is due to the presence of free and peptide-bound proline in this portion of the column eluate [4]. On the basis of the 16:1 ratio of proline to hydroxylysine in Fr I [4], we believe this material represents the collagen-related polypeptides which were described by Krane et al. [9]. Three percent of the Hyl present in the reference urine sample was present in Fr I. Askenasi has demonstrated that approximately 10% of the total urine hydroxylysine is present in these polypeptides [10, 11].

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Note

Le comportement du 16 α-glucuronide d'oestriol sur resine Amberlite XAD-2

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L'Amberlite XAD-2 est une résine polystyrène, macromoléculaire, neutre, qui a trouvé de nombreuses applications en raison de ses capacités d'adsorption. Celles-ci ont été mises à profit, notamment pour l'extraction des médicaments [1—4], des acides biliaires [5, 6] et des hormones stéroïdes [7—15] à partir des échantillons biologiques. L'utilisation de ce support a été ainsi préconisée pour l'extraction des oestrogènes de l'urine, en vue de leur dosage colorimétrique [11, 12] ou fluorimétrique [13] par la réaction de Kober, ou par chromatographie en phase gazeuse [14, 15].

Cherchant à évaluer l'intérêt réel des méthodes ainsi proposées, et notamment de celles qui excluent l'hydrolyse des oestrogènes conjugués [11—13], nous avons testé le comportement, sur cette résine, de l'oestriol (Δ 1,3,5(10) oestratriène 3,16 α,17 β-triol et surtout celui de 16 α-glucuronide d'oestriol qui représente, au cours de la grossesse, la principale forme d'élimination de l'oestriol dans l'urine.

MATERIELS ET MÉTHODES

La résine Amberlite XAD-2, 300—1000 μm (Serva, Heidelberg 1, G.F.R.), est purifiée par lavages successifs avec 3 fois 3 volumes d'eau, 2 volumes de méthanol, puis 3 fois 3 volumes d'eau. Elle est conservée dans l'eau, à 4°. Tous les réactifs utilisés sont de qualité analytique: Oestriol et 16 α glucuronide d'oestriol (Sigma, St. Louis, Mo., U.S.A.); Hydroquinone, paranitrophènol,

*À qui adresser toute la correspondance.

éthanol, méthanol et acide sulfurique (Merck, Darmstadt, G.F.R.); Chloroforme R.P. (Prolabo, Paris, France)

Chromatographie

La chromatographie est réalisée dans de petites colonnes en polypropylène (Bio-Rad, Richmond, Calif., U.S.A.). Le volume utile (4 × 0.7 cm) est garni de 1.5 ml de résine purifiée (ceci correspond à 0.4 g de résine sèche).

Un ml d'urine ou des divers échantillons contenant l'oestriol ou le 16 α-glucuronide d'oestriol est déposé sur chaque colonne dont le débit est réglé à environ 0.3 ml/min.

Les colonnes sont ensuite lavées avec 8 ml de solution aqueuse (de force ionique variable) au débit de 3 ml/min, puis éluées par 5 ml de méthanol (débit: 1 ml/min).

Les dosages des oestrogènes éventuellement entraînés dans les eaux de lavage, et celui des oestrogènes élués, sont effectués selon la réaction colorimétrique de Kober-Ittrich [16], comportant l'extraction du chromophore par une solution chloroformique de paranitrophènol à 2 g pour 100 ml.

RÉSULTATS ET DISCUSSION

Dans un premier temps, la capacité d'adsorption de la résine pour l'oestriol et le 16 α-glucuronide d'oestriol, a été contrôlée. Cette étude permet de confirmer les résultats de Bradlow [7] et d'Osawa et Slaunwhite [11]; aucune perte d'oestrogène n'est enregistrée dans les eaux de lavage après dépôt de 10 μg d'oestriol ou de son dérivé 16 α-glucuroconjugué, en solution dans l'eau ou dans l'urine. La capacité d'adsorption pour l'oestriol paraît très grande puisqu'après dépôt de 100 μg sur les colonnes décrites, on n'en retrouve que 0.6 μg dans les eaux de lavage.

L'élution par le méthanol a été ensuite étudiée. Celle de l'oestriol paraît excellente puisque le pourcentage de récupération est supérieur à 95% avec 5 ml de méthanol. Par contre, dans le cas du 16 α-glucuronide d'oestriol, l'élution dépend fortement des conditions opératoires, comme le montre le tableau I; l'influence de la force ionique du milieu utilisé pour solubiliser le glucuronide d'oestriol ou pour laver la colonne de résine est bien mise en évidence puisque l'addition de chlorures de sodium ou de potassium permet d'obtenir un pourcentage de récupération dans l'éluat méthanolique voisin de 95%. L'addition d'urée, par contre, ne modifie pas le comportement de l'oestriol 16 α-glucuroconjugué.

L'effet de la force ionique qui vient d'être rapporté, présente un intérêt particulier dans le cadre du dosage des oestrogènes conjugués urinaires, puisqu'il affecte le pourcentage de récupération des oestrogènes dans ce milieu, comme le montre le tableau II. Cet effet ne semble pas avoir été mentionné par d'autres auteurs [7, 11, 15]. Ceux-ci préconisent généralement le lavage de la résine par un volume d'eau plus faible (par rapport au volume d'urine déposé) que dans les conditions décrites ici, de sorte qu'il persiste vraisemblablement sur la résine

TABLEAU I

MODALITÉ D'ÉTUDE DE L'ÉLUTION PAR 5 ML DE MÉTHANOL DU 16 α-GLUCURONIDE D'OESTRIOL APRÈS SON DÉPÔT SUR LA COLONNE ET LAVAGE DE LA RÉSINE DANS DIVERSES CONDITIONS

Composition des solutions de 16 α-glucuronide d'oestriol (10 μg déposés par colonne)	Solution de lavage (8 ml)	Récupération après élution par 5 ml de méthanol* (%)
Eau	Eau	6.9 ± 2
Urée 0.33 *M*	Urée 0.33 *M*	1.5 ± 0.5
NaCl 0.14 *M*	Eau	29.3 ± 20
NaCl 0.85 *M*	Eau	44 ± 11
NaCl 1.29 *M*	Eau	73 ± 3
NaCl 1.72 *M*	Eau	89.5 ± 5.5
Eau	NaCl 0.14 *M*	90 ± 2
NaCl 0.14 *M*	NaCl 0.14 *M*	96 ± 3
KCl 0.13 *M*	KCl 0.13 *M*	94.5 ± 3.5

*Pour chaque éssai, les valeurs indiquées représentent les moyennes calculées à partir de trois résultats au minimum.

TABLEAU II

EFFET DE LA FORCE IONIQUE SUR L'ÉLUTION DU 16 α-GLUCURONIDE D'OESTRIOL EN SOLUTION DANS L'URINE

Composition des solutions urinaires de 16 α-glucuronide d'oestriol (10 μg déposés par colonne)	Solution de lavage (8 ml)	Récupération après élution par 5 ml de méthanol* (%)
Urine pure	Eau	84.4 ± 4.5
Urine—eau (1:1)	Eau	55 ± 8
Urine—NaCl 3.44 *M* (1:1)	Eau	87 ± 8.5
Urine—NaCl 0.14 *M* (1:1)	NaCl 0.14 *M*	93.5 ± 3

*Pour chaque éssai, les valeurs indiquées représentent les moyennes calculées à partir de trois résultats au minimum.

une certaine quantité résiduelle de sels apportés par l'urine; l'irréversibilité de la fixation du 16 α-glucuronide d'oestriol par la résine, serait alors masquée partiellement.

En fait, le défaut d'élution du 16 α-glucuronide d'oestriol par le méthanol en l'absence de sels est surprenant puisque, normalement, la glucuroconjugaison des stéroïdes, en renforçant leur hydrophilie, devrait diminuer leur affinité pour la résine XAD-2. Ainsi, selon Osawa et Slaunwhite [11], les oestrogènes conjugués peuvent être élués assez sélectivement de l'amberlite XAD-2, par de

l'éthanol à 30% alors que l'essentiel des formes libres, reste fixé sur la résine dans ces conditions.

Il semble que l'effet de la force ionique puisse être expliqué par la présence possible de liaisons électrostatiques entre le radical glycuronate et la résine XAD-2. En effet, bien que les mesures titrimétriques classiques ne permettent pas de mettre en évidence la présence de charges sur la résine, Puon et Cantwell [17] concluent à l'existence d'un très petit nombre de charges cationiques et anioniques pour expliquer l'adsorption particulière de certains solutés. Ces observations sont aussi compatibles avec celles de Zaika [18] qui note une certaine difficulté à éluer l'ion hydroxyle (OH^-) de l'Amberlite XAD-2.

Les mesures de la conductibilité de la phase aqueuse en équilibre avec cette résine, sont aussi en faveur d'une libération lente de composés ionisés, comme le montre le tableau III. Quel qu'en soit le mécanisme, l'adsorption irréversible de l'oestriol 16 α-glucuroconjugué sur la résine XAD-2, à faible force ionique, doit être efficacement contrôlée pour permettre l'utilisation de ce support dans des conditions satisfaisantes, en vue du dosage des oestrogènes conjugués urinaires.

Pour mettre en évidence l'intérêt de la purification des oestrogènes urinaires réalisée dans ces conditions, 30 urines de grossesse âgées de 26—42 semaines ont été dosées, en double, par la méthode colorimétrique d'Ittrich [16] avec et sans extraction chromatographique des oestrogènes sur résine XAD-2. Les résultats obtenus (Fig. 1) montrent que les points figuratifs du nuage de corrélation sont très dispersés et que la pente de la droite de régression (a = 0.794± 0.302) est relativement faible et très variable; ceci traduit une importante erreur liée à l'une des deux méthodes. On observe aussi que l'ordonnée à l'origine (b = 2.905) est élevée et reflète une erreur systématique constante qui n'est pas négligeable. Ces erreurs sont liées, nous le pensons, à la méthode

TABLEAU III

MESURE DE LA CONDUCTIBILITÉ DE LA PHASE AQUEUSE EN ÉQUILIBRE AVEC LA RÉSINE XAD-2

Les conductibilités exprimées en Siemens par cm (Scm^{-1}) ont été corrigées en tenant compte de la constante de cellule C = 0.84. Les valeurs indiquées représentent les moyennes des résultats de 3 essais. La conductibilité de l'eau bidistillée est de 2.1 Scm^{-1}.

	Conductibilité		
	1 g de résine préalablement purifiée et séchée, remise en suspension dans 40 ml d'eau bidistillée. Mesure de la conductibilité après agitation	La suspension de résine précédente est décantée, puis reprise par 40 ml d'eau bidistillée. Mesure:	
		avant agitation	après agitation
Résine neutre XAD-2	11.50 Scm^{-1}	2.35 Scm^{-1}	3.80 Scm^{-1}

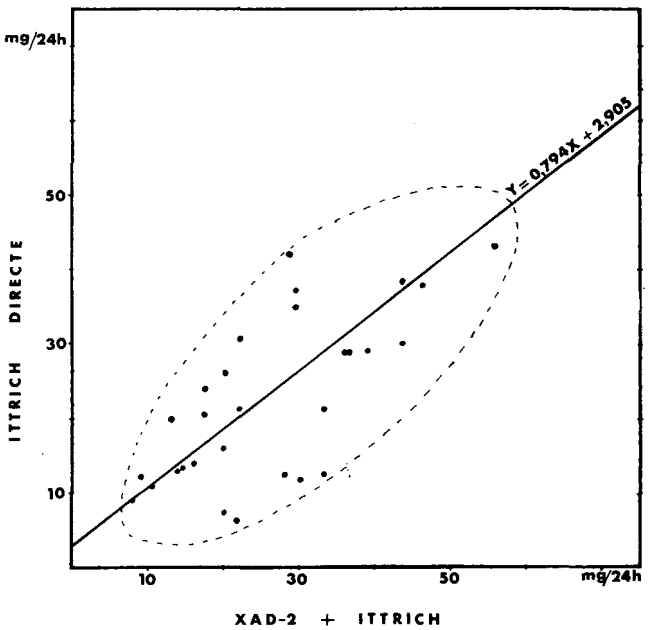

Fig. 1. Comparaison des valeurs de l'oestrogénurie, exprimées en oestriol et obtenues par la méthode d'Ittrich pratiquée directement ou après extraction par l'Amberlite XAD-2. Les urines ont été dosées deux fois par chaque méthode et chaque point figuratif est obtenu à partir de la valeur moyenne des deux résultats.

directe d'Ittrich et s'expliquent par la présence dans l'urine de nombreux composés susceptibles de gêner le développement de la réaction de Kober, et par celle de chromogènes interférents. Ces résultats confirment, par conséquent, le manque de fiabilité de la méthode directe d'Ittrich signalé par plusieurs auteurs, notamment par Scholler et al. [19] et soulignent l'intérêt de la méthode proposé.

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Note

High-performance liquid chromatographic separation of esters of 4-hydroxymethyl-7-methoxy-coumarin

A method for the determination of acidic compounds in the picomole range

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(Received December 16th, 1977)

4-Bromomethyl-7-methoxy-coumarin (Br-Mmc) has been shown to form fluorescent esters with monocarboxylic acids [1,2] and a variety of other acidic compounds of biomedical interest [3]. Although carboxylic acids are readily measured by gas–liquid chromatographic methods [4], a sensitive high-performance liquid chromatographic (HPLC) method may nevertheless find certain applications. In this paper, methods are described for the separation of a series of common fatty acids and of a barbiturate in blood as examples for the application and the limitations of the method.

MATERIALS AND METHODS

Usual laboratory chemicals were of analytical grade. They were purchased from E. Merck (Darmstadt, G.F.R.). Br-Mmc was from Regis (Morton Grove, Ill., U.S.A.). The micro-refluxer [5] and appropriate glassware can be obtained from Regis, or from the Forschungsinstitut Berghof (Tübingen, G.F.R.).

Derivative formation

This was performed by refluxing acetone solutions of the reaction components in the presence of crystalline water-free K_2CO_3 either with [1] or without crown ether as catalyst [2]. Extraction of the barbiturate from small blood samples was performed according to a published procedure [6].

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HPLC Equipment

A Varian 8500 high pressure liquid chromatograph was used in combination with a Perkin-Elmer fluorescence spectrophotometer Model 204 and a Perkin-Elmer spectrophotometer LC55 as detectors. Separations were achieved with methanol—water gradients, using a 25 cm long column with C_{18}-brushes on a 10-μm silica core (Nucleosil 10C-18; Macherey, Nagel & Co., Düren, G.F.R.). (For details of the separation procedure see legends to the figures).

RESULTS

Using a linear water—methanol gradient, 50—100% (Δ = 1% methanol/min) a mixture of the Mmc-esters of the saturated unbranched aliphatic fatty acids between formic and stearic acid can be completely separated within 60 min. (Fig. 1A). Starting with 40% of methanol, a steeper methanol gradient (Δ =

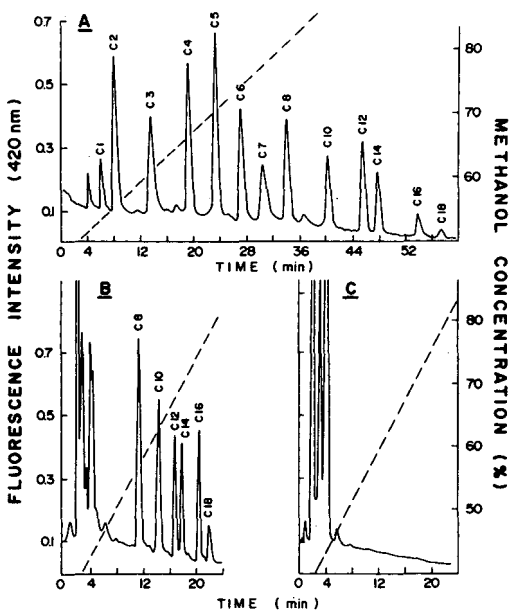

Fig. 1. HPLC separation of Mmc-esters of aliphatic, saturated fatty acids. Equipment: Varian 8500 high-pressure liquid chromatograph; Column: 250 × 2 mm I.D., Nucleosil 10 C_{18}, Macherey Nagel & Co. Detector: Perkin-Elmer fluoroscence spectrophotometer Model 204, with 10-μl flow-cell. Fluorescence activation at 340 nm and fluorescence emission measurement at 420 nm. (A) Mixture of authentic Mmc-esters (about 0.2 nmoles) of formic acid (C_1), acetic acid (C_2) propionic acid (C_3), *n*-butyric acid (C_4), *n*-valeric acid (C_5), caproic acid (C_6), caprylic acid (C_8), capric acid (C_{10}), lauric acid (C_{12}), myristic acid (C_{14}) palmitic acid (C_{16}) and stearic acid (C_{18}). Separation conditions: 3 min elution with 50% methanol, then linear increase of methanol: (Δ = 1%/min). Flow-rate 1 ml/min. (B) Separation of a reaction mixture of fatty acids with Br-Mmc. 10 μl of the solution contained caprylic acid (C_8; 0.16 nmole), capric acid (C_{10}; 0.14 nmole), lauric acid (C_{12}; 0.12 nmole); myristic acid (C_{14}; 0.12 nmole); palmitic acid (C_{16}; 0.17 nmole) and stearic acid (C_{18}; 0.12 nmole). Separation conditions: 3 min elution with 40% methanol, then linear increase of methanol (Δ= 2%/min). Flow-rate 1 ml/min. (C) Blank reaction mixture, containing only reagents, but no fatty acids. Separation conditions as in (B).

2% methanol/min) is still sufficient to separate within about 30 min all homologues up to C_{12} and the even-numbered members of the series up to C_{18}. Higher homologues of the saturated aliphatic fatty acids have not been included in these experiments. Side products of the derivative-forming reaction normally interfere under these conditions with the separation of the first four members of the series so that only the fatty acids with $C_{n>5}$ are separable by immediate application of an aliquot of the reaction mixture to the reversed-phase column. It should be pointed out, moreover, that Br-Mmc, the derivative-forming reagent, elutes in this region as well. Although it is non-fluorescent, it causes fluorescence-quenching, due to its relatively high concentration in the reaction mixture. Fig. 1 B shows the separation of caprylic, capric, lauric, myristic, palmitic and stearic acids, which were reacted with Br-Mmc in 0.275 nmole amounts. One fifth of the reaction mixture was applied immediately to the column. It is obvious from this figure, and it was confirmed by numerous repetitions of the reaction with amounts of fatty acids varying between 60—275 pmoles, that the peak areas of the Mmc-derivatives of the homologous fatty acids, as recorded by fluorescence were not identical, however, they were readily reproducible. Even peak height measurements gave satisfactory quantitative results, if the time of column equilibration with 40% methanol was kept constant at 10 min.

TABLE I

RELATIONSHIPS BETWEEN AREAS OF THE RECORDED PEAKS, SUBSTANCE AMOUNT AND LENGTH OF THE CARBON CHAIN OF THE ALIPHATIC FATTY ACIDS

(For details of the separation see legend to Fig. 1). The figures in the table are the mean values ± S.D. (peak height × width at half height) of four measurements of four reactions. The amounts of the fatty acids refer to the amount present in the total reaction mixture.

Amount of fatty acid (pmole)	Carbon chain length					
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}
60	72 ± 1	63 ± 15	45 ± 5	42 ± 9	47 ± 9	18 ± 4
180	201 ± 22	157 ± 16	123 ± 9	112 ± 9	131 ± 6	49 ± 5
200	231 ± 17	173 ± 15	138 ± 10	128 ± 7	144 ± 9	53 ± 5
275	324 ± 13	252 ± 14	199 ± 11	187 ± 10	205 ± 14	76 ± 1

As can be derived from the data of Table I, mean standard deviation in the range of 60—300 pmole was less than ± 10%. The recorded peak areas were directly proportional to the amounts of fatty acids in the reaction mixtures. Fig. 2 shows this for caprylic and palmitic acid. Fluorescence quantum yields of the Mmc-esters have not yet been established. The reasons for the differences in peak areas, starting derivatization with equimolar amounts of the acids are, therefore, not known.

Another example of application of the method is the determination of a

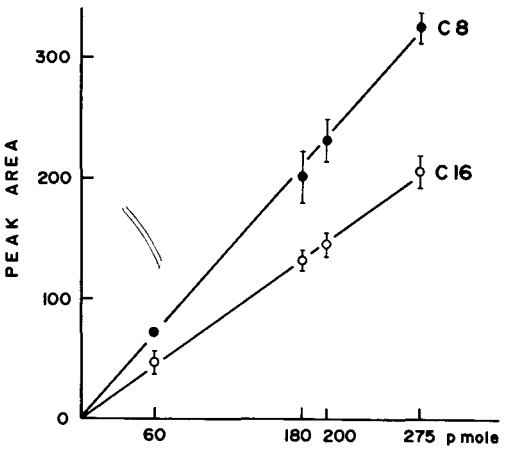

Fig. 2. Standard graph for Mmc-caprylate (C_8) and Mmc-palmitate in the range of 60—275 pmoles; (Table I).

barbiturate in blood. Since details of the extraction and derivatization with Br-Mmc, and thin-layer chromatographic separation have been described [6] only the HPLC-separation of the extract of a 40-μl blood sample containing 2 nmoles of barbital is shown (Fig. 3). The separation of a blood extract free of barbital (blank sample) shows that blood constituents do not interfere with barbital separation. Absorbance recorded for the same sample shows the higher sensitivity of the fluorescence measurement. Preliminary results with HPLC separations of the Mmc-derivatives of some prostaglandins are promising. Due to its high affinity for the reversed-phase used in the present work, Mmc-arachidonic acid was eluted only with methanol, well behind palmitic acid. Its separation from other fatty acids by this method should render a simple and sensitive procedure for its estimation.

DISCUSSION

Mmc-esters are the first fluorescent derivatives suited for liquid chromatographic determination of acidic compounds [7, 8]. Although their properties allow thin-layer chromatographic separation, a system which avoids exposure to light on an active surface is preferable, especially since Br-Mmc is rapidly decomposed under these conditions to fluorescent products. HPLC was therefore the method of choice. It is shown in the present work that a simple, rapid, reproducible separation system is suitable for the estimation of Mmc-derivatives. Since the derivatization reaction can be scaled down to a reaction volume of 5 μl it is possible to determine a few picomoles of the derivatized compounds. This range of sensitivity is comparable with advanced gas—liquid chromatographic methods; it is considerably higher than the post column reaction with Ce(IV) in combination with UV absorptimetry or fluorimetry [8]. The strongly absorbing *p*-bromo-phenacyl esters [9, 10] and the *p*-nitrophenacyl esters [11, 12] are detectable in 0.1 nmole amounts. Dansyl derivatives of barbiturates can be estimated at a comparable level of sensitivity as their Mmc-

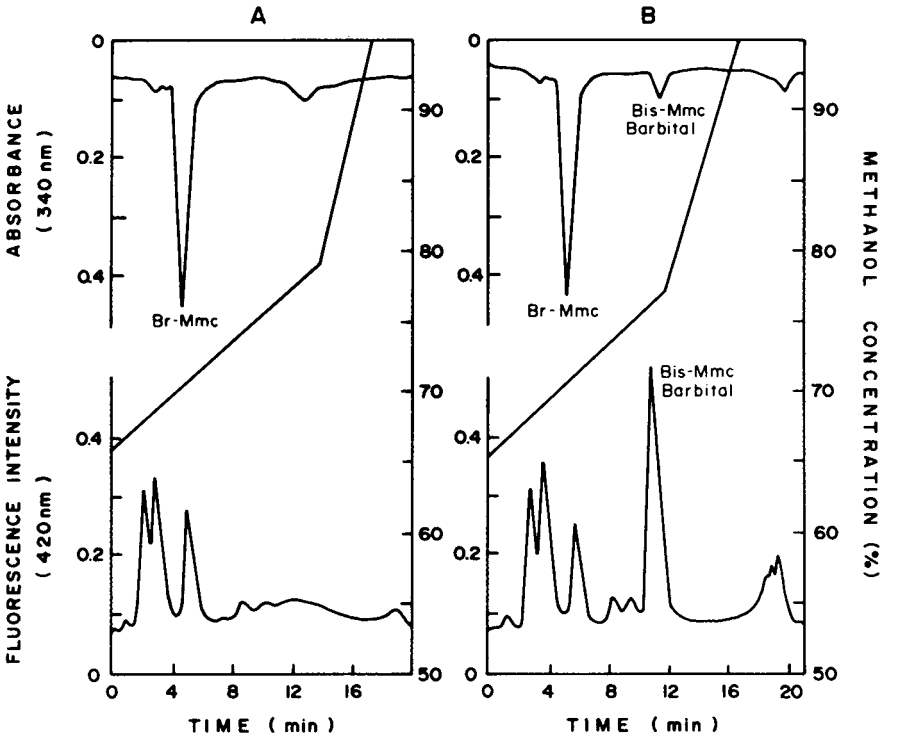

Fig. 3. Determination of barbital in a 40-μl blood sample, by extraction and derivatization with Br-Mmc. (A) Blood sample without barbital (blank); (B) blood sample with 2 nmoles barbital. The reaction volume was 5 μl and 2 μl of the reaction mixture was applied to HPLC separation. Separation conditions: linear water—methanol gradient, starting with 65% methanol ($\triangle$ 2%/min); flow-rate 1 ml/min. After the appearance of the bis-Mmc-barbital peak (13 min) the methanol concentration is rapidly increased, in order to wash out impurities from the column. (See also legend to Fig. 1 and Materials and methods).

derivatives [13]. It should be mentioned that UV detectors are well suited to monitor (at 340 nm) Mmc-derivatives. The sensitivity, as compared with the fluorometric method, is lower by about one order of magnitude, if a flow cell of 10 mm path length is used (Fig. 3).

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Note

Rapid method for the high-performance liquid-chromatographic determination of acephylline in human serum

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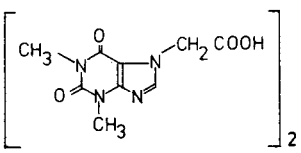
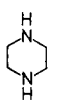
Acephyllinepiperazine (Acepiphylline, Etaphylline ®) has been used for the treatment of asthma. Acephylline is a derivative of theophylline and is chemically described as 1,3-dimethylxanthine-7-acetic acid (7-theophylline-acetic acid).



acephylline



piperazine

Until now no specific method for the determination of acephylline has been described in the literature. Turner-Warwick [1] used the spectrophotometric assay of theophylline introduced by Schack and Waxler [2]. This method is not specific and involves solvent extraction of the drug. The author interpreted the results as theophylline levels. There is no evidence that acephylline is metabolized to theophylline and the results of this author therefore give a false picture of the real blood levels. The UV absorption curves are different, as can be seen from Fig. 1.

A new high-performance liquid-chromatography (HPLC) procedure for the determination of acephylline and study of its absorption and pharmacokinetics in serum has been developed. Proteins are removed by precipitation with per-

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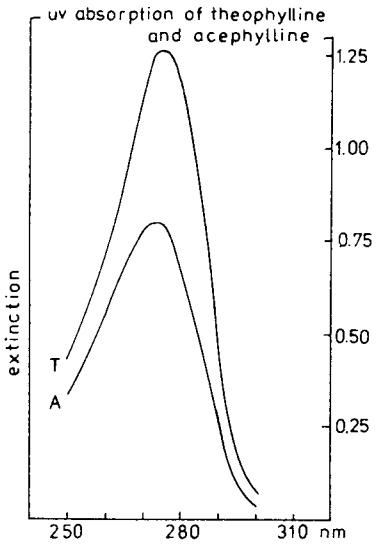

Fig. 1. The UV absorption curves of theophylline (T, 20 μg/ml) and acephylline (A, 26.4 μg/ml). The concentrations are molar equivalents.

chloric acid. The supernatant is neutralized by potassium carbonate. Excess perchloric acid is thus precipitated as potassium perchlorate.

PROCEDURE

A 0.5-ml serum sample was pipetted into a tube containing 50 μl of 70% perchloric acid. After mixing for 30 sec on a whirl-mixer, the tube was centrifuged to precipitate the denatured proteins. This was followed by adding 100 μl of a saturated potassium carbonate solution and further mixing. The potassium perchlorate was precipitated by centrifuging for 10 min. An aliquot was injected on to the column.

Analyses were performed using a Waters Assoc. Model 6000 pump and Modet 440 absorbance detector. A reversed-phase system was used, consisting of a Bondapak C_{18} column (30 cm × 4 mm I.D.) with a particle size of 10 μ (Waters Assoc.), and methanol 4% in 0.01 *M* sodium dihydrogen phosphate, at a flow-rate of 2.0 ml/min. Absorbance was monitored at 280 nm. The detector was operated at a sensitivity of 0.02 a.u.f.s. Peak heights were used for quantitation.

RESULTS AND DISCUSSION

Chromatograms of serum samples (Fig. 2) demonstrate that no contamination peaks occur. The retention time of acephylline is 10 min. Decreasing this time by increasing the methanol concentration in the eluent causes the serum and acephylline peaks to merge. Dietary xanthines, caffeine, theophylline and theobromine and metabolites did not interfere with the assay. The standard curve of acephylline added to serum was linear over the range 1—50 μg/ml and

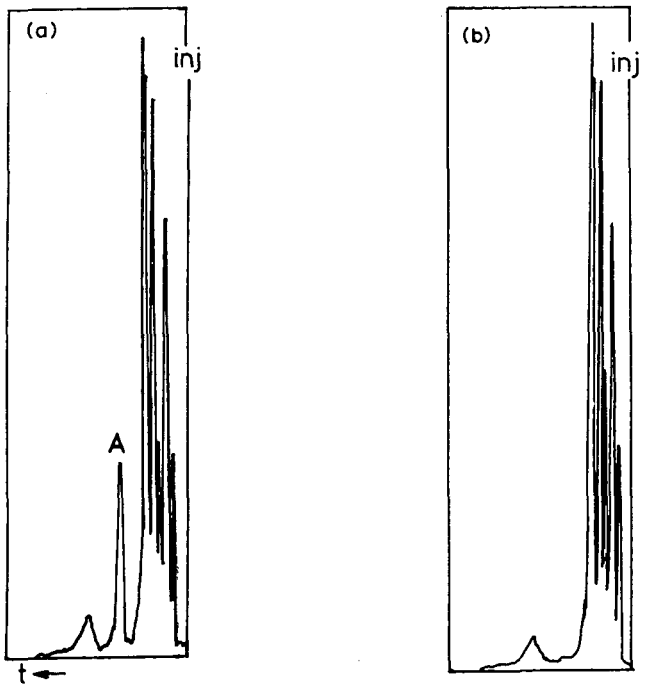

Fig. 2. a, representative chromatogram of the HPLC of acephylline in human serum. A, acephylline. b, chromatogram of a blank human serum.

passed through the origin. The correlation coefficient was $r = 0.997$, and the standard deviation 2.8% ($n = 6$ at 10 μg/ml.) The lower detection limit was 1 μg/ml serum.

This HPLC method is easy to perform, involves no extraction or derivatization procedures, and can be successfully performed with 50 μl of serum. The standards were made in serum, so the recovery was 100%.

A white Wener rabbit was administered acephyllinepiperazine 4 mg/kg as an intravenous bolus. Venous blood samples were taken every 5 min. From the decline of the concentration curve an elimination half-life of 15 min was found. After 1 h neither acephylline nor theophylline could be detected in the serum. These investigations will be continued with volunteers and patients.

ACKNOWLEDGEMENT

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Note

Analysis of thiopentone in human plasma by high-performance liquid chromatography

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As part of a study of perinatal drug transfer, we are investigating the placental transfer of drugs administered just prior to delivery, especially in caesarian section. The drug of choice for induction of anaesthesia is thiopentone [5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid], an ultra short-acting barbiturate. Numerous methods of analysis for thiopentone have been presented in the literature [1—5]. Oroszlan and Maengwyn-Davies [1] reported a spectrophotometric assay involving three different wavelengths and having a sensitivity of 1 μg/ml. Scoppa [2] described a spectrofluorimetric determination with a sensitivity of 0.5 μg/ml. Neither method is specific for thiopentone. Bogan and Smith [3] used extraction followed by gas—liquid chromatography to separate and quantitate thiopentone specifically. Sensitivity was improved by both Sennello and Kohn [4] and Schepens and Heyndricks [5] by the use of a nitrogen-sensitive detector. Concentrations of 0.1 μg/ml were quantitated by this method.

The gas chromatographic determination of thiopentone is preferable to other previously reported analytical methods owing to its accurate quantitation and high specificity for low levels of drugs. It does, however, require time-consuming extraction procedures followed by lenghthy derivatization to enable the sample to be chromatographed at a low enough temperature to stop desulphuration.

Accordingly, a high-performance liquid chromatographic (HPLC) assay for plasma levels of free thiopentone was developed since the extraction and derivatization procedures could be eliminated, leading to the rapid and accurate assay of a large number of plasma samples. To simplify preparation of the sample prior to chromatographing a precolumn was inserted between the injection port and the main analytical column to remove proteins and other particulate matter from the plasma samples. Using this method, pretreatment of the sample involved a single dilution of plasma with internal standard to produce a sample suitable for chromatographing.

EXPERIMENTAL

Instrumentation

Analysis of samples was performed on a Perkin-Elmer 1220 liquid chromatograph using a reversed-phase 25 cm × 4.6 mm I.D. Partisil 10/25 ODS column (Whatman, Maidstone, Great Britain) protected by a 3 cm × 2.8 mm I.D. precolumn separated from the main column by a 2-μm stainless-steel frit.

The mobile phase [methanol–0.1% sodium citrate buffer (pH 6.5), 45:55] was eluted at 0.5 ml/min. The detector was a Perkin-Elmer LC-55 operated at 290 nm and its output led through a Perkin-Elmer M-2 calculating integrator which gave peak area ratios for thiopentone to internal standard.

Sample preparation

At 1-min intervals after the induction of anaesthesia with intravenous thiopentone, 2-ml samples of whole blood were collected from the contralateral median cubital vein of women undergoing delivery by ceasarian section. The blood was stored in sequestrene (EDTA) tubes at 4° until analysed. Just prior to analysis the tubes were thoroughly mixed on a Vortex mixer for 30 sec and a 0.5-ml portion was centrifuged at 1600 *g* for 5 min. A 95-μl portion of the supernatant was withdrawn and mixed with 5 μl of internal standard (0.02% quinoline) in glass vials. Aliquots (10 μl) were withdrawn for analysis.

RESULTS AND DISCUSSION

Specificity

A representative chromatogram is shown in Fig. 1. Analysis of samples from a number of patients has shown that a variety of drugs used pre-operatively, postdelivery, and as premedication did not interfere. These include folic acid, penicillin V, hyoscine, nitrazepam, morphine, ergometrine, phenobarbitone, pethidine and chlorpromazine.

A typical plasma profile from a patient who received intravenously 250 mg of thiopentone is shown in Fig. 2. The well-known rapid decrease in thiopentone plasma levels is evident.

Precision

A standard curve was constructed with points ranging from 0 to 20 μg/ml of thiopentone and the appropriate amount of internal standard. A plot of the

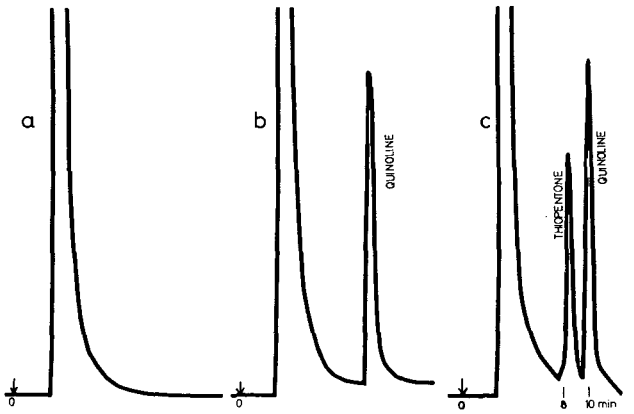

Fig. 1. Liquid chromatograms of human plasma following direct injection. (a) Drug-free plasma; (b) plasma with internal standard; (c) drug-containing plasma with internal standard.

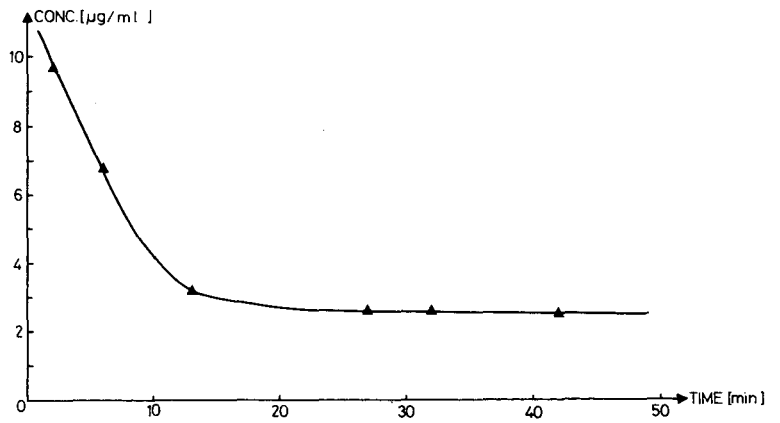

Fig. 2. Maternal plasma concentrations of thiopentone as a function of time.

ratio of peak areas (y) versus concentration of thiopentone (x) gave a line which fitted the equation

$$y = 0.0349\,x - 0.0060$$

with a coefficient of correlation of 0.9993. The lower limit of detection (signal-to-noise ratio 10:1) was 0.5 µg/ml for a 10-µl injection and the curve was linear over the range 0.5 to 20 µg/ml.

Precolumn

In developing this assay such pretreatments as extraction and ultrafiltration were considered in addition to the use of a precolumn. Extraction was found to be time-consuming while ultrafiltration was inappropriate owing to both extensive binding of thiopentone to the membrane and the filtration time required to obtain a suitable sample. The precolumn offered the only viable alternative.

The column was constructed of 3 cm × 2.8 mm I.D. (¼ in. O.D.) 316 stainless steel and was packed with 10-μm Partisil 10/25 ODS packing. Plasma samples were then injected through the septum directly on to the precolumn. The backpressure of the system was monitored and when this pressure rose above ∼2500 p.s.i.g., the precolumn was replaced.

Approximately 500 μl of plasma (fifty 10-μl injections) could be filtered by the one precolumn before it required replacement.

CONCLUSION

A simple HPLC method for the determination of plasma levels of thiopentone has been described. Sample pretreatment is minimized by the use of a precolumn inserted between the injection port and the analytical column. In a 10-μl injection the minimum detectable concentration of free thiopentone in plasma is 0.5 μg/ml.

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Note

Rapid determination of amoxycillin (Clamoxyl®) and ampicillin (Penbritin®) in body fluids of many by means of high-performance liquid chromatography

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The determination in body fluids of antimicrobial drugs of biological origin is mainly carried out using microbiological techniques [1—6]. This guarantees the determination of the microbiologically active principles, including active metabolites. The relatively long time required for analysis by such techniques is a disadvantage when the results are urgently needed by clinicians. Frequently, in such cases more than one antibiotic drug is administered, which sometimes results in difficulties when measuring concentrations by means of the microbiological assay.

The analysis of amoxycillin and ampicillin by means of high-performance liquid chromatography (HPLC) was developed in order to obtain information about the pharmacokinetics and characteristics of penetration into tissue fluids. This type of analysis has been reported previously, but only for non-biological applications such as the quality control of pure substances [7].

Additional pharmacokinetic information on amoxycillin and ampicillin plasma concentrations will be useful in optimizing therapy with antibiotics for the treatment of patients with a variety of susceptible bacterial infections for which the minimum inhibitory concentration has been established. Such a study would serve in the formulation of guidelines for rational treatment. The HPLC method may be of use in pharmacokinetic studies, giving evidence that the antibiotic drug reaches the infected tissues in an adequate concentration.

EXPERIMENTAL

A spectra Physics 3500 B high-performance liquid chromatograph was used. The column (15 cm × 4.6 mm I.D.) was packed with LiChrosorb RP-8 (particle sice 5 μm) obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection was effected at 225 nm.

The solvent for amoxycillin is a potassium dihydrogen phosphate buffer (Sörensen buffer, pH 4.6, 0.067 *M*) at a flow-rate of 1.2 ml/min. The solvent for ampicillin is a mixture of 425 ml of the KH_2PO_4 buffer (pH 4.6) and 75 ml of methanol at a flow-rate of 1.2 ml/min.

Drugs

Amoxycillin (100% chromatographically pure, 86% activity) and ampicillin (100% chromatographically pure, 86.5% activity) were obtained as pure compounds from Beecham Pharmaceuticals (Amstelveen, The Netherlands). 6-Aminopenicillanic acid and benzylpenicilloic acid were gifts from Gist-Brocades (Delft, The Netherlands).

Subjects

Amoxycillin and ampicillin were administered to volunteers from the Department of Clinical Pharmacy and patients from various departments of the St. Radboud Hospital. Blood samples of 0.2 ml were collected frequently by finger-tip puncture (Microlance No. 433, Becton Dickinson, U.S.A.). Urine was collected as spontaneously voided. The pH of the urine was kept alkaline in some subjects by the regular daily intake of 10 g of sodium bicarbonate. Saliva was collected at regular time intervals by spontaneous production.

Sample preparation

For both drugs the same procedure was followed for the analysis of plasma and saliva concentrations. 0.1 ml of plasma or saliva was mixed with 0.4 ml of perchloric acid (0.33 *N*) on a Vortex mixer. The mixture was centrifuged at ca. 2600 *g* for 5 min (Heraus Christ centrifuge). 100 μl of the clear supernatant were injected into the high-performance liquid chromatograph.

Urine was treated as follows. 10 μl of urine were mixed with 0.5 ml of perchloric acid (0.33 *N*) on a Vortex mixer. 100 μl were injected into the high-performance liquid chromatograph.

Before each series of determinations a calibration curve was constructed (peah height vs. concentration). The precision of the method was established as 100 ± 2%. The lowest concentration that can be measured accurately is 0.5 μg/ml. The total time required for one analysis is 15 min.

RESULTS

Ampicillin and amoxycillin are well separated from other endogenous compounds present in plasma, saliva and urine (Fig. 1A,B). Fig. 2 shows the pharmacokinetics of amoxycillin in a volunteer who took 750 mg of the antibiotic orally. The maximum plasma concentration was 14 μg/ml, this maximum being reached after 1.5 h. Amoxycillin was eliminated with a half-life of 1.1 h.

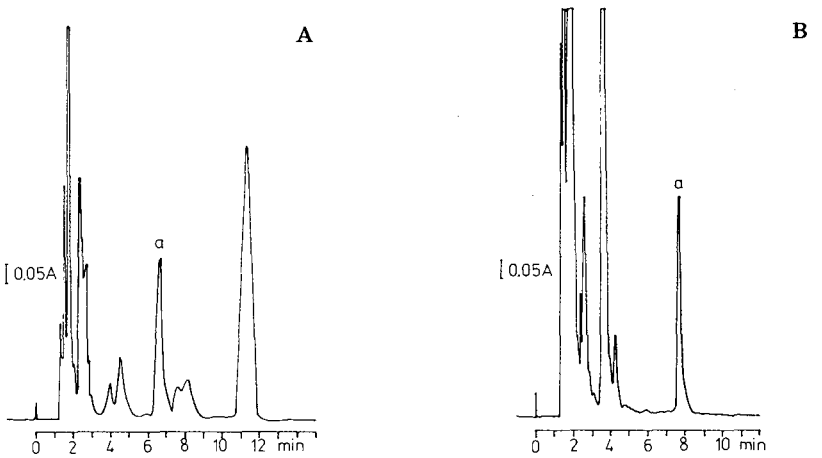


Fig. 1. A. Chromatogram of amoxycillin in human urine. The column used was LiChrosorb RP-8 (5 μm) and the solvent 0.067 *M* KH_2PO_4 (pH 4.6). The flow-rate is 1.2 ml/min. B. Chromatogram of ampicillin in human plasma ($\approx$ 32 μg/ml). The column used was LiChrosorb RP-8 (5 μm) and the solvent a mixture of 425 ml of KH_2PO_4 buffer (pH 4.6) and 75 ml of methanol. Flow-rate, 1.2 ml/min.

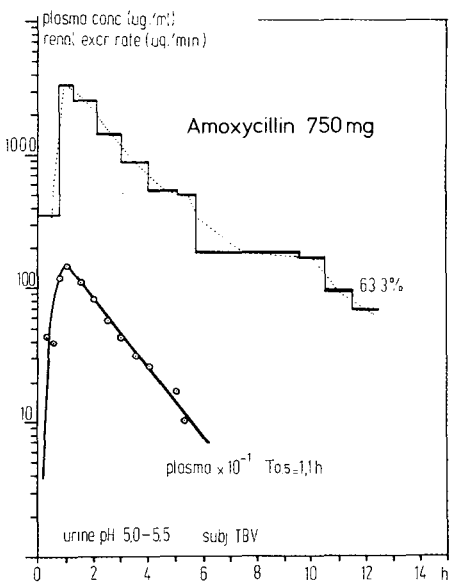
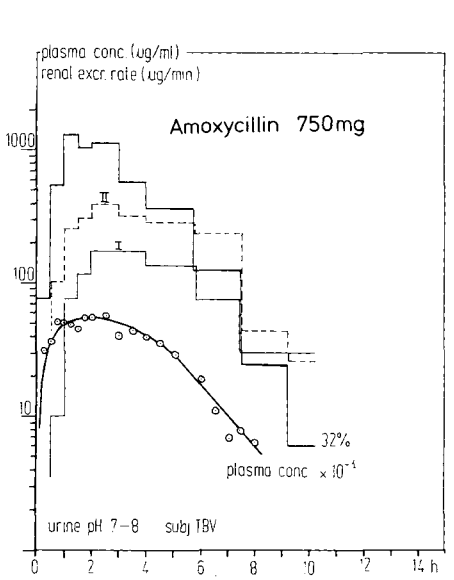


Fig. 2. Plasma concentration—time curve and renal excretion rate of amoxycillin in a human volunteer who took 750 mg of the drug orally. The maximum plasma concentration is 14 μg/ml. The urine pH was 5.3 $\pm$ 0.2 throughout the experiment.

The renal excretion rate reached a maximum value of 3500 μg/min. The excretion curve is parallel to the plasma concentration—time curve. The renal clearance constant was calculated to be 250 ml/min. 63.3% of the dose administered was excreted in the urine as the parent drug. No influence of the urinary flow-rate (ml/min) on the renal excretion rate was observed. The pH of the urine of the volunteers was measured and found to be acidic (pH 5.3 ± 0.2 throughout the experiment). The subjects experienced excitement and nervousness. The same effects are observed after the intake of high amounts of ammonium chloride for the purpose of rendering the urine acidic (8 g/day). Amoxycillin is an acidic drug, the pK_a values of the COOH, NH_2 and OH group are 2.4, 7.4 and 9.6 respectively, and a solution of 0.2% (w/v) of the drug in CO_2-free water has a pH of 3.5—5.5 [8].

Fig. 3 shows the pharmacokinetics of amoxycillin in the same volunteer after intake of 750 mg of the drug. During the experiment the urine pH was kept alkaline (pH 7.5—8.2) by the regular intake, four times a day, of 2.5 g of sodium bicarbonate. The maximum plasma concentration was only 7.5 μg/ml. The maximum remains for a longer period than with acidic urine (Fig. 4). The half-life of elimination was measured again as 1.1 h. The maximum renal excretion rate was lower (1400 μg/ml). The total percentage of the drug



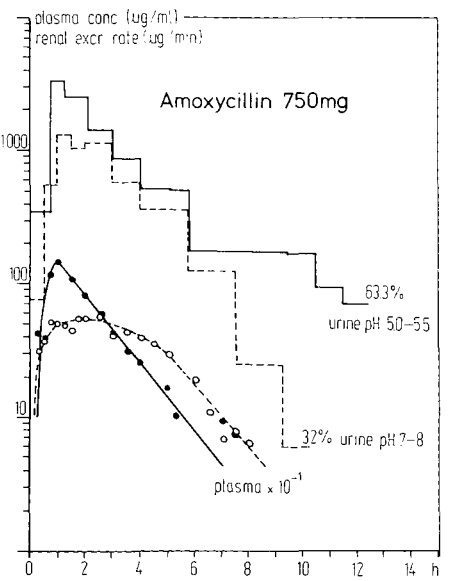


Fig. 3. Plasma concentration—time curve and renal excretion rate of amoxycillin in a human volunteer who took 750 mg of the drug orally. The urine is kept alkaline (pH 7.5—8.2). The maximum plasma concentration is 7.5 μg/ml. The compounds I and II are unidentified (see Table I).

Fig. 4. Plasma concentration—time curve and renal excretion rate of amoxycillin in the same volunteer under different urinary pH conditions (acidic and alkaline). 63.3% of the drug was excreted unchanged with acidic urine while 32% was excreted unchanged under alkaline conditions. The area under the plasma concentration—time curve under alkaline conditions is 87% of that obtained with acidic conditions.

excreted as the parent drug appeared to be considerably lower, 32%.

In the chromatogram of the urine two components (I and II) could be measured. The renal excretion rate of both unidentified compounds showed a pharmacokinetic behaviour, i.e. increase and decrease of concentration in the time course of the experiment as a result of amoxycillin intake (Fig. 3). The compounds elute under different conditions from the RP-8 column (Table I), thus the structures are different from 6-aminopenicillanic acid and benzylpenicilloic acid. The renal excretion rates of these two compounds, characterized by the initials I and II referring to their retention times and given in Table I, are shown in Fig. 3. The kinetic profile of these metabolites under acidic urine conditions is different, the renal excretion rates of both compounds are almost identical.

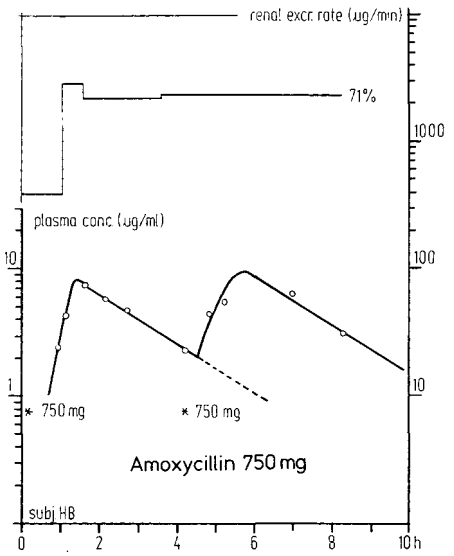


Fig. 5. Plasma concentration—time curve and renal excretion rate of amoxycillin at the start of treatment of 750 mg orally, twice daily.

Fig. 5 shows the start of a plasma concentration—time profile of a patient who took amoxycillin orally twice a day (750 mg twice daily). The maximum concentration appears to be 8.5 µg/ml, the maximum being reached after 1.75 h. The elimination half-life is found to be 1.5 h.

Ampicillin taken by three volunteers reached a maximum plasma concentration of 11 µg/ml after an oral dose of 1250 mg and shows a half-life of 1.5 h. 15% of the oral dose was recovered as the parent compound in the urine.

No ampicillin or amoxycillin could be detected in saliva.

DISCUSSION

The HPLC method for ampicillin and amoxycillin presented here is rapid, sensitive and reproducible. The results are comparable with those obtained with the microbiological methods, as reported earlier [9,10]. Both methods

TABLE I

RELATIVE RETENTION TIMES OF AMOXYCILLIN AND RELATED COMPOUNDS

Compound	Relative retention time	
	Solvent: KH_2PO_4	Solvent: KH_2PO_4 + methanol
Amoxycillin	1.00	0.22
Ampicillin	—	1.00
I in urine	0.52	—
II in urine	0.63	—
6-Aminopenicillanic acid	0.35	0.01
Benzylpenicilloic acid	—	0.01

are complementary to each other, one measuring the drug as a chemical entity, the other as the active principle.

As an aid to therapy, the HPLC method may be the preferred technique when the bacteria have been identified and the appropriate drug has been chosen. The low renal excretion of amoxycillin under alkaline conditions is not yet fully understood and is the subject of further research. A possible explanation might be that the phenolic hydroxyl group is the dominating group in the regulation of the renal excretion. The lower maximum plasma concentration of amoxycillin under alkaline conditions is compensated by the extended time course of the plasma concentration. The area under the plasma concentration—time curve with alkaline conditions is only 87% of that obtained with acidic conditions, which implies that about the same amount of drug is absorbed. The differences in the absorption kinetics are too small to be responsible for the big differences in renal excretion rates as shown in Fig. 4.

Ampicillin and amoxycillin, as acidic compounds, are excreted in the saliva at extremely low rates. This behaviour has been reported earlier for acidic compounds [11].

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Biomedical Applications

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Note

Dosage sensible et rapide du chloramphenicol dans le serum par chromatographie liquide haute pression

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(Reçu le 16 novembre 1977)

Le développement de l'utilisation du chloramphénicol (CAP) en thérapeutique humaine et animale, soulève le problème de la détection et de la mesure des taux sériques de CAP libre, donc actif, tant sur le plan de la surveillance clinique des patients que sur celui des études de pharmacocinétique du CAP. Parmi les nombreuses méthodes décrites, aucune ne donne satisfaction sur les trois aspects de spécificité, sensibilité et facilité de mise en oeuvre recherchés. Les méthodes classiques de dosage microbiologique [1] présentent l'inconvénient majeur d'un manque de spécificité et de sensibilité. Un progrès a été apporté par les techniques spectrophotométriques [2, 3]. Elles sont fondées sur la mesure de l'amine formée après réduction du groupement nitré du cycle aromatique du CAP et de ses analogues, et pêchent là encore par manque de spécificité et de sensibilité. La chromatographie en phase gazeuse [4, 5] a permis de franchir un degré supplémentaire pour l'abtention d'une bonne spécificité et d'une grande sensibilité. Cependant les résultats obtenus dans une publication récente [6] portant sur des concentrations sériques de CAP de l'ordre de 5—20 mg/l font apparaître un taux de récupération limité à 64 %. Enfin deux méthodes très sensibles ont été récemment décrites, l'une est enzymatique [7], l'autre fluorimétrique [8] . La première, très sensible, présente l'inconvénient d'utiliser des isotopes radioactifs et de nécessiter l'emploi d'un compteur à scintillation liquide; la seconde quant à elle, est limitée par les phénomènes de fluorescence non spécifique, importants et variables, observés sur les échantillons témoins. C'est pourquoi nous nous proposons de décrire une méthode simple et rapide, spécifique et sensible de dosage du CAP dans le sérum. Elle est basée sur une analyse

en chromatographie liquide haute pression après extraction du CAP par l'acétate d'éthyle. Spécifique du CAP libre, à l'exclusion de ses analogues (esters succinique et palmitique, thiamphénicol) et de son principal métabolite (glucuroconjugué), elle permet de détecter, pour une prise d'essai de sérum de 0.5 ml, des taux de l'ordre de 250 ng/ml.

MATÉRIEL ET MÉTHODES

Réactifs

Tous les solvants utilisés sont purs pour analyse (E. Merck, Darmstadt, R.F.A.). Les produits standard de référence sont: chloramphénicol (Calbiochem, San Diego, Calif., U.S.A.: B grade), thiamphénicol (Parke Davis, Ann Arbor, Mich., U.S.A.), esters palmitiques et succiniques de CAP préparés à partir de CAP standard.

Extraction

0.5 ml de sérum sont dilués dans un volume égal de tampon phosphate 1 *M* pH 6.5. L'extraction du CAP est réalisée par deux fois 2 ml d'acétate d'éthyle dans des fioles coniques de 5 ml bouchées PTFE. Après filtration sur sulfate de sodium les phases organiques sont réunies et évaporées sous vide. Le résidu est repris par trois fois 1 ml d'acétonitrile saturé en isooctane dans une fiole conique de 5 ml. Après addition d'1 ml d'isooctane saturé en acétonitrile, agitation suivie d'une décantation, la phase supérieure (isooctane) est éliminée par aspiration. La phase acétonitrile est évaporée à sec dans la fiole sous un courant d'azote.

Chromatographie liquide haute pression

L'extrait sec est repris par 0.1 ml d'acétate d'éthyle. 10 μl de cette solution sont analysés en chromatographie liquide haute pression dans les conditions suivantes:

L'appareil utilisé est le modèle SP 3500 B Spectra-Physics, équipé d'un détecteur ultraviolet à 254 nm. La colonne (25 cm × 2.1 mm I.D.) est du type C_{18} Spherisorb ODS phase inversée, granulométrie 5 μm (Spectra Physics).

L'injection de l'échantillon est effectuée au moyen d'une microseringue Valco de 50 μl dans une vanne équipée d'une boucle de capacité 10 μl.

L'élution est réalisée par le mélange eau—méthanol (70:30) avec un débit de 0.8 ml/mn. La sensibilité du détecteur est établie à 0.005 unité d'absorbance pleine échelle.

Les pics sur le chromatogramme sont identifiés par leur temps de rétention, leur hauteur est mesurée et les concentrations en CAP des échantillons sont établies par comparaison avec une solution de référence de CAP standard dans l'acétate d'éthyle, de concentration connue et de valeur avoisinante, chromatographiée dans les mêmes conditions.

RÉSULTATS ET DISCUSSION

Le chromatogramme obtenu à partir de la solution de référence de CAP dans l'acétate d'éthyle (5 μg/ml) est représenté à la Fig. 1. Dans les condi-

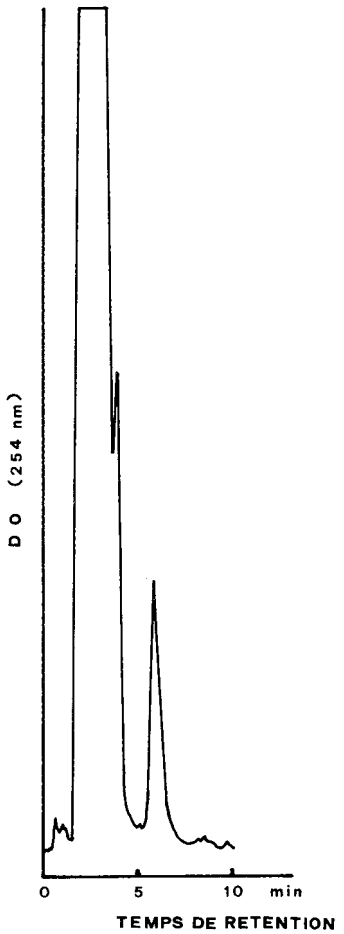

Fig. 1. Chromatogramme de CAP standard (50 ng injectés).

tions décrites le temps de rétention du CAP est de 6 min.

La linéarité de la réponse a étè vérifiée pour des doses injectés de 10 ng à 100 ng.

La reproductibilité de l'analyse chromatographique a été estimée par 6 injections successives de 10 μl de la solution de référence (50 ng de CAP standard). Le coefficient de variation mesuré est de 3 %.

La spécificité de la réponse a été testée pour le CAP, les esters succinique et palmitique et le thiamphénicol. Les conditions d'extraction du CAP restent valables pour le succinate et le palmitate ainsi que pour le thiamphénicol. Cependant dans les conditions chromatographiques décrites le palmitate est retenu sur la colonne alors que succinate et thiamphénicol sont élués dans le front du solvant. De plus, pour ce dernier la réponse spécifique est beaucoup plus faible que pour le CAP. Les glucuroconjugués du CAP, eux, ne sont pas extraits à l'acétate d'éthyle. Le pic observé est donc caractéristique du CAP libre et permet de le distinguer de ses analogues.

Détermination dans le sérum

Dans les conditions décrites, les chromatogrammes obtenus avec des extraits, d'une part de sérum témoin normal, d'autre part du même sérum surchargé à 1 μg de CAP par ml sont respectivement représentés aux Fig. 2a et b. Le temps de rétention et la réponse spécifique sont les mêmes que pour la solution standard. L'analyse du sérum témoin ne permet de mettre en évidence aucune interférence mesurable, qui viendrait limiter la sensibilité de la méthode.

Le taux de récupération a été mesuré sur 6 échantillons de sérum surchargés avec 1 μg de CAP standard par ml.

Le taux de récupération moyen est de 98.7 % ± 5.8. La variabilité du dosage, exprimée par son coefficient de variation est donc inférieure à 6 %.

L'analyse d'un sérum surchargé de 1 μg de CAP standard/ml se traduit par un pic de 6.5 cm de hauteur. Il est aisément possible d'apprécier et de mesurer avec précision un pic de hauteur quatre fois moindre. L'absence d'interférence sur le chromatogramme d'un extrait de sérum témoin permet

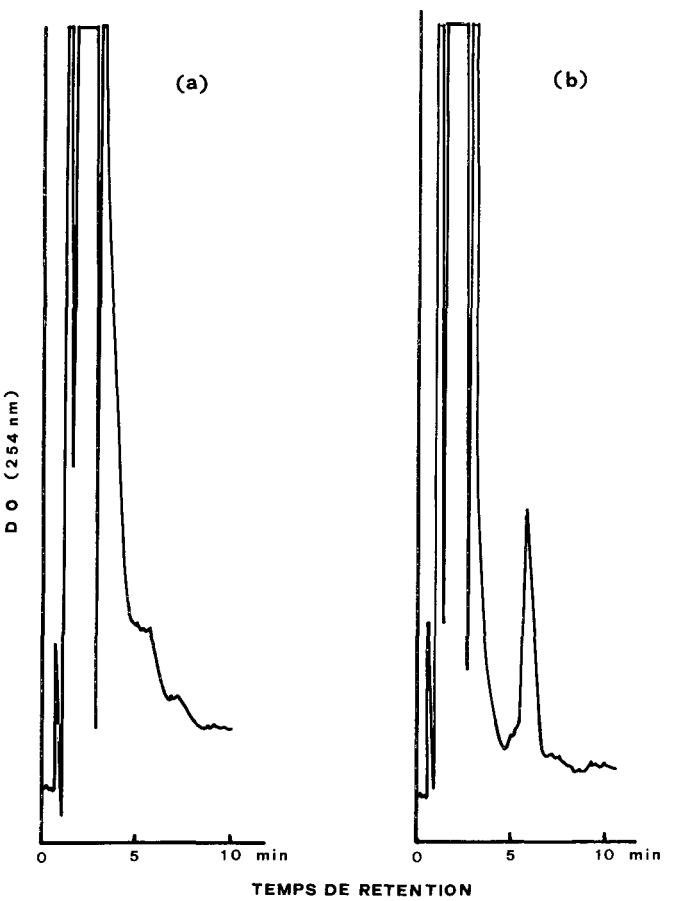

Fig.2. (a) Chromatogramme d'un extrait de sérum témoin. (b) Chromatogramme d'un extrait du même sérum surchargé à 1 μg/ml de CAP standard.

d'affirmer en toute sécurité qu'un tel pic correspond à une dose de CAP injectée de 12.5 ng, qui se situe dans la zone de linéarité de la réponse. La présente méthode, telle qu'elle est décrite, avec une prise d'essai de 0.5 ml de sérum, permet ainsi facilement de détecter et de mesurer le CAP libre dans le sérum au taux de 250 ng/ml. Or, la surveillance clinique de patients soumis à un traitement thérapeutique au CAP, a permis à de nombreux auteurs de préciser les taux sanguins alors atteints. 2 h après l'administration orale de 2 g de CAP, la concentration sérique atteint un pic de 20—40 μg/ml; le CAP est ensuite éliminé avec une demi vie de 1.5—3.5 h [5, 6].

CONCLUSION

La méthode de dosage décrite allie aux qualités de spécificité d'une méthode chromatographique, la simplicité et la rapidité nécessaires à une mise en oeuvre de routine. L'absence d'interférence observée au niveau des sérums témoins permet d'autre part de tirer parti au maximum de la grande sensibilité de cette méthode. Si la limite de détection ne constitue pas le facteur limitant, ce qui peut être le cas pour la détermination, lors des examens de biologie clinique, des taux sériques de CAP chez des patients sous thérapeutique, le volume d'essai peut être facilement réduit. Par contre, les études de pharmacocinétique et du métabolisme fin du CAP chez l'homme ou l'animal nécessitent des seuils de détection beaucoup plus bas. La méthode décrite ici permet alors, sur des prises d'essai plus importantes, d'abaisser la limite de détection à quelques ng/ml.

REMERCIEMENTS

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Biomedical Applications

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Note

Estimation of the hypoxic cell-sensitiser misonidazole and its O-demethylated metabolite in biological materials by reversed-phase high-performance liquid chromatography

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It is widely held that the failure of radiotherapy to cure many human tumours is due in part to the presence of poorly oxygenated malignant cells which are comparatively resistant to radiation [1, 2]. Considerable interest has therefore been shown in hypoxic cell-sensitisers [3] of which one of the most promising is the 2-nitroimidazole, misonidazole (Roche) (for review see ref. 3). Preliminary clinical trials with this compound have demonstrated radiosensitisation in man [4] and it is now under investigation at a number of radiotherapy centres.

As both the radiosensitising effect and the toxicity of misonidazole are dose-dependent [5], a knowledge of the pharmacokinetics of the drug in individual patients is particularly desirable. Five techniques for the assay of misonidazole have been described previously. Reported UV spectrophotometric [6] and

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polarographic [7] techniques have the disadvantage that they determine total nitroimidazole concentration, failing to discriminate between misonidazole and its O-demethylated metabolite (Ro 05-9963). This separation has been achieved by paper chromatography [8], thin-layer chromatography (TLC) [9] and gas—liquid chromatography (GLC) [9]. However, the two former methods do not lend themselves to accurate routine analysis and the latter method requires a time-consuming extraction and derivatisation procedure.

It has been shown that C_{18}-bonded silica is the best of the commercially available bonded supports for the reversed-phase separation of misonidazole and its O-demethylated metabolite [10]. More recent work has, however, indicated that even better resolution can be achieved on a C_{22}-bonded silica [11].

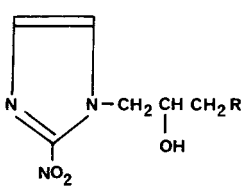
The present paper describes a rapid reversed-phase high-performance liquid chromatographic (HPLC) method for the assay of misonidazole and its O-demethylated metabolite in biological materials.

EXPERIMENTAL

Essentially the same method has been employed in four separate laboratories, each using different HPLC equipment. To avoid repetition, the present communication gives precise details of the technique used in one laboratory.

Reagents

The following nitroimidazoles were synthesised and supplied by Roche Products (Welwyn Garden City, Herts., Great Britain): 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (misonidazole, Ro 07-0582) (I); 1-(2-nitroimidazol-1-yl)-2,3-propandiol (Ro 05-9963) (II); 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269) (III); and 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913) (IV). The structural formulae of these compounds are shown in Fig. 1.



R = OCH_3	Ro 07-0582 (misonidazole)	I
R = OH	Ro 05-9963	II
R = Cl	Ro 07-0269	III
R = OCH_2CH_3	Ro 07-0913	IV

Fig. 1. Structural formulae of nitroimidazoles.

HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain). Water was twice-distilled in glass and both solvents were passed through appropriate 0.45 μm Millipore filters and de-aerated under vacuum before use.

Sample preparation

The following procedure was found to be suitable for samples of urine, tissue homogenate (10—20%, w/v) and heparinised blood plasma. The sample was treated with 9 vol. methanol containing the internal standard Ro 07-0269 (11.1 μg/ml) and mixed thoroughly. After centrifugation (2000 *g*, 10 min) the super-

natant was removed for HPLC analysis. Alternatively, the plasma was deproteinated by passage through Amicon CF50A filter cones at 1000 *g* (60 min). The ultrafiltrate was then diluted with methanol to the concentration of the eluant prior to chromatography. Standards, prepared by adding known amounts of misonidazole and Ro 05-9963 in methanol to plasma collected before drug administration, were analysed as for experimental samples.

Chromatography

Samples (10 μl) of the methanolic extracts were chromatographed at ambient temperature using a Waters Model ALC/GPC-244 liquid chromatograph equipped with a U6K sample loop injector and a μBondapak C_{18} (octadecylsilane) column (30 cm × 3.9 mm I.D., particle size = 10 μm) (Waters Assoc., Milford, Mass., U.S.A.). The mobile phase, consisting of 19% methanol—water, was delivered at a constant flow-rate of 2 ml/min (pressure = 2000 p.s.i.). The column effluent was monitored at 313 nm using a Waters Model 440 (absorbance detector coupled to a Servoscribe chart recorder. Peak areas were determined using a Varian CDS 111 computer.

RESULTS

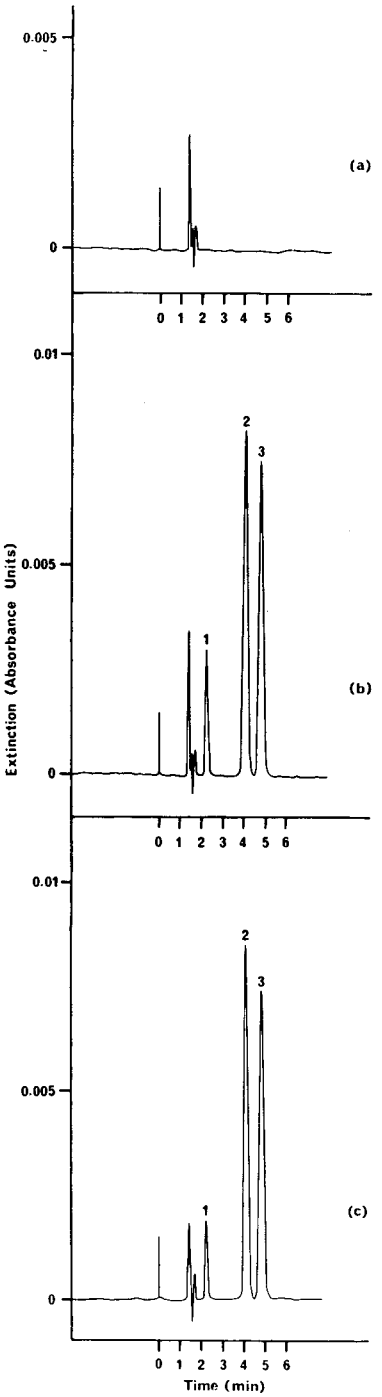
Fig. 2a shows a typical chromatogram of a methanol extract of blood plasma taken from a cerebral tumour patient (CA) immediately before administration of misonidazole. The internal standard was deliberately omitted. The chromatogram illustrated in Fig. 2b is of a methanol extract of the same plasma spiked with misonidazole (peak 2) and Ro 05-9963 (peak 1) and containing the internal standard Ro 07-0269 (peak 3). Ro 05-9963, misonidazole and Ro 07-0269 had retention times of 2.3 min, 4.1 min and 4.8 min respectively. The three peaks were completely resolved from each other and from material eluting at the solvent front. Typical height equivalents to a theoretical plate for misonidazole, Ro 05-9963 and Ro 07-0269 were respectively 0.15 mm, 0.25 mm and 0.15 mm.

Comparison of Fig. 2a and b shows that the blank plasma contained no components capable of interfering with peaks 1—3. Similar results were obtained for extracts of control urine and tissue homogenate.

Fig. 2c shows a chromatogram of the methanol extract of plasma taken from the same patient 2 h after the administration of misonidazole (3 g/m^2). It may be seen that the chromatogram contains peaks corresponding to misonidazole and Ro 05-9963.

The efficiency of recovery of misonidazole, Ro 05-9963 and Ro 07-0269 from biological media after protein precipitation was always >95%. Plots of normalised peak area ratio (peak area:peak area internal standard) against concentration were linear over the concentration ranges studied (2—1000 μg/ml misonidazole; 2—50 μg/ml Ro 05-9963) and had zero intercepts. Plots of peak height ratio were also linear. The coefficient of variation calculated for ten replicate analyses (peak height ratio) was 2.9% for misonidazole and 1.9% for Ro 05-9963.

Allowing a minimum signal-to-noise ratio of two, the lower limit of sensitivity of the protein precipitation method was approximately 5 μg/ml sample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(a)
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0.005
0
1
2
3
(b)
0 1 2 3 4 5 6
0.01
0.005
0
1
2
3
(c)
0 1 2 3 4 5 6
Time (min)
Extinction (Absorbance Units)

(plasma, urine or tissue homogenate) for misonidazole and 2 μg/ml for the O-demethylated metabolite. This represents an on-column injection of 5 ng and 2 ng respectively.

In some studies Ro 07-0913 was used as the internal standard. This compound had a retention time of 7.3 min and was completely resolved from the other nitroimidazoles. To check the precision of the HPLC method some test samples were analysed by both HPLC and GLC [9]. Good agreement was observed for both misonidazole and Ro 05-9963.

An application of the present HPLC technique is demonstrated in Fig. 3

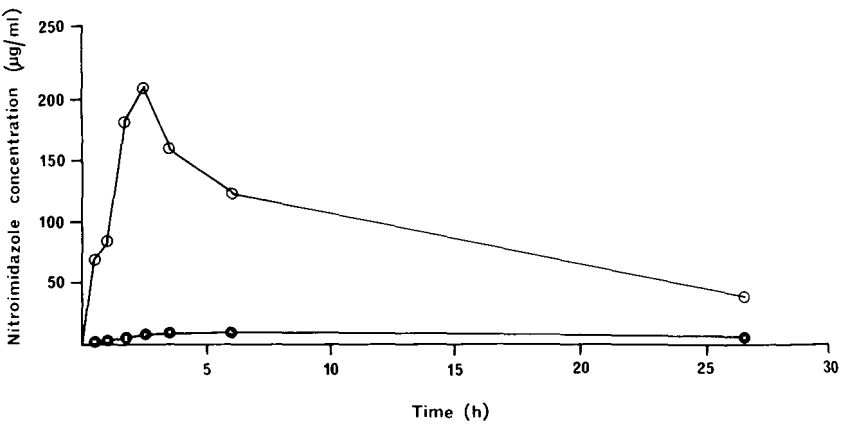

Fig. 3. Plasma time—course of misonidazole (○) and Ro 05-9963 (●) for a patient with a cerebral glioma after administration of 3 g/m² misonidazole orally. This patient exhibited abnormally high plasma levels of misonidazole.

which shows the plasma time-course of misonidazole and Ro 05-9963 for a cerebral tumour patient (EI) receiving 3 g/m² misonidazole orally. This patient exhibited abnormally high plasma levels of misonidazole, thus illustrating the importance of drug monitoring. It may also be seen that the O-demethylated metabolite constitutes a small, but not insignificant, proportion of the total plasma nitroimidazole concentration.

DISCUSSION

The rapid HPLC technique described in the present paper has several advantages. Firstly, unlike previous spectrophotometric [6] and polarographic [7] techniques, the method is specific for both misonidazole and its O-demethylated metabolite Ro 05-9963. Secondly, unlike the reported GLC technique [9]

Fig. 2. HPLC of methanol extracts of blood plasma from a patient (CA) with a cerebral glioma. Chromatographic conditions: column, μ Bondapak C_{18} (20 cm × 3.9 mm I.D.; particle size = 10 μm); mobile phase, methanol—water (19:81); flow-rate, 2ml/min; column pressure, 2000 p.s.i.; temperature, ambient; detection, absorbance at 313 nm; sample volume, 10 μl. (a) Sample taken immediately before administration of misonidazole. Internal standard was omitted. (b) Same sample as (a) but spiked with Ro 05-9963 (peak 1, 20 μg/ml plasma) and misonidazole (peak 2, 100 μg/ml plasma). Peak 3 corresponds to the internal standard (11.1 μg/ml methanol). (c) Sample taken 2 h after administration of 3 g/m² misonidazole orally.

it requires only one simple protein precipitation step and no derivatisation prior to analysis. Thirdly, unlike the previous paper chromatography and TLC methods [8, 9], it lends itself readily to accurate quantitation on a routine basis. Finally, the sensitivity of the method is comparable with, or better than, these alternative techniques. This is adequate for the assay of misonidazole and Ro 05-9963 following the administration of therapeutic doses of misonidazole. Furthermore, the sensitivity of the method could be improved by concentration of the methanol extract or by using the Amicon filter cones.

The HPLC method is currently being employed to determine the levels of misonidazole and Ro 05-9963 in tissues and body fluids of patients and experimental animals. Digestion with β-glucuronidase has also allowed the estimation of glucuronide derivatives of these compounds in urine. The present method does not, however, allow the estimation of the nitro-reduction product 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol (Ro 07-7273) which has been identified as a metabolite [8, 9]. This compound does not possess the UV absorption properties of the nitroimidazoles and we are currently evaluating various alternative methods for its detection.

The HPLC method described here has also proved useful for the assay of various other nitroimidazoles including the 5-nitroimidazole, metronidazole (2-(2-methyl-5-nitro-1-imidazolyl)-ethanol, Flagyl, May and Baker), used clinically both as an anti-trichomonal agent and as a hypoxic cell-sensitiser.

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Biomedical Applications

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Book Review

Chromatographic and electrophoretic techniques, Vol. II, Zone electrophoresis, edited by I. Smith, William Heinemann, London, 4th ed., 1976, IX + 485 pp., price £ 9.00, ISBN 0-433-30505-5.

This book is called the fourth edition, to put it in line with its companion volume, but it is preceded by only two editions.

In the new version of the whole two-volume treatise, the chapter on the "Biobit" models of peptides, proteins and nucleic acids (48 pages) has been updated and moved from the chromatographic volume to the electrophoretic volume. Thus the electrophoretists will follow the chromatographers in getting some knowledge of this visual and tactile educational aid.

The total number of pages of the new edition has nevertheless been reduced from 524 to 485. Material included in 4 new chapters has been more than balanced by the exclusion of many of the clinical applications. This is justified, as the emphasis is on techniques.

Only 8 of the 23 former co-authors have been retained: A. Feinstein, W.B. Gratzer, J. Kohn, E.G. Richards, W.J. Ritschard, C.R. Shaw, A.L. Tárnoky (shortening his á into a in the list of authors) and Jacqueline B. Weiss. Nine new names have been added: D. Beale, A. Brownstone, C.F. Doré (both from NIMR), D. Leaback, J.W. Payne, M. Siciliano, Margaret J. Smith, J.M.B. Versey and C.W. Wrigley. As in the preceding edition, the authors of minor sections do not appear in the list of co-authors.

Ivor Smith's sections on general principles and on general techniques for low-voltage paper electrophoresis and starch-gel electrophoresis, Feinstein's on agar-gel electrophoresis and immunoelectrophoresis (practically limited to the latter) and Weiss' on preparative block electrophoresis are only slightly changed. Some 50 pages which had been devoted to the separation of various high- and low-molecular solutes by low-voltage paper electrophoresis, including clinical interpretation of the serum-protein patterns, have been left out. This will make the preceding edition still useful side by side with the new one. The same can be said for the omitted 79 pages on the application of starch-gel electrophoresis (serum and urine proteins, haemoglobins). The chapter on high-voltage paper electrophoresis has been completely re-written; examples of the separation of amino acids and partial protein hydrolysates ("fingerprints") abound. Ritschard's chapter on thin-layer electrophoresis has been supplemented by a five-page appendix on fluorescamine. The authoritative

treatise on celluloseacetate membrane electrophoresis by its inventor, Kohn, has followed the line of the previous version, but the bibliography has been reduced from 9 pages to 1. The chapter on starch-gel zymograms has been modernized and given a more general title "Separation and visualization of enzymes on gels" (Shaw and Siciliano).

Polyacrylamide disc gel electrophoresis is enriched by the description of gels which can be solubilized; among the applications, milk and CSF proteins have been omitted, but serum proteins (Tárnoky) have survived.

Excellent new chapters have been added: Quantitative immunoelectrophoresis (Versey, 20 pages), concentration gradient polyacrylamide gel electrophoresis (Leaback, 12 pages), isoelectric focusing of proteins (Leaback and Wrigley, 49 pages — with emphasis on density-gradient columns, but also describing gel media), and electrophoresis of proteins on SDS—polyacrylamide gels (Payne, 36 pages). Flat slab (polyacrylamide) gel electrophoresis, except for the latter three modifications, has been dropped. The chapter on preparative acrylamide disc electrophoresis is still divided into two articles (by I. Smith and, for higher loads, by Brownstone). Electrophoresis of RNA has been thoroughly revised by Gratzer and Richards; formamide gels are among the innovations.

On the whole, the exclusion of some parts which could hardly have been updated without enlarging them (discussion of clinical significance) and the inclusion of new chapters (gradient gel, focusing) have refreshed the book. Some outdated techniques, such as paper electrophoresis of lipoproteins, have remained. In some respects the book lags behind the state of the art. Agarose gels, which Fredrickson's studies have made popular for the separation of lipoproteins according to Noble, are not mentioned, except for quantitative immunoelectrophoresis, the separation of RNA, and mixed polyacrylamide—agarose gels. The "cellogel" family of gels which, unlike cellulose acetate membranes, allows the staining of lipoproteins by fat dyes has also remained outside the scope of the book. In practice, this staining is not quantitatively inferior to that of Kohn's ozone—Schiff reagent method which is rightly recommended in the book for cellulose acetate membranes.

Lucid style, wealth of illustrations, great attention to relevant detail are among the prominent features of this and the preceding editions.

The design of the dust-cover is more elegant than that of Edition 2. The quality of paper and illustrations is also higher. Some of the new figures (reprinted from advertizing pamphlets) show the Biobit models in colour; they are too small and diffuse to be fully appreciated. Fig. 1.1, though a line-drawing, has been printed as a half-tone picture.

The running titles well serve the purpose of confusing the readers. Thus, e.g., concentration gradient PAGE is called "polyacrylamide gel electrophoresis", isoelectric focusing becomes "electrophoresis of proteins", but switches over arbitrarily to "focusing of proteins"; "electrophoresis of proteins" then reappears to accompany the chapter on SDS—PAGE. Preparative acrylamide disc electrophoresis becomes "acrylamide disc electrophoresis"; models for macromolecules become "macromolecules".

Hradec Králové (Czechoslovakia) Z. ZADÁK and I.M. HAIS

Author Index

Subject Index

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
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