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Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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

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or to:
The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands.
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CHROMBIO. 391

TRENNUNG UND CHARAKTERISIERUNG SAURER HARNBESTANDTEILE

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(Eingegangen am 23. April 1979)

SUMMARY

Separation and characterization of acidic urine constituents

The acidic compounds of urine were separated by thin-layer chromatography in eight fractions. Each fraction was investigated separately by the combination glass capillary gas chromatography—mass spectrometry. About 500 compounds were detected, 2/5 of these could be characterized by their mass spectra. Retention data and key fragments of the mass spectra were tabulated. Many of the detected compounds are still unknown.

EINLEITUNG

Endprodukte physiologischer und pathologischer Stoffwechselprodukte sind vielfach Säuren, die durch Aufnahme von "Profilen" gaschromatographisch fassbar sind [1—14] . Da einige Säuren hohe Wasserlöslichkeit besitzen, ist eine direkte Analyse der Gesamtfraktion, z.B. durch Ausschütteln mit organischen Lösungsmitteln und anschliessender Gaschromatographie (GC)-Analyse, nicht möglich. Zweckmässigerweise wird die Gesamtsäurefraktion enzymatisch hydrolysiert, dann adsorbiert man die Säuren an Ionenaustauschern und überführt sie nach Elution mit Diazomethan in die Methylester, um sie so als flüchtige Derivate gaschromatographisch trennen zu können. Da die enzymatische Hydrolyse im wesentlichen dazu dient, phenolische Säuren, die als Glucuronide und Sulfate vorliegen können, zu spalten, kann man, wenn man diese nicht vollständig erfassen will, auf den Schritt der enzymatischen Hydrolyse verzichten. Teilweise werden bei diesem Verfahren auch Phenole herausgeholt, doch stören diese nicht sehr.

Mit Hilfe der Gaschromatographie lässt sich zwar eine weitgehende Trennung der komplexen Säurefraktion erzielen, doch gelingt diese selbst unter Verwendung von Glaskapillarsäulen nicht vollständig: Massenspektren, die von einheitlich erscheinenden GC-Peaks aufgenommen werden, zeigen oft die

Überlagerung mehrerer Komponenten. Dies macht selbst eine halbquantitative Auswertung der Gaschromatogramme problematisch und erschwert vor allem die Strukturbestimmung der zahlreichen uns noch unbekannten Harnbestandteile. Deshalb ist eine Vortrennung des Säuregemisches — etwa durch Chromatographie an Dünnschichtplatten — für eine nachfolgende Identifizierung in der Kombination GC—Massenspektrometrie (MS) notwendig.

Ziel unserer Arbeiten ist es, diese unbekannten Verbindungen in ihrer Struktur aufzuklären, ihre physiologische Bedeutung abzuklären und durch Kenntnis ihrer Massenspektren die Voraussetzungen für eine quantitative Erfassung mit der multiple ion detection (MID)-Methode zu schaffen.

Um besseren Einblick in das komplexe Säuregemisch erhalten zu können, trennten wir die Säurefraktion dünnschichtchromatographisch in einzelne Zonen auf und untersuchten dann diese mit Hilfe der Kombination Glaskapillargaschromatographie—Massenspektrometrie. Interessante Verbindungen wurden durch präparative GC soweit angereichert, dass zur Aufnahme von Massenspektren mit Hochauflösung durch die peak-matching Technik zur Bestimmung von Bruttoformeln ausreichende Probenmengen zur Verfügung standen. Liess sich trotz Kenntnis der Bruttoformel eine Struktur nicht bestimmen, wurden Proben zu Messungen von anderen spektroskopischen Daten und für Abbaureaktionen im Mikromassstab am präparativen Gaschromatographen gesammelt.

AUFARBEITUNG

Harnproben wurden in frisch gereinigten und mit aqua dest. ausgespülten Polyethylenflaschen gesammelt und entweder sofort weiterverarbeitet oder bei —25° gelagert. (Bereits nach kurzer Lagerung bei Raumtemperatur beobachtete man die Zersetzung von Aminosäurekonjugaten; als Indikator für Zersetzung wurde das Auftreten von Benzoesäure gewertet, die in frischem Harn nur in Spuren oder gar nicht vorhanden ist.)

Während für die Aufnahme einzelner Übersichtschromatogramme die Aufarbeitung von 5—10 ml Urin genügt, wurden zur Identifizierung von Nebenbestandteilen durch Dünnschichtchromatographie und zur Substanzisolierung jeweils mehrere Portionen von je 800 ml Urin aufgetrennt.

Da die für die Spaltung der Konjugate einzusetzenden Enzyme durch Harnbestandteile gehemmt werden, wurden diese und vorhandene Salze zunächst durch Überlaufen über eine XAD 4 Säule entfernt. Beim Nachwaschen der Säule mit Wasser werden nach Elution der Salze auch stark polare Stoffe (z.B. Zitronensäure) von der Säule mit heruntergewaschen. Deshalb müssen — wenn das Salz entfernt ist — die angegebenen Lösungsmittel-Volumina eingehalten werden.

Zur Entsalzung des Urins wird ein Aliquot von 10 ml mit 2 *N* HCl auf pH 1 eingestellt und auf eine mit XAD 4 beschickte Säule gebracht. Die Glassäule hat einen Durchmesser von 1 cm, die Länge beträgt 80 cm, am Auslauf befindet sich ein silanisierter Glaswollepfropfen. Die Säule ist mit einem Teflonhahn bestückt. Das Bettvolumen beträgt 40 ml, das entspricht einer Füllhöhe von 50 cm. Die Säule wird vor Gebrauch mit 50 ml 5% Natriumchlorid-Lösung gewaschen.

Die Adsorptionsgeschwindigkeit für die Urinprobe wird so gewählt, dass eine Durchlaufrate von 0.5 ml/min eingehalten werden kann. Danach wird portionsweise so lange mit Wasser (2 ml/min) nachgewaschen, bis am Auslauf der Säule kein Chlorid mehr nachgewiesen werden kann. Die zurückgehaltenen Stoffe werden mit 150 ml über Weinsäure destilliertem Methanol mit einer Tropfgeschwindigkeit von 3 ml/min eluiert. Sowohl die wässrige Phase des Urins als auch das nachgespülte Wasser werden getrennt aufgefangen. Zur Verbesserung des Adsorptionsergebnisses wird die wässrige Phase noch einmal über die Säule geschickt. Das Methanoleluat wird dem ersten hinzugefügt. Zur Erfassung von leichter flüchtigen Säuren kann mit Ether statt mit Methanol eluiert werden, da beim anschliessenden Eindampfen des Ethereluats am Rotationsverdampfer geringere Verluste an leicht flüchtigen Komponenten auftreten als bei Verwendung des schwerer flüchtigen Methanols. Hierbei wird die Säule vor dem Eluieren trocken gesaugt und nach Zugabe einer kleinen Menge Ether so lange geschüttelt, bis alle Luftblasen verschwunden sind. Um beim Schütteln keine Verunreinigungen einzuschleppen, ist es günstig, Säulen zu verwenden, die mit einem Schliffstopfen verschliessbar sind.

Der Extrakt wird in 30 ml 0.5 *M* Natriumacetatpuffer (41 g Natriumacetat + 30 ml Eisessig auf 1 l Wasser) aufgenommen und mit 0.1 ml Helicase (0.052 U β-Glucuronidase, 0.026 U Arylsulfatase pro ml) unter einem Stickstoffstrom drei Tage bei 37° im Wasserbad inkubiert. Die Abtrennung des Enzyms erfolgt wiederum über XAD 4 in der geschilderten Weise.

Zur Abtrennung der Neutralteile und Basen wird die methanolische bzw. etherische Lösung auf einen Ionenaustauscher (Amberlite A-26, Serva, Heidelberg, B.R.D.) gebracht. Die Tropfgeschwindigkeit beträgt 0.5 ml/min. Der Ionenaustauscher wird vor der Benutzung mit 0.1 *N* HCl und 0.1 *N* NaOH regeneriert. Für 10 ml Urin reichen 10 cm^3 Amberlite A-26 in der OH^--Form in methanolischer bzw. etherischer Lösung aus. Die Neutralstoffe und Basen werden mit 50 ml Methanol entfernt und die Säuren dann mit 100 ml Ether bzw. Methanol, das mit HCl-Gas bis zu einer 0.1 *N* Lösung angereichert ist, eluiert. Während der Elution wird der pH-Wert am Auslauf der Säule mit Pyridin auf pH 5 eingestellt.

Zur Aufarbeitung grösserer Urinmengen wird in etwas abgeänderter Weise vorgegangen: 800—1000 ml Urin werden mit 6 *N* HCl auf pH 1 eingestellt und mit 500 g XAD 4 in einem 2-Liter Rundkolben am Rotationsverdampfer für 2 h bei 0°gerührt. In einer geeigneten Glassäule werden die Salze wie oben beschrieben abgetrennt und die Neutralteile und Säuren mit 1.5 l Methanol eluiert. Der eingedampfte Extrakt wird mit 1 ml Helicase versetzt und bei pH 4.5 drei Tage inkubiert. Das Enzym wird wie oben beschrieben abgetrennt und die methanolische Lösung mit 70 ml Ionenaustauscher Amberlite A-26 auf Säuren aufgearbeitet. Zur Elution werden 1.5 l methanolischer 0.1 *N* HCl benötigt. Das Lösungsmittel wird vorsichtig am Rotationsverdampfer abgezogen und der Rückstand im Kühlschrank bis zur Derivatisierung aufbewahrt.

DERIVATISIERUNG

Die Methylierung wird mit Diazomethan [1, 3] ausgeführt. Dazu wird die

methanolische Lösung mit so viel Diazomethan in etherischer Lösung übergossen, bis die gelbe Farbe bestehen bleibt. Nach 30 Sekunden wird vorsichtig unter einem Stickstoffstrom eingedampft. Zur Methylierung von phenolischer OH-Gruppen lässt man die Diazomethanlösung im grossen Überschuss ca. 12 h bei 5° einwirken.

DÜNNSCHICHTCHROMATOGRAPHIE

Eine verdünnte Lösung der Methylester wurde entweder auf eine selbst präparierte Dünnschichtplatte 20 × 20 cm, 1 mm Schichtdicke (Kieselgel 60 $HF_{254+366}$) oder auf eine Merck Fertigplatte (Kieselgel 60 $F_{254+366}$) aufgetragen und in Ether—Cyclohexan (4:5) zweimal chromatographiert.

Die Dünnschichtplatten wurden unter UV-Licht bei 366 nm in 8 Zonen eingeteilt:

Zone	R_F-Wert
DC 0	0.96—0.87
DC 1	0.87—0.77
DC 2	0.77—0.66
DC 3	0.66—0.56
DC 4	0.56—0.46
DC 5	0.46—0.29
DC 6	0.29—0.21
DC 7	0.21—0.04

Die Zonen wurden einzeln von der Platte isoliert und das Kieselgel im Soxhlet mit Methanol 5 h extrahiert.

Zur Isolierung von Reinsubstanzen wurden die Fraktionen DC 0 bis DC 7 entweder mehrfach dünnschichtchromatographisch aufgetrennt oder die gesuchte Komponente über einen präparativen Gaschromatographen angereichert. In manchen Fällen wurden beide Trennverfahren kombiniert.

AUFNAHMEBEDINGUNGEN DER MASSENSPEKTREN UND GASCHROMATOGRAMME

Massenspektrometrie—Gaschromatographie

LKB-2091 Gerät mit getrennten Öldiffusionspumpen (150 l/sec Saugleistung) für Quelle und Einlass. E.I.-Ionenquelle, 250°, Elektronenenergie 70 eV, Beschleunigungsspannung 3.5 kV, TIC-Signal bei 20 eV registriert. Separator: 2-stufiger Molekül-Jet-Separator (nach Becker-Ryhage) und "sliding valve" zur Trennung von GC- und MS-Teil, Temperatur 250°.

Gaschromatograph: Pye-Unicam Ein-Säulengerät, Temperaturprogramm: 2°/min, Säule: Dünnfilm-Glaskapillarsäule 25 m, OV-101, Trägergas: Helium (2 ml/min).

Schreiber: UV-Schreiber 1600 Hz mit drei Empfindlichkeitsspuren 1:10:100, oder Potentiometer-Schreiber LKB-Biocal 2066; Bereich 100 mV, Papiervorschub 0.5 cm/min; Papierbreite 25 cm.

Datensystem: LKB 2130, PDP-11 Rechner (16-bit Memory) mit Disk-System der Firma Digital Equipment Corporation, Sichtschirm Tektronix 4012 und Versatec-Plotter.

Gaschromatographie

Carlo Erba Modell 2301; Doppelsäulengerät mit Flammenionisationsdetektor. Trägergas: Wasserstoff (2 ml/min). Säule: 25 m OV-101 Dünnfilmglaskapillarsäule mit Platinkapillaranschluss. Injizierte Menge: 0.2—0.8 µl, Injektor-Temperatur: 275°, Detektor-Temperatur: 275°, Ofen-Temperatur: 75°, 7 min isotherm, Temperaturprogramm: 2°/min bis 280°; Splitverhältnis: 1:20, Abschwächer (attenuator): 32.

Präparativer Gaschromatograph: Carlo Erba Fractovap 2400 T. Säule: 1.5 m, 6 mm Durchmesser, gefüllt mit 3% SE-30 auf Supelcoport (80—100 mesh). Injizierte Menge: 30 µl, Injektor-Temperatur: 275°, Detektor-Temperatur: 275°, Ofen-Temperatur: 100°, Temperaturprogramm: 100°, 2 min isotherm, 3°/min bis 270°; Splitverhältnis: 1:100, Abschwächer (attenuator): 64.

ERGEBNISSE

Fig. 1 zeigt das Glaskapillargaschromatogramm einer aus Harn isolierten Gesamtfraktion nach dem Umsatz mit Diazomethan. Die angezeigten Komponenten wurden — soweit sie durch Massenspektren erfasst werden konnten — nach steigenden Retentionsindices durchnummeriert. Retentionsindices, Molekulargewicht und Schlüsselionen (Intensitätswert in Klammern) sind in Tabelle I zusammengestellt. Die Tabelle enthält ferner — soweit bekannt — die vollständige oder teilweise Struktur und Summenformel sowie Literaturangaben. Strukturen, die durch Vergleichsmessungen eindeutig abgesichert wurden, sind durch Sternchen gekennzeichnet, vermutete Strukturen wurden mit einem Fragezeichen versehen. Das Vorliegen von Gemischen ist, soweit dies erkennbar ist, ebenfalls vermerkt.

Im Gaschromatogramm der Gesamtfraktion sind viele in geringer Menge vorliegende Verbindungen von anderen überlagert, so dass sie nicht auffindbar sind. Sie erscheinen aber, wenn man die auf einer Dünnschichtplatte getrennte Säurefraktion isoliert und in gleicher Weise wie die Hauptfraktion untersucht.

In der ersten und zweiten Zone (Fig. 2 und 3) DC 0 und DC 1, sind hauptsächlich Dicarbonsäuren, Cresole, die Pentylurofuransäure und andere wenig polare Verbindungen angereichert. Die Zonen drei und vier (Fig. 4 und 5) DC 2 und DC 3, enthalten vorwiegend aromatische und heterocyclische Säuren und nicht abgetrennte Phenole. In den Zonen fünf und sechs (Fig. 6 und 7) DC 4 und DC 5, findet man vorzugsweise Hydroxysäuren, Citronensäure und Hippursäure, während in den Zonen sieben und acht (Fig. 8 und 9) DC 6 und DC 7, Aminosäurekonjugate überwiegen.

Über die Strukturuntersuchung der einzelnen Verbindungsklassen wird an anderer Stelle berichtet [64].

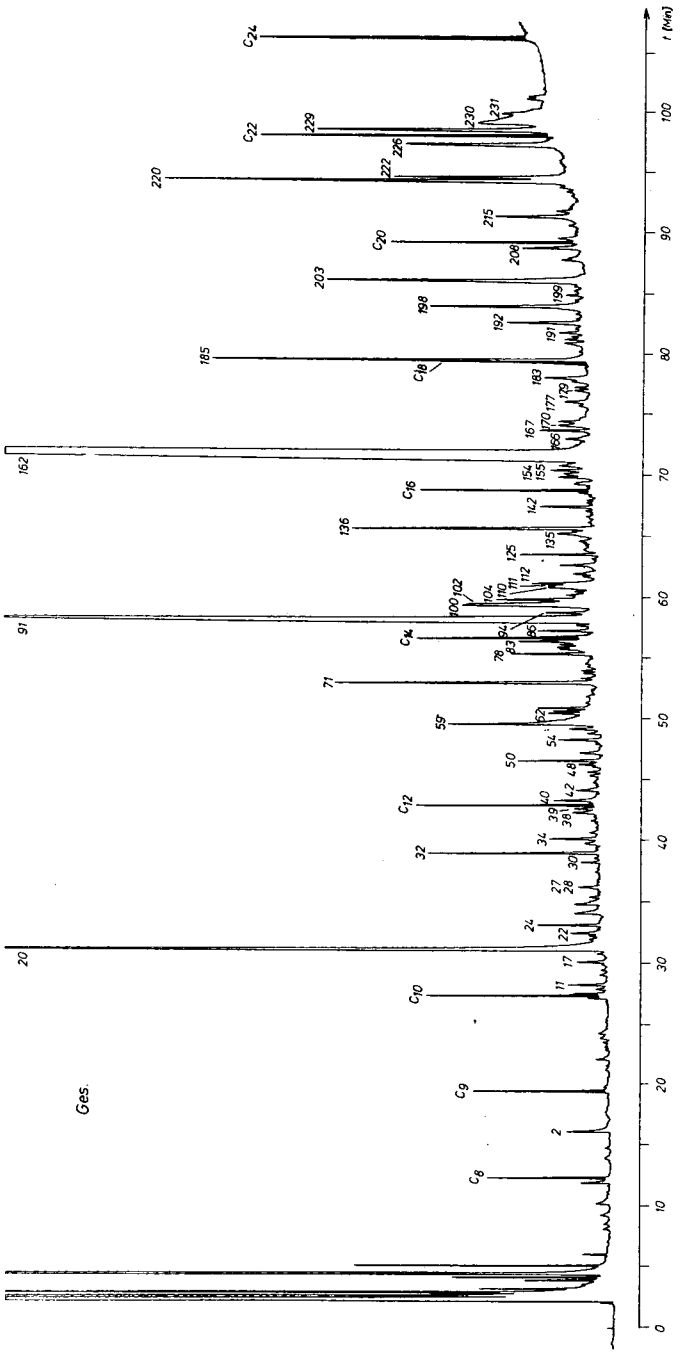


Fig. 1. Glaskapillargaschromatogramm einer aus Harn gewonnenen Säurefraktion in Form der Methylester.

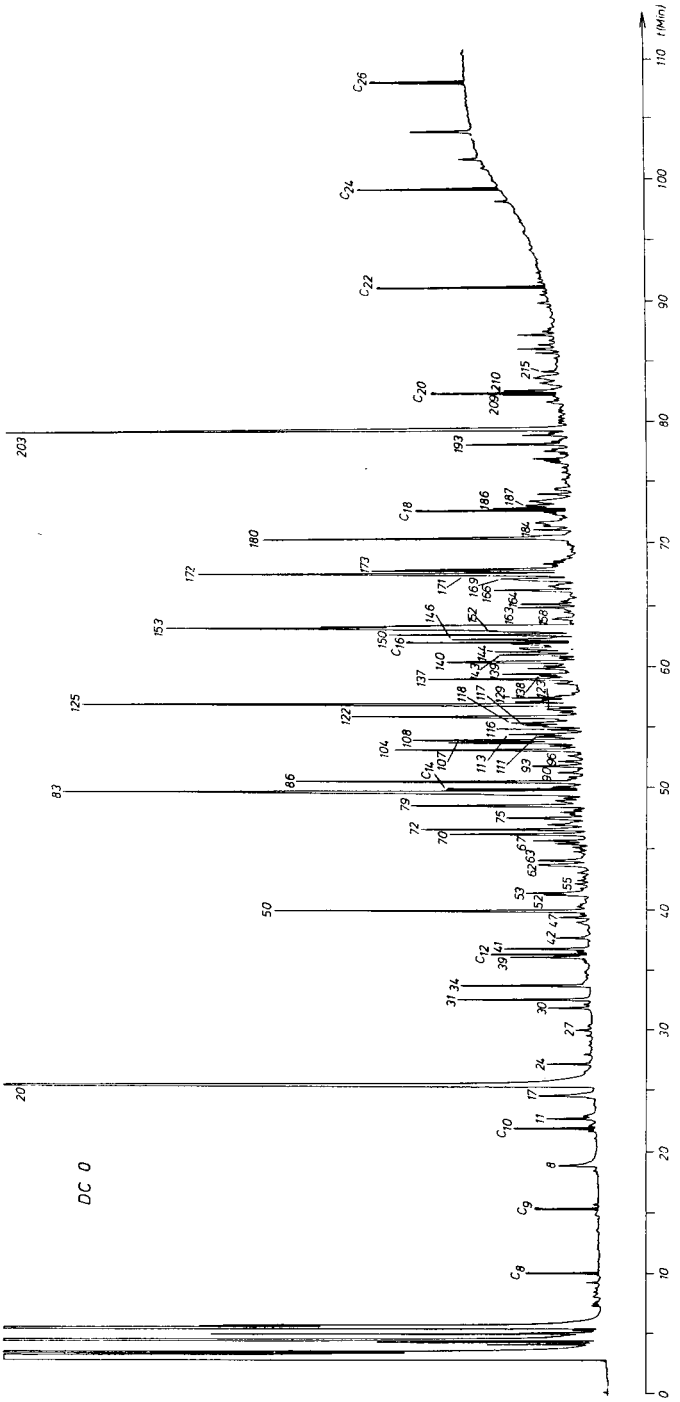


Fig. 2. Glaskapillargaschromatogramm der Fraktion DC 0.

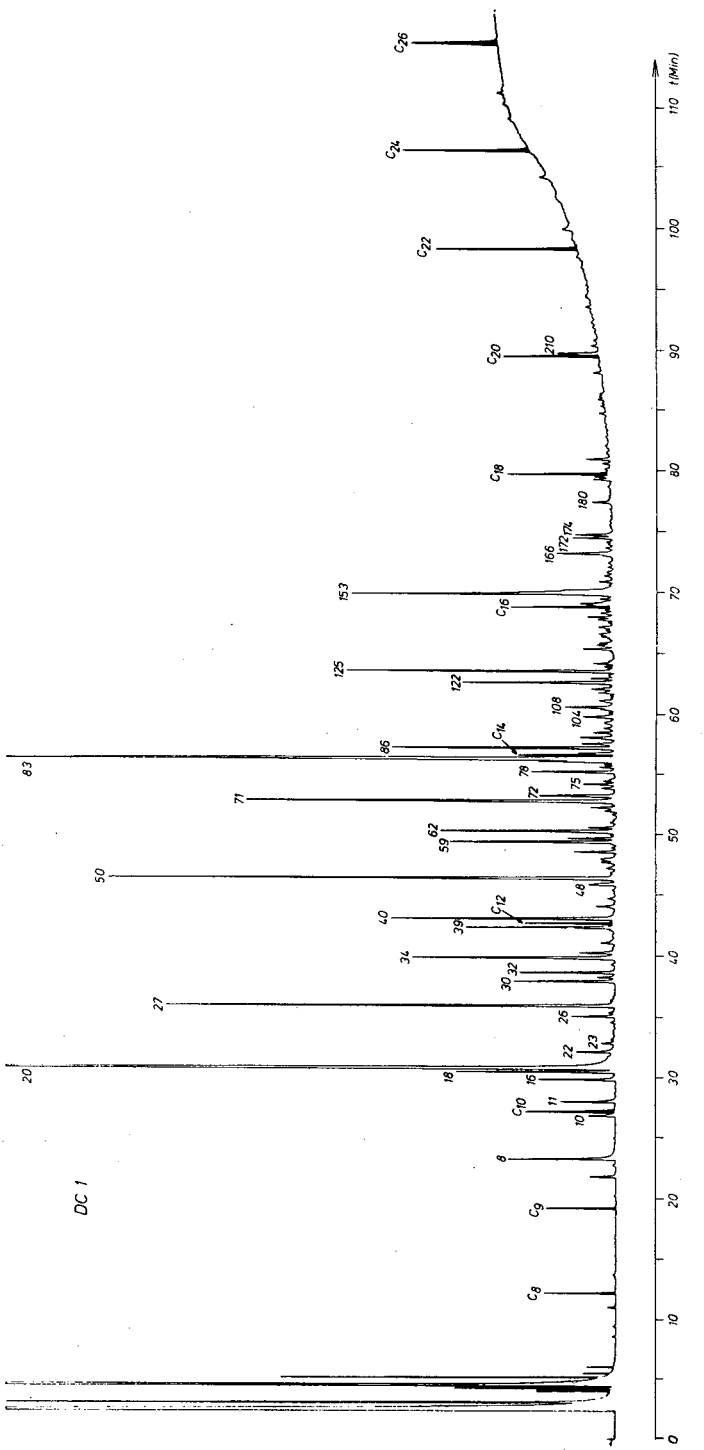


Fig. 3. Glaskapillargaschromatogramm der Fraktion DC 1.

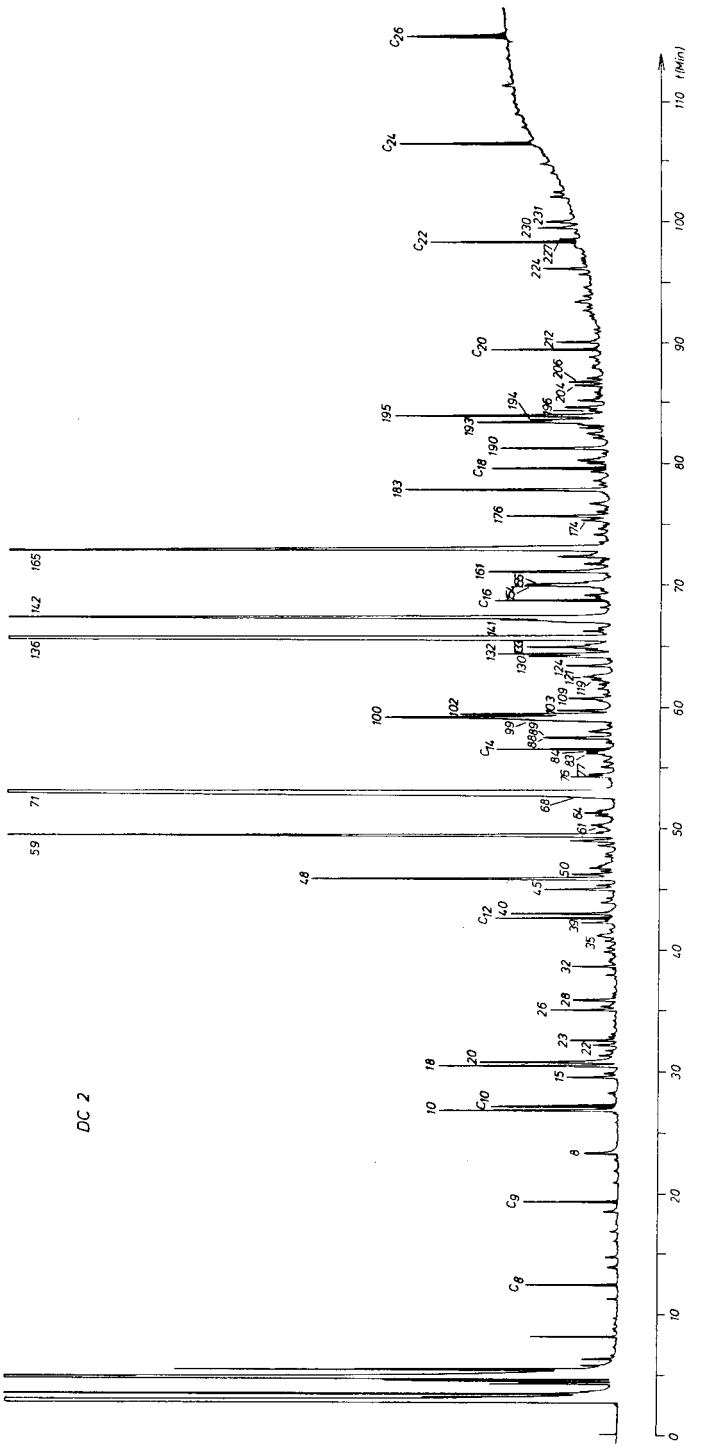


Fig. 4. Glaskapillargaschromatogramm der Fraktion DC 2.

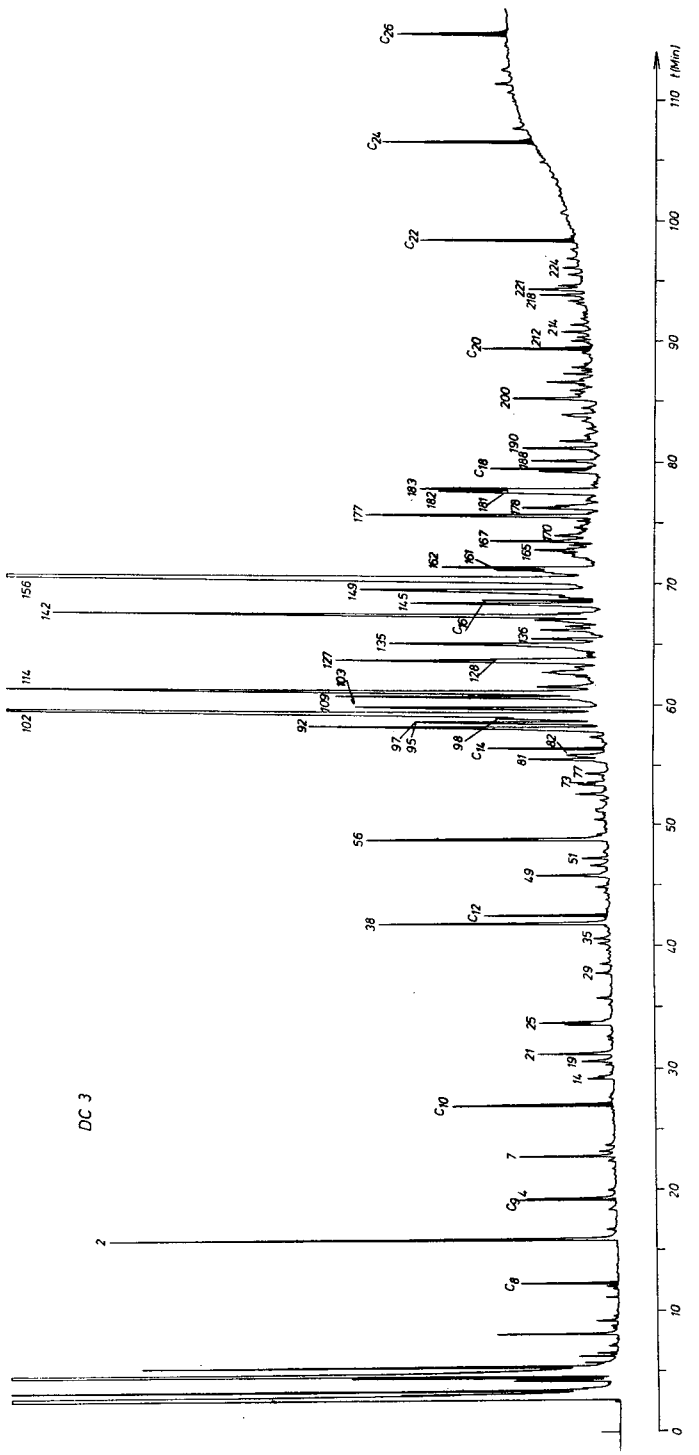


Fig. 5. Glaskapillargaschromatogramm der Fraktion DC 3.

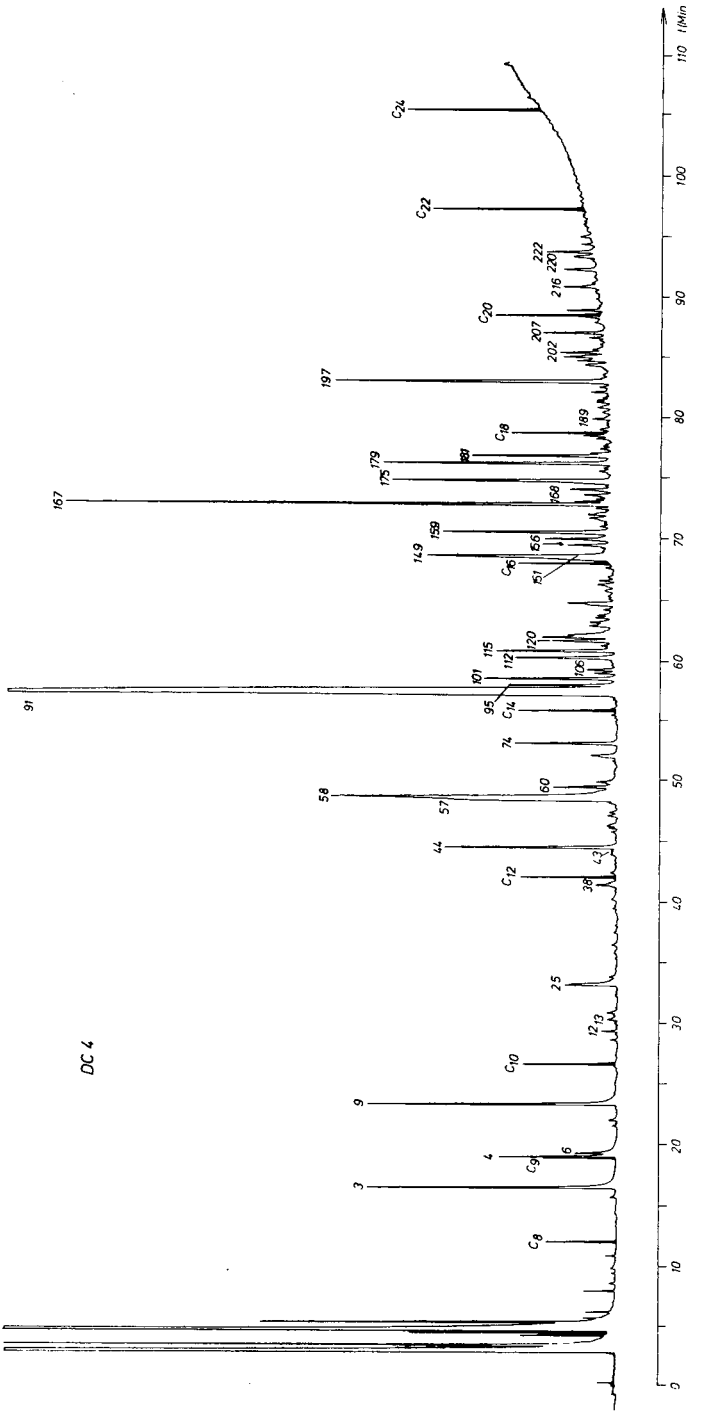


Fig. 6. Glaskapillargaschromatogramm der Fraktion DC 4.

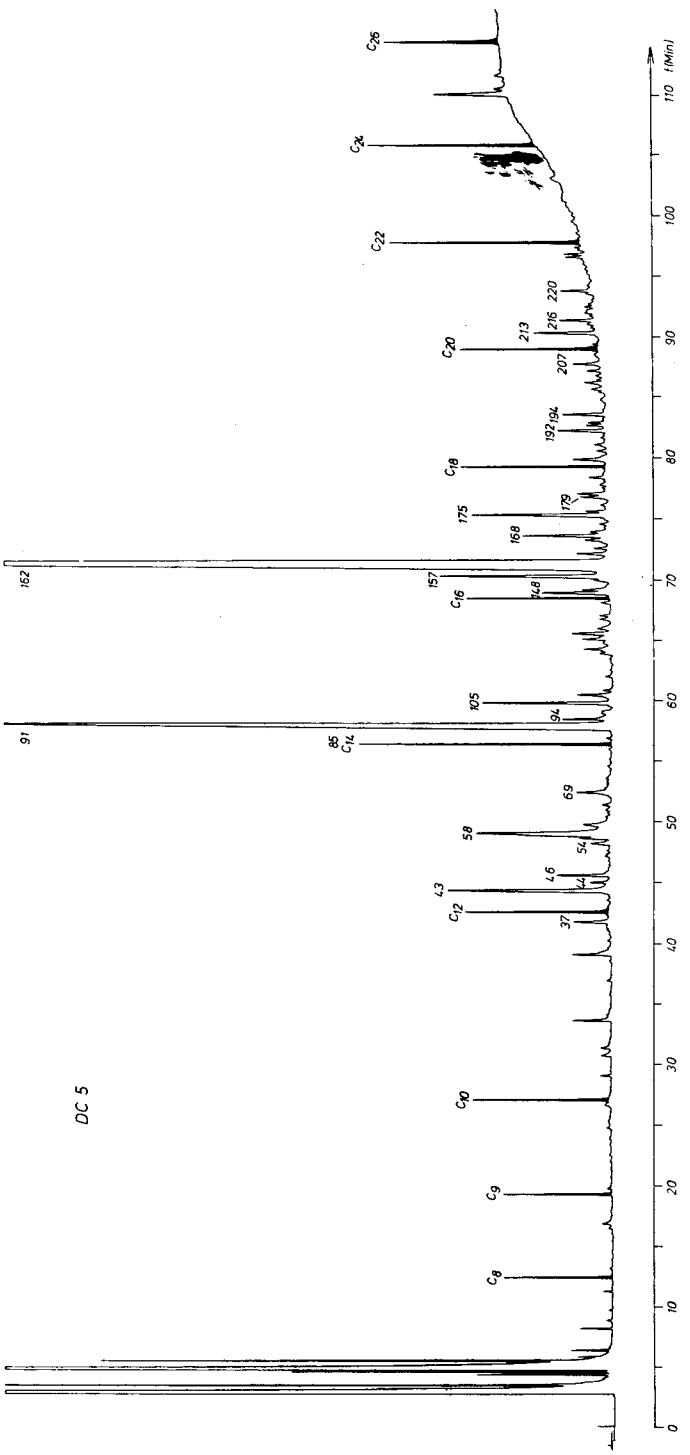
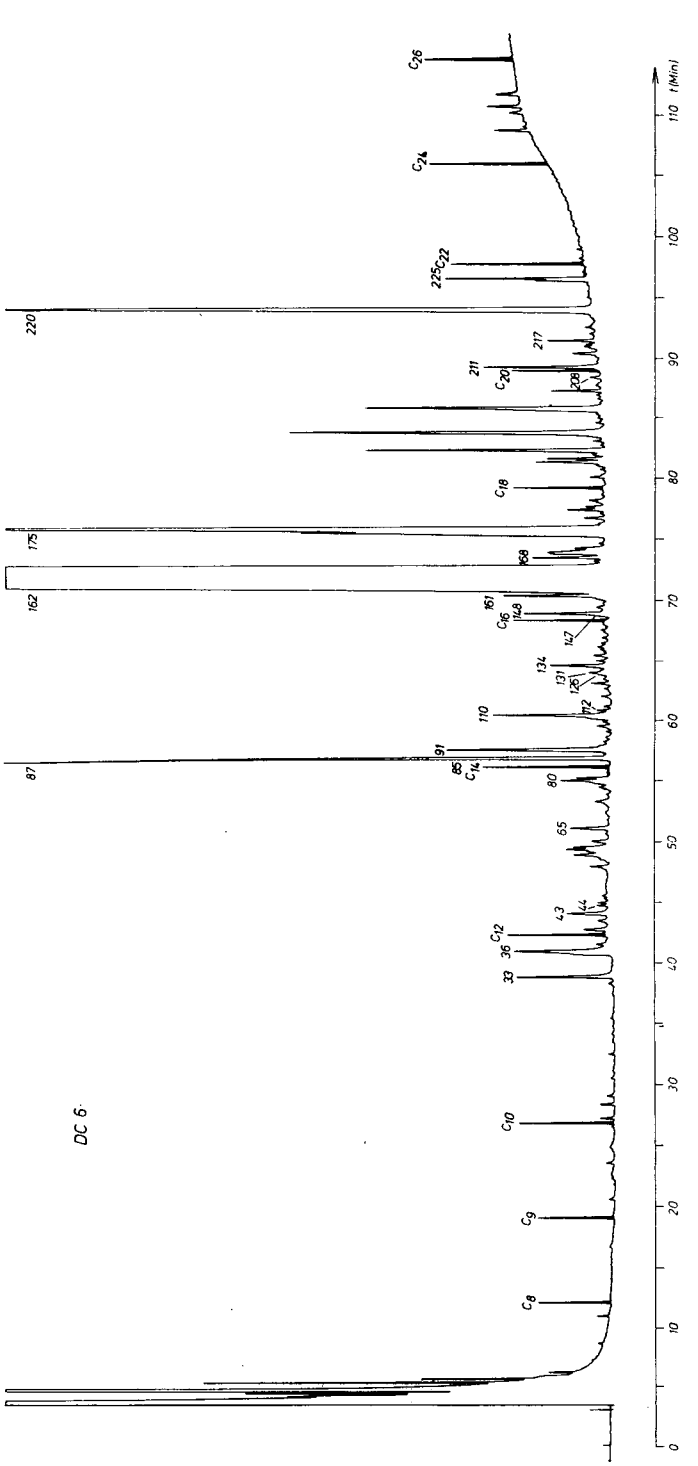


Fig. 7. Glaskapillargaschromatogramm der Fraktion DC 5.


DC 6
C8
C9
C10
C12
C14
C16
C18
C20
C22
C24
C26
87
91
110
162
175
220
225
t (Min)
0
10
20
30
40
50
60
70
80
90
100
110

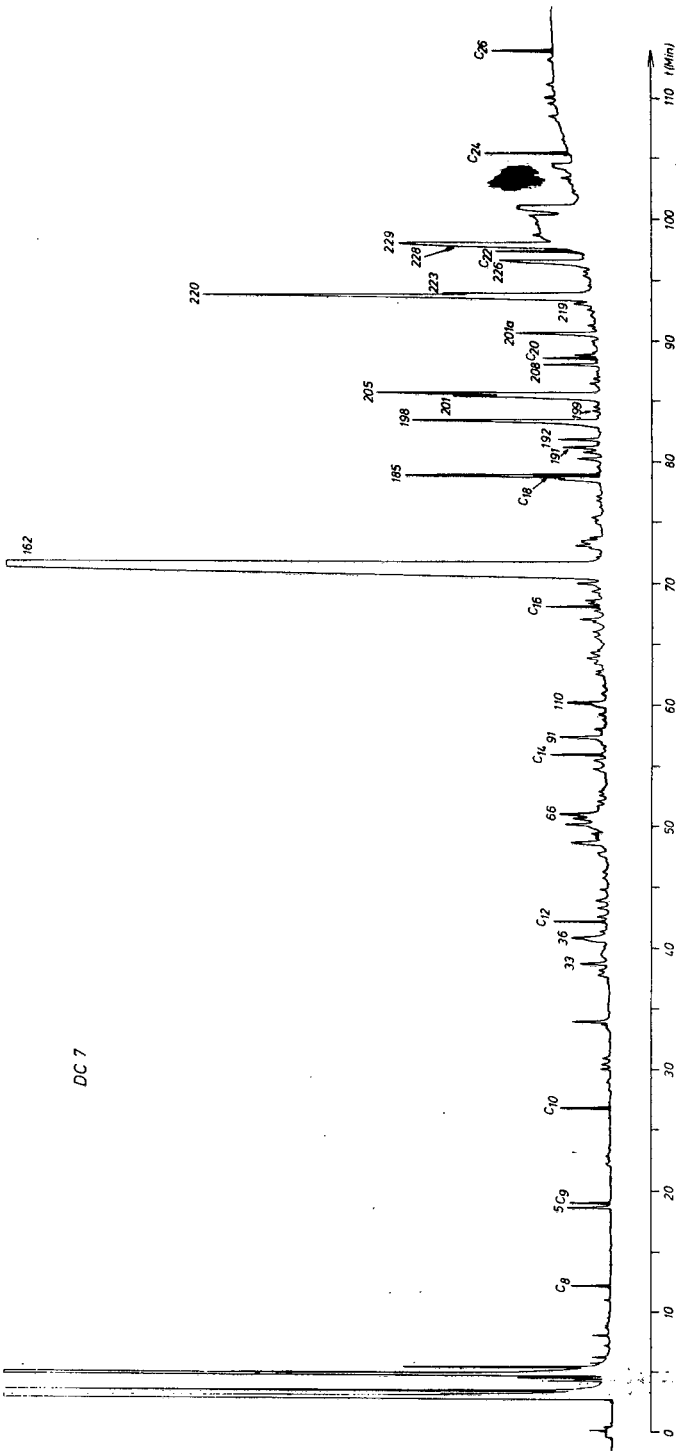


Fig. 9. Glaskapillargaschromatogramm der Fraktion DC 7.

TABELLE I

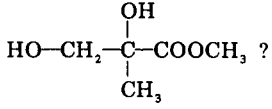
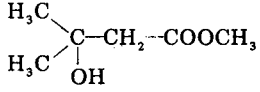
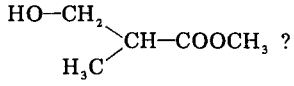
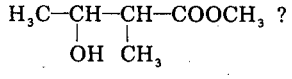
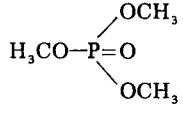
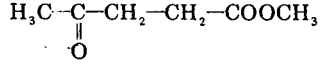
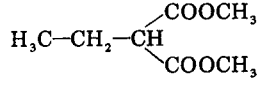
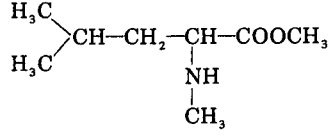
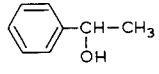
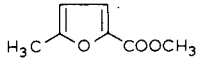
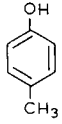
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
1		$C_5H_{10}O_4$		134?	104(2%), 88(100), 75(28), 59(93), 45(18), 43(10), 31(33)		
2	849	$C_6H_{12}O_3$		132	117(52%, M^+–15), 101(8), 85(73), 75(17), 74(37), 73(12), 59(80), 43(100), 42(24), 41(18), 39(12), 31(40), 29(17), 27(18)	3, 14, 22, 23	
3	860	$C_5H_{10}O_3$		118	103(8%, M^+–15), 100(10), 88(100), 87(41), 85(8), 71(7), 69(14), 59(52), 57(60), 56(44), 55(20), 41(28), 31(84)		
4	894	$C_6H_{12}O_3$		132?	117(7%, M^+–15), 114(3), 101(18), 88(100), 87(18), 85(22), 73(8), 61(9), 59(19), 57(84), 56(57), 45(58), 43(33)		
5*	894	$C_3H_9O_4P$		140	140(15%, M^+), 110(100), 109(33), 95(27), 80(25), 79(29)	14	
6	898				134(2%), 100(7), 99(5), 88(8), 85(57), 57(27), 56(100), 45(10), 43(50), 41(44), 29(60), 28(75)		
7	943	$C_6H_{10}O_3$		130	130(2%, M^+), 115(15), 99(22), 88(8), 71(7), 59(12), 57(9), 55(16), 45(7), 43(100)	3	

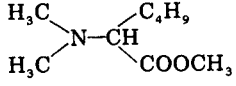
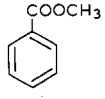
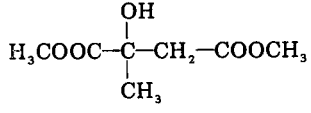
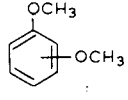
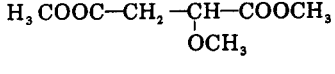
TABELLE I *(Fortsetzung)*

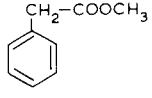
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
8*	953	C_6H_6O		94	94(100%,M^+),66(26), 65(21),63(8),39(24)	15	
9	955	$C_7H_{14}O_3$?		146?	114(4%),103(20), 102(48),101(21), 87(100),70(20),55(95), 43(29),41(20),39(14), 31(37)		
10*	996	$C_6H_{10}O_4$	$H_3COOC-(CH_2)_2-COOCH_3$	146	115(100%,M^+–31), 114(27),87(19),59(59), 55(74)	14,16–20	
11*	1011	$C_7H_{12}O_4$		160	132(59%),129(37), 101(30),100(24), 69(30),59(100),55(24)	21	
12*	1014	$C_8H_{17}NO_2$		159	100(100%,M^+),58(46), 44(23)	3	
13	1024			102	102(100%),88(5), 71(12),70(12),44(16), 42(55)		
14	1029	$C_7H_{10}O_4$?		158	126(9%),122(10), 111(100),99(32),98(28), 83(24),71(54),67(52), 55(85),43(71),41(33), 39(30)		Gemisch, Dicarbon- säure

15	1031	$C_7H_{12}O_4$		160?	142(56%),129(12), 128(8),71(16),69(21), 59(37),55(23),43(100), 41(10),27(29)	
16*	1035	$C_7H_{12}O_4$	$H_3COOC-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-COOCH_3$	160	129(31%,M^+--31), 128(18),101(19), 100(18),87(13),74(7), 69(18),59(100),55(8), 41(22)	18,19
17*	1036	$C_8H_{10}O$		122	122(33%,M^+),107(94), 105(12),79(100),78(23), 77(49),51(23)	
18*	1043	$C_7H_8O_3$		140	140(64%,M^+),125(7), 109(100),95(10),81(9), 54(10)	
19	1047	$C_8H_{16}O_3$	$C_4H_9-\underset{\displaystyle OH}{\underset{\vert}{CH}}-CH_2-COOCH_3$	160?	142(8%),103(61), 102(17),100(21),74(57), 71(71),61(35),55(35), 43(100)	
20*	1047	C_7H_8O		108	108(94%,M^+),107(100), 91(4),90(7),79(18), 77(22),53(10)	3,14
21	1054	$C_9H_{16}O_2$	Lacton	156	156(2%,M^+),113(15), 99(100),73(24),71(12), 69(13),57(17),56(23), 55(20),43(87)	

(Fortsetzung S. 270)

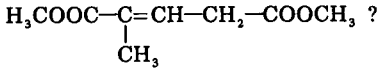
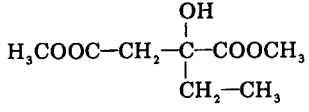
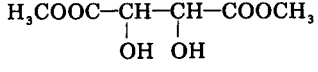
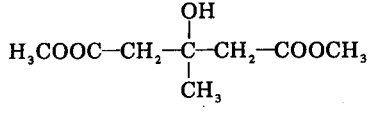
TABELLE I *(Fortsetzung)*

Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
22	1065				129(12%),101(68), 69(10),59(100),55(8), 42(11),41(19)		
23*	1070	$C_9H_{19}NO_2$		173	173(2%,M^+),116(15), 115(8),114(100),72(36)		
24*	1072	$C_8H_8O_2$		136	136(33%,M^+), 105(100), 77(59),51(25),50(12)	3,9,24	
25*	1087	$C_7H_{12}O_5$		176	127(4%,M^+–49),117(44), 99(7),85(36),74(33), 59(10),43(100)		*m/e* 117 $C_5H_9O_3$
26*	1102	$C_7H_{12}O_4$	$H_3COOC-CH_2-CH_2-CH_2-COOCH_3$	160	129(54%,M^+–31), 128(28),101(48), 100(70),97(10),87(20), 74(10),69(9),59(100), 55(55)	20,25	
27	1111	$C_8H_{10}O_2$		138	138(100%,M^+),123(43), 95(52),80(9),77(35), 65(19),52(20),51(18), 41(19)		
28*	1113	$C_7H_{12}O_5$		176	117(38%),85(7), 75(100),59(22),55(10), 47(11),43(10)		

29	1137	$C_8H_{12}O_4$	$H_3COOC-C_4H_6-COOCH_3$	172	141(44%,M^+−31), 140(44), 113(35), 112(100), 97(28), 85(20), 82(18), 81(44), 59(52), 55(32), 53(35)		
30*	1137	$C_8H_{14}O_4$	$H_3COOC-CH_2-CH(CH_3)-CH_2-COOCH_3$	174	143(65%,M^+−31), 142(22), 115(38), 114(77), 107(8), 101(72), 99(9), 83(18), 82(24), 74(26), 73(45), 71(18), 69(71), 59(100), 55(49), 43(41), 42(28), 41(48), 39(24)	18, 19	
31	1148	$C_8H_{12}O_4$	$H_3COOC-C_4H_6-COOCH_3$	172	141(55%,M^+−31), 140(93), 113(40), 112(100), 109(38), 108(15), 97(46), 85(12), 82(24), 81(25), 59(51), 57(36), 55(37), 53(53), 45(44), 41(40), 39(46)		Isomer zu Nr. 34
32*	1149	$C_9H_{10}O_2$		150	150(24%,M^+), 91(100), 65(13), 63(5), 59(7), 51(4), 39(8)	3, 9, 24, 26	
33	1155	$C_7H_8O_3$?		140?	140(55%), 125(100), 122(11), 111(9), 110(7), 97(11), 95(10), 94(7), 83(6), 82(6), 71(10), 69(24), 54(11), 43(56)		

(Fortsetzung S. 272)

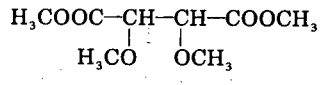
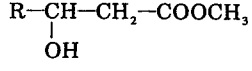
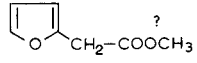
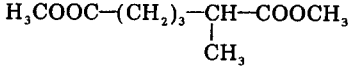
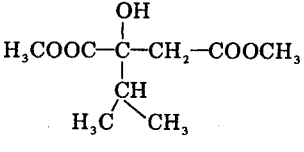
TABELLE I *(Fortsetzung)*

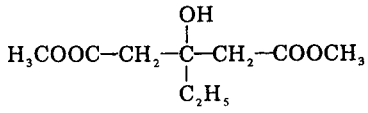
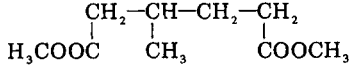
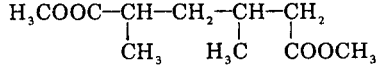
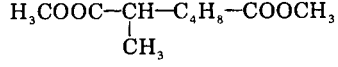
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
34	1163	$C_8H_{12}O_4$	 ?	172	141(53%,M^+–31), 140(100),113(36), 112(84),109(31), 108(17),97(32),85(8), 82(18),81(24),69(8), 59(35),55(24),53(42), 41(24),39(36)		
35*	1176	$C_8H_{14}O_5$		190	161(2%,M^+–29),141(2), 131(40),101(10),99(22), 59(11),57(100),43(24), 29(27)		
36	1183	$C_6H_{10}O_6$		178	119(26%,M^+–59),101(2), 90(100),75(8),73(12), 60(13),59(21),45(10), 42(12),33(91),31(25), 29(32)	1	
37	1190				128(60%),117(7),85(7), 73(53),58(29),44(14), 43(93),42(100)		Amino-säure
38*	1191	$C_8H_{14}O_5$		190	175(3%,M^+–15),157(5), 143(14),141(7),117(41), 101(11),85(35),74(10), 59(12),43(100)	27,28	

39	1195	$C_8H_{12}O_4$	$H_3COOC-C_4H_6-COOCH_3$	172	141(42%,M^+–31), 140(100),113(47), 112(89),109(22), 108(17),99(9),98(74), 97(33),85(10),82(23), 81(33),59(46),55(35), 53(51),45(17),42(17), 41(18),39(36)		
40*	1206	$C_8H_{14}O_4$	$H_3COOC-(CH_2)_4-COOCH_3$	174	143(35%,M^+–31), 115(17),114(60), 111(58),101(55),87(14), 83(22),74(46),73(30), 69(7),59(100),55(73), 43(40),41(33)	16–20, 29	
41	1207	$C_9H_{12}O_2$		152	152(100%,M^+),137(49), 109(39),107(7),94(12), 91(22),81(13),79(15), 77(18),66(16),65(15), 51(9),39(17)		substituiertes Phenol
42	1220	$C_9H_{16}O_4$	$H_3COOC-C_5H_{10}-COOCH_3$	188	157(38%,M^+–31), 156(18),136(23), 135(31),129(45), 128(96),115(11), 113(33),102(25), 100(21),97(50),87(54), 74(35),69(100),68(38), 59(61),55(70),41(57)		Gemisch aus MG 188 und MG 168 α-Ethyl-verzweigt
43*	1226	$C_7H_{12}O_6$	$H_3COOC-\underset{\underset{OH}{\vert}}{CH}-\underset{\underset{OCH_3}{\vert}}{CH}-COOCH_3$	192	143(4%,M^+–49), 133(20),105(13), 104(68),103(74), 89(17),77(19),75(27), 74(24),73(64),59(36), 45(100),33(28),31(32)	1	

(Fortsetzung S. 274)

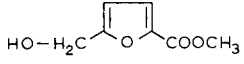
TABELLE I *(Fortsetzung)*

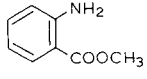
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
44*	1235	$C_8H_{14}O_6$		206	206(2%,M^+),174(5), 159(4),147(12), 119(68),103(100), 88(20),85(19),75(37), 73(36),59(20),47(10), 45(36)	1	
45	1234				178(7%),146(12), 119(10),103(76),74(55), 71(50),61(32),59(20), 55(24),45(33),43(100), 41(30)		
46	1244	$C_7H_8O_3$		140?	140(32%),126(29), 108(43),102(30),98(35), 87(76),81(100),80(45), 74(68),70(35),59(17), 57(24),55(44),54(31), 43(37),41(43)		Gemisch Dicarbonsäure und Furylessigsäure
47*	1245	$C_9H_{16}O_4$		188	157(24%,M^+–31), 154(27),129(31), 128(57),126(19), 115(24),101(18), 97(38),88(76),87(20), 74(32),69(92),68(22), 59(100),55(44),43(28), 41(57)		
48*	1247	$C_9H_{16}O_5$		204	161(7%,M^+–43), 145(50),130(7),129(7), 113(20),102(17), 101(24),74(14),71(100), 59(20), 43(91)	18,19	

49*	1248	$C_9H_{16}O_5$		204	172(4%), 143(7), 131(28), 129(48), 117(18), 101(12), 99(20), 87(43), 85(17), 57(100), 55(18), 45(93), 43(35)		nicht ganz rein
50*	1253	$C_9H_{16}O_4$		188	157(38%, M^+-31), 128(43), 125(52), 115(100), 101(11), 97(18), 96(17), 87(24), 83(43), 74(53), 73(77), 69(70), 59(73), 55(77), 43(43), 41(52)	16–18,20	
51	1268				130(20%), 127(38), 117(19), 107(10), 103(14), 88(56), 87(77), 71(37), 59(77), 57(30), 55(100), 45(73), 43(43)		α-Methyl-carbonsäure
52*	1272	$C_{10}H_{18}O_4$		202	171(13%, M^+-31), 143(24), 142(28), 129(63), 115(12), 111(22), 101(24), 97(11), 96(10), 88(100), 83(67), 74(23), 73(58), 69(97), 59(70), 55(28), 41(64)		
53	1275	$C_{10}H_{18}O_4$		202	171(23%, M^+-31), 143(28), 142(38), 129(63), 115(13), 111(23), 101(14), 97(10), 96(10), 88(100), 83(67), 74(23), 73(58), 69(98), 59(69), 55(24), 41(64)		

(Fortsetzung S. 276)

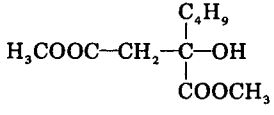
TABELLE I *(Fortsetzung)*

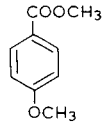
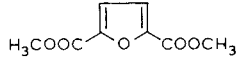
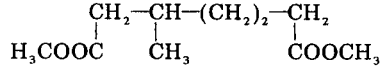
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
54	1281	$C_{10}H_{16}O_2$	Lacton	168?	168(2%),157(9),154(10), 140(8),129(14),99(100), 85(32),71(19),68(22), 59(18),56(21),55(24), 43(91),41(13)		siehe Nr. 21
55	1286	$C_9H_{14}O_4$	$H_3COOC—C_5H_8—COOCH_3$	186	155(30%,M^+−31), 154(43),127(38), 126(100),111(11), 95(18),94(20),85(23), 67(43),59(59),53(21)		
56	1291	$C_8H_{12}O_5$	$H_3COOC—\underset{\displaystyle OCH_3}{\underset{\vert}{C}}=CH—CH_2—COOCH_3$	188	188(3%,M^+), 158(4), 141(6),129(35), 128(13), 125(10), 115(69),101(11), 97(9), 87(10), 69(20), 59(59), 55(82), 45(100)	14,45	
57*	1292	$C_7H_8O_4$		156	156(24%,M^+),139(9), 127(10),125(37), 123(18),97(100), 95(10),79(8),69(37), 41(49),39(23)	16,20,30, 31	
58	1295	$C_9H_{17}NO_4$		203	203(1%,M^+),172(8), 144(100),139(6), 116(11),84(73),42(22)		

59	1298	$C_9H_{14}O_4$	$H_3COOC-C_5H_8-COOCH_3$	186	186(2%,M^+),155(12), 154(14),127(37), 126(30),122(44), 113(41),100(14), 99(16),95(20),85(77), 84(46),81(18),71(47), 67(80),59(100),55(22), 53(18),43(21),41(61), 39(32)		
60	1307	$C_{10}H_{16}O_2$	Lacton	168	168(9%),159(9),151(7), 140(8),131(26),119(11), 99(100),74(10),71(56), 59(12),57(11),55(34), 43(29)		Gemisch
61	1307				144(89%),115(12), 103(20),102(35), 95(12),88(100),74(13), 71(28),59(49),58(24), 43(93),41(54)		Gemisch
62*	1309	$C_9H_{16}O_4$	$H_3COOC-(CH_2)_5-COOCH_3$	188	157(38%,M^+-31), 128(35),125(39), 115(97),111(18), 100(12),97(30),87(25), 83(45),74(100),73(40), 69(71),59(85),55(86), 43(71),41(57)	62	
63*	1314	$C_8H_9NO_2$		151	151(56%,M^+),120(32), 119(100),92(53),65(25), 52(7),39(12)	32	

(Fortsetzung S. 278)

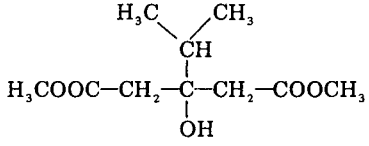
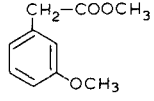
TABELLE I *(Fortsetzung)*

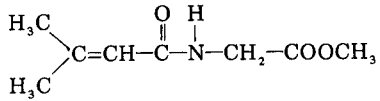
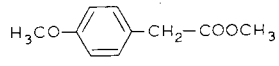
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
64	1324	$C_9H_{10}O_3$		166	166(20%,M^+),144(14), 108(100),107(37), 91(22)		
65	1327	$C_7H_{13}NO_3$	acetylierte Aminosäure	159	159(7%,M^+),141(6), 127(12),117(24), 114(12),103(24), 88(29),85(100),74(18), 71(28),61(9),59(24), 57(34),43(58)		Gemisch, acetylierte Aminosäure
66	1328			235?	235(7%),217(3),191(9), 175(14),162(13), 161(30),147(36), 131(8),119(7),105(9), 89(45),75(21),73(100), 59(18),45(17)		
67	1338	$C_{10}H_{18}O_4$	$H_3COOC-C_6H_{12}-COOCH_3$	202	171(23%,M^+-31), 142(24),139(33), 129(100),110(13), 105(14),97(59),87(28), 83(24),74(58),69(91), 59(38),55(56),43(24), 41(44)		
68*	1341	$C_{10}H_{18}O_5$		218	162(8%,M^+-56), 159(54),141(9), 130(20),127(10), 102(25),101(21), 85(96),74(22),59(18), 57(100),43(33),41(26)		

69	1343				154(4%), 123(9), 122(18), 101(11), 96(20), 90(34), 81(88), 80(50), 74(100), 59(24), 55(34), 43(58), 41(53)		Gemisch
70*	1345	$C_9H_{10}O_3$			166	166(45%, M^+), 135(100), 107(14), 92(17), 77(27), 64(13), 63(10), 50(6)	9, 26, 33
71*	1346	$C_8H_8O_5$			184	184(34%, M^+), 153(100), 126(6), 125(5), 95(8), 69(7), 66(5), 59(11), 53(5), 38(17)	3, 16, 31, 34
72*	1351	$C_{10}H_{18}O_4$			202	171(27%, M^+-31), 142(18), 139(17), 129(80), 111(18), 110(10), 101(18), 97(63), 87(23), 83(19), 82(23), 74(64), 69(100), 59(56), 55(42), 43(37), 41(56)	18, 19
73	1357				220?	189(5%, M^+-31), 161(90), 157(18), 156(17), 153(12), 133(14), 129(50), 101(100), 99(18), 98(14), 71(17), 69(26), 59(30), 55(35), 41(36)	
74	1359				184?	184(2%), 169(4), 156(51), 154(24), 141(12), 99(74), 98(47), 96(26), 84(13), 71(32), 69(74), 68(33), 56(45), 41(100)	Gemisch

(Fortsetzung S. 280)

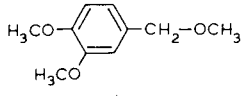
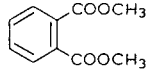
TABELLE I *(Fortsetzung)*

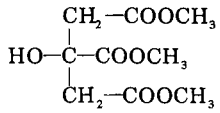
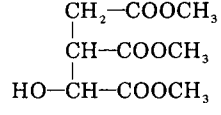
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
75	1366	$C_{10}H_{18}O_4$?	$H_3COOC-C_6H_{12}-COOCH_3$	202	171(22%,M^+–31), 155(4),143(77), 139(19),129(53), 127(18),125(20), 116(38),111(60), 101(11),97(57),87(15), 83(58),74(50),73(37), 69(71),59(49), 55(100),43(42),41(51)		2-Propyl-glutarsäure ?
76*	1367	$C_{10}H_{18}O_5$		218	175(8%,M^+–43), 145(49),143(96), 113(18),101(54), 74(9),71(100),69(9), 59(28),43(62)		
77	1370	$C_{10}H_{18}O_4$?		202?	187(78%),186(31), 159(18),154(24), 127(100),126(55), 113(23),101(24), 99(37),95(27),85(12), 75(24),59(83),55(23), 41(41)		
78*	1380	$C_{10}H_{12}O_3$		180	180(45%,M^+),149(4), 148(4),121(100), 91(23),88(10),78(13), 77(9),65(7),59(7), 51(6)	9	
79	1380	$C_{11}H_{20}O_4$	$H_3COOC-CH(CH_3)-CH_2-CH(CH_3)-CH_2-CH_2-COOCH_3$	216	185(9%,M^+–31), 184(3),157(14), 129(38),125(10), 114(8),101(11),97(32), 88(100),83(7),73(11), 69(34),59(38),55(45),		

80	1386	$C_8H_{13}NO_3$		171	171(14%,M^+),156(3), 139(5),120(7), 112(11),88(10), 83(100),82(25),55(83)	35—38
81	1388	$C_8H_{10}N_2O_4$		198?	198(14%,M^+),167(100), 166(25),137(28), 109(6),81(10),80(9)	
82	1393	$C_{11}H_{20}O_4$?	$H_3COOC-C_7H_{14}-COOCH_3$?	216?	185(4%,M^+-31), 184(12),157(8),156(7), 153(14),143(100), 129(23),111(85), 101(51),83(65),69(55), 59(70),55(66),41(38)	
83*	1393	$C_{10}H_{12}O_3$		180	180(15%,M^+),121(100), 78(7),77(6),59(8),55(7)	9
84	1397	$C_{10}H_{16}O_4$	$H_3COOC-C_6H_{10}-COOCH_3$	200	200(3%,M^+),169(12), 168(5),152(10), 140(38),136(60), 127(18),126(16), 109(23),108(24), 99(22),85(70),81(62), 80(38),74(46),67(80), 59(82),55(44),53(30), 45(26),43(48),41(93), 39(42),15(100)	
85	1400			134?	134(34%,M^+),119(5), 102(100),91(8),77(5), 70(4),67(5),65(7), 51(4),42(39)	Heterocyclische Verbindung 2N?

(Fortsetzung S. 282)

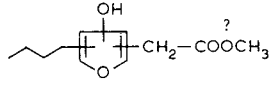
TABELLE I *(Fortsetzung)*

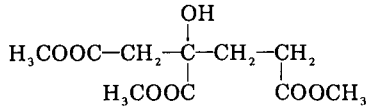
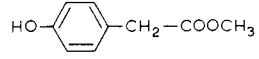
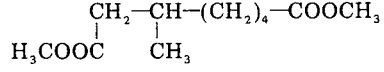
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
86★	1410	$C_{10}H_{18}O_4$	$H_3COOC-(CH_2)_6-COOCH_3$	202	171(50%, M^+—31), 142(10), 139(13), 138(65), 129(82), 114(11), 111(23), 110(17), 101(9), 97(64), 87(36), 83(39), 74(100), 69(90), 59(63), 55(78), 43(64), 41(65)	18—20	
87★	1413	$C_{10}H_{14}O_3$ ★		182	182(48%, M^+), 167(4), 166(5), 151(100), 139(4), 135(3), 107(10), 91(6), 77(8), 65(9), 53(3), 51(7), 45(6)		
88★	1414	$C_{10}H_{10}O_4$		194	194(9%, M^+), 163(100), 135(8), 133(5), 92(11), 77(23)		
89	1416	$C_{11}H_{20}O_3$		200?	198(3%), 170(4), 163(8), 143(18), 123(7), 111(25), 97(10), 87(18), 83(19), 74(19), 71(12), 69(23), 55(27), 43(100)		
90	1423	$C_9H_{12}O_5$		200?	200(5%, M^+), 182(12), 169(17), 154(27), 151(53), 150(23), 141(24), 140(52), 125(11), 122(13), 109(25), 108(29), 99(31), 95(32), 81(100), 80(21), 74(18), 67(27), 59(43), 55(16), 53(17), 43(22), 41(39), 39(23)		

91*	1424	$C_9H_{14}O_7$		234	175(14%,M^+–59), 153(7),143(100), 111(6),101(82),84(5), 74(7),69(13),59(36), 57(12),43(23)	1,14,17	
92	1428	$C_{12}H_{22}O_4$	Methylaconitsäuretrimethylester (Artefakt)	230	199(38%,M^+–31), 198(20),171(72), 167(36),166(81), 157(22),139(61), 129(18),127(16), 111(54),101(9),83(17), 81(13),75(18),59(74), 55(23), 53(41), 15(100)		
93	1431				196(12%,M^+),174(10), 165(8),163(20), 160(11),139(46), 133(24),132(22), 131(61),115(12), 105(23),104(18), 103(20),91(100), 85(17),77(20),65(23), 55(11),51(13)		Gemisch
94*	1435	$C_9H_{14}O_7$		234	175(4%,M^+–59),171(3), 159(3),143(100), 115(90),111(12), 101(13),99(9),87(8), 84(9),83(53),59(53), 55(75)	14,17	
95	1435	$C_{12}H_{22}O_4$	$H_3COOC-C_8H_{16}-COOCH_3$	230	199(38%,M^+–31), 198(52),171(17), 170(47),167(53), 166(77),155(13), 139(85),138(31), 127(7),123(8),111(45), 83(54),59(71),53(56), 15(100)		

(Fortsetzung S. 284)

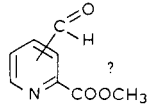
TABELLE I *(Fortsetzung)*

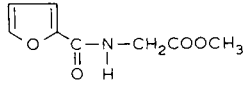
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
96	1438	$C_{11}H_{18}O_4$	$H_3COOC-C_7H_{12}-COOCH_3$	214	182(25%, M^+-32), 155(12), 154(31), 151(34), 150(32), 140(12), 130(14), 125(11), 123(23), 122(43), 115(14), 113(11), 109(21), 99(24), 98(33), 95(41), 85(100), 81(53), 79(23), 74(28), 68(19), 67(28), 59(60), 55(43), 43(42), 41(44)		
97	1435			179?	184(48%), 133(10), 116(100), 105(7), 103(6), 91(9), 77(7), 65(6), 56(9), 42(18)		
98	1440	$C_{12}H_{22}O_4$	Methylaconitsäuretrimethylester (Artefakt)	230	215(7%, M^+-15), 199(32), 171(75), 167(38), 166(70), 157(41), 139(45), 129(11), 127(11), 125(10), 111(52), 101(8), 85(7), 83(11), 81(10), 75(9), 59(67), 55(11), 53(38), 15(100)		
99	1441	$C_{11}H_{16}O_4$		212	212(24%, M^+), 153(100), 111(68), 94(30), 79(10), 66(9), 59(28)		

100	1443	$C_{10}H_{16}O_7$		248?	189(10%), 157(100), 125(30), 59(23)		
101	1445	$C_{11}H_{18}O_5$?	$H_3COOC-C_7H_{12}O-COOCH_3$	230?	215(7%, M^+-15), 199(38), 198(13), 171(100), 167(47), 166(90), 157(28), 139(62), 129(21), 127(17), 117(38), 111(65), 85(37), 81(18), 53(46)		
102★	1446	$C_9H_{10}O_3$		166	166(23%, M^+), 107(100), 78(8), 77(20), 59(11), 51(10)	3, 9, 17, 24, 26, 30, 33, 39	
103	1452			178	160(100%, M^+-18), 119(31), 101(21), 100(54), 87(23), 59(59), 42(33)		
104★	1453	$C_{11}H_{20}O_4$		216	185(43%, M^+-31), 170(10), 156(12), 153(23), 152(43), 143(97), 139(24), 125(27), 111(83), 101(27), 97(22), 87(18), 83(100), 82(32), 74(93), 69(70), 59(72), 55(99), 43(63), 41(70)	18, 19	zweite Verb. mit MG 212

(Fortsetzung S. 286)

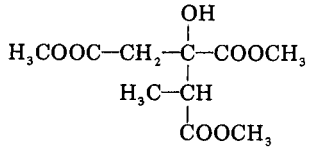
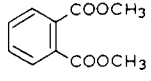
TABELLE I *(Fortsetzung)*

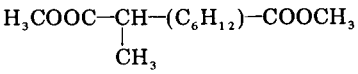
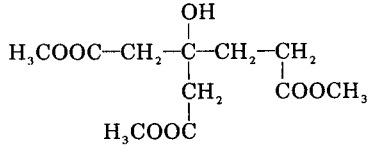
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
105	1456	$C_9H_{14}O_7$	Isomeres der Isocitronensäure	234	202(3%,M^+–31), 175(28),174(8),171(7), 146(13),143(86), 115(100),114(23), 111(8),101(7),87(13), 83(47),59(42),55(74)	14,17	
106	1456	$C_8H_7NO_3$		165	165(38%),164(62), 137(55),136(70), 107(89),106(27), 93(18),79(97),78(100), 77(32),65(9),51(18), 50(33)		
107	1463	$C_{11}H_{18}O_4$	$H_3COOC—C_7H_{12}—COOCH_3$	214	182(38%,M^+–32), 155(12),151(18), 150(9),140(12), 127(23),123(18), 122(17),113(9), 112(16),99(8),95(100), 88(36),67(27),59(28), 57(18),55(23)		
108	1466	$C_{11}H_{18}O_4$	$H_3COOC-C_7H_{12}—COOCH_3$	214	214(3%,M^+),182(35), 155(20),154(25), 151(34),150(36), 141(9),140(12), 123(28),122(34), 109(24),108(17), 99(20),95(60),94(24), 88(9),85(18),81(100), 79(19),74(31),67(33), 59(38),55(44),53(24), 43(27),41(41)		

109	1469	$C_{11}H_{20}O_4$	$H_3COOC{-}C_7H_{14}{-}COOCH_3$	216	185(11%,M^+–31), 184(8),156(13), 143(75),124(7), 116(100),111(73), 101(34),84(20),83(63), 74(27),69(23),59(67), 55(74),43(57)	
110*	1471	$C_8H_9NO_4$		183	183(11%,M^+),124(40), 95(100),67(4),43(5), 39(11)	3,31,40
111	1471	$C_{12}H_{20}O_4$	$H_3COOC{-}C_8H_{14}{-}COOCH_3$	228	228(7%,M^+),194(24), 168(18),165(33), 164(28),163(97), 141(23),136(20), 135(19),127(62), 123(24),109(30), 108(18),104(9), 103(15),99(10), 88(100),85(38),81(77), 59(38),55(42),41(45)	

(Fortsetzung S. 288)

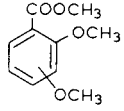
TABELLE I *(Fortsetzung)*

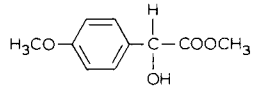
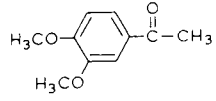
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
112*	1473	$C_{10}H_{16}O_7$		248	189(12%,M^+–59), 157(100), 143(7), 125(8), 115(61), 101(30), 88(28), 83(8), 59(21), 57(23), 56(17)		*m/e* 189 $C_8H_{13}O_5$ *m/e* 157 $C_7H_9O_4$
113	1475	$C_{12}H_{20}O_4$	$H_3COOC-C_8H_{14}-COOCH_3$	228	228(6%,M^+), 197(8), 196(37), 169(23), 168(48), 165(53), 164(31), 154(24), 139(44), 137(25), 136(20), 123(31), 122(25), 109(76), 99(46), 95(100), 93(27), 88(23), 81(64), 67(50), 59(68), 55(56), 41(66)		
114	1476	$C_{11}H_{20}O_4$	$H_3COOC-C_7H_{14}-COOCH_3$	216	184(8%,M^+–32), 156(13), 143(93), 116(100), 111(64), 101(31), 83(57), 74(30), 69(28), 59(62), 55(72), 43(52)		Isomer mit 109
115	1483	$C_{10}H_{16}O_7$		248	189(12%,M^+–59), 161(6), 157(100), 125(11), 115(62), 101(33), 88(30), 59(32), 57(24), 56(22)		Isomer mit 112
116*	1481	$C_{10}H_{10}O_4$		194	194(23%), 163(100), 135(12), 120(8), 103(8), 95(17), 77(10), 76(15), 50(8)		

117	1487	$C_{12}H_{22}O_4$		230	199(11%,M^+–31), 192(13), 171(18), 166(23), 157(40), 143(22), 125(18), 111(17), 101(15), 97(55), 90(10), 88(100), 83(21), 74(28), 69(54), 59(42), 57(19), 55(67), 41(53)	Gemisch
118	1490	$C_{11}H_{18}O_4$	$H_3COOC-C_7H_{12}-COOCH_3$	214	208(8%), 177(13), 176(12), 157(38), 151(56), 150(100), 140(32), 125(33), 97(29), 83(54), 81(75), 80(67), 74(74), 55(73), 43(49), 41(73)	
119	1493	$C_{10}H_{14}O_5$	$H_3COOC-C_6H_8O-COOCH_3$	214	214(6%,M^+), 196(8), 165(26), 143(16), 107(25), 101(74), 88(50), 82(100), 75(23), 69(33), 67(26), 55(25), 43(28), 41(56)	
120	1496	$C_{11}H_{18}O_7$		262	189(22%,M^+–73), 185(4), 175(10), 157(82), 143(100), 115(30), 111(17), 101(79), 87(9), 74(18), 69(18), 59(29), 43(60), 29(44)	
121	1498				220(16%,M^+), 198(10), 189(8), 183(19), 174(100), 151(24), 147(29), 146(25), 141(27), 115(27), 114(34), 101(9), 91(13), 87(33), 74(17), 71(36), 59(44)	

(Fortsetzung S. 290)

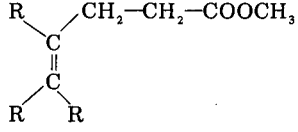
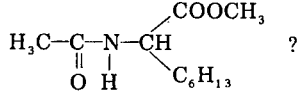
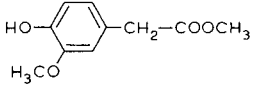
TABELLE I *(Fortsetzung)*

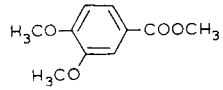
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
122	1498	$C_{11}H_{18}O_4$	$H_3COOC{-}C_7H_{12}{-}COOCH_3$?	214	183(7%,M^+−31),182(8), 155(13),154(15), 151(29),150(90), 141(17),140(24), 123(37),122(36), 114(20),109(29), 99(17),95(50),81(98), 80(68),74(55),67(60), 59(100),55(67),54(37), 43(44),41(81),39(43)		
123	1508	$C_{12}H_{20}O_4$	$H_3COOC{-}C_8H_{14}{-}COOCH_3$	228	197(9%,M^+−31), 196(19),169(8), 168(8),165(10), 164(20),136(18), 109(32),95(28),74(12), 73(100),59(14),55(12), 53(11),41(13)		
124	1513	$C_{10}H_{12}O_4$		196?	196(100%,M^+),181(52), 165(83),163(46), 151(14),150(13), 135(12),125(17), 123(18),122(15), 107(45),92(13), 79(28),77(27),63(10)	26,33,41	
125*	1514	$C_{11}H_{20}O_4$	$H_3COOC{-}C_7H_{14}{-}COOCH_3$	216	185(48%,M^+−31), 152(70),143(38), 125(21),124(20), 111(55),101(11), 98(20),97(29),87(37), 84(33),83(66),74(99), 69(38),59(57),55(100), 43(57),41(60)	14	

126	1514				159(5%),132(10),117(9), 101(35),88(69),85(25), 75(100),74(30),73(20), 71(17),59(12),45(23)		Gemisch
127*	1517	$C_{10}H_{12}O_4$		196	196(6%,M^+),171(4), 137(100),135(8), 109(22),107(5), 94(18),77(19),66(9), 51(7)	9,26,33, 39	
128*	1520	$C_{10}H_{12}O_3$		180	180(49%,M^+),165(100), 122(12),107(12), 94(16),79(16),77(30), 43(22)		
129	1523	$C_{12}H_{20}O_4$	$H_3COOC{-}C_8H_{14}{-}COOCH_3$	228	228(3%,M^+),197(44), 196(79),168(100), 165(12),164(22), 154(20),153(76), 140(57),137(22), 136(24),125(43), 121(39),112(74), 109(81),108(81), 95(84),94(45),83(37), 67(43),59(82),55(72), 53(44),41(82),39(50)		
130	1524	$C_{10}H_{12}O_3$?		180?	180(30%),179(75), 152(32),151(100), 138(18),123(11), 122(8),107(7), 106(5),95(23),94(24), 67(51),66(44),53(23), 41(23)		Gemisch

(Fortsetzung S. 292)

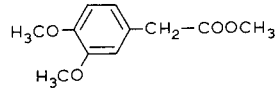
TABELLE I *(Fortsetzung)*

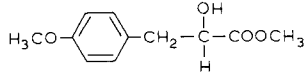
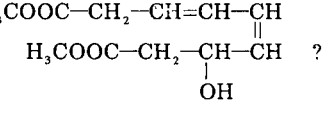
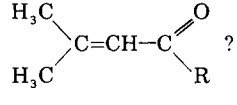
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
131	1528				155(8%),126(11), 117(16),99(11),85(97), 75(100),74(48),59(16), 55(20)		Gemisch
132	1528	$C_{12}H_{20}O_4$	$H_3COOC-C_8H_{14}-COOCH_3$	228?	228(8%,M^+),198(11), 179(15),170(8),168(12), 157(13),155(37), 129(10),126(13), 125(15),101(15), 100(18),96(23),95(73), 94(40),84(24),81(100), 74(37),67(48),59(70), 55(23),43(37),41(60)		
133	1537	$C_{11}H_{16}O_4$		212	212(27%,M^+),181(32), 180(62),153(33), 152(100),151(80), 139(78),135(28), 124(17),121(94), 111(14),94(16),93(15), 81(16),79(18),66(19), 65(20),61(14),59(22), 51(20)		Gemisch
134	1539	$C_{11}H_{21}NO_3$	 ?	215?	215(1%,M^+),172(3), 156(37),114(100), 99(11),88(14),60(8), 56(13),55(15),43(47), 30(30)		
135★	1540	$C_{10}H_{12}O_4$		196	196(23%,M^+),155(12), 153(4),137(100), 122(11),94(9)	42—46	

136*	1548	$C_{10}H_{12}O_4$		196?	196(100%,M^+),181(13), 165(94),153(18), 149(8),137(40), 125(10),107(23), 79(26),77(20),59(18), 51(20)	26,33
137	1550	$C_{12}H_{22}O_4$	$H_3COOC-C_8H_{16}-COOCH_3$	230	199(22%,M^+-31), 169(11),157(67), 137(48),125(82), 111(30),109(18), 101(17),97(57),87(29), 83(64),81(26),74(63), 69(64),59(55),55(100), 43(50),41(71)	
138	1555	$C_{12}H_{22}O_4$	$H_3COOC-C_8H_{16}-COOCH_3$	230	199(31%,M^+-31), 166(32),157(62), 139(19),138(22), 125(67),101(23), 98(24),97(52),87(23), 84(19),83(48),81(17), 74(80),69(50),59(58), 55(100),43(58),41(55)	
139	1557	$C_{12}H_{20}O_4$	$H_3COOC-CH(CH_3)-C_6H_{10}-COOCH_3$	228	197(11%,M^+-31), 196(30),169(12), 168(16),164(43), 149(37),136(43), 124(18),109(100), 108(29),101(17), 96(24),95(52),88(18), 82(44),81(43),69(23), 67(37),59(57),55(58), 53(23),43(22),41(53), 39(37)	

(Fortsetzung S. 294)

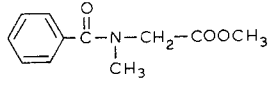
TABELLE I *(Fortsetzung)*

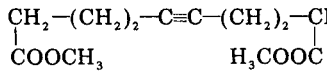
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
140	1573	$C_{12}H_{20}O_4$	$H_3COOC-C_8H_{14}-COOCH_3$	228	197(11%,M^+–31), 196(28),169(8), 168(11),165(22), 164(40),155(31), 143(18),136(24), 123(37),122(23), 111(13),101(7), 95(100),81(32),79(18), 74(22),69(19),67(34), 59(40),55(38),43(26), 41(41),39(23)		
141	1574			197?	197(66%,M^+),169(13), 133(88),132(56), 105(57),104(81), 94(18),77(46),76(95), 74(32),50(73),43(100)		
142*	1579	$C_{11}H_{14}O_4$		210	210(34%,M^+),195(3), 151(100),135(7), 107(12),91(4),90(4), 78(6),77(6),65(7), 59(3),51(4).	9,26,33	
143	1583	$C_{12}H_{20}O_4$	$H_3COOC-C_8H_{14}-COOCH_3$	228	196(18%,M^+–32), 165(27),164(36), 136(17),123(18), 122(17),119(33), 95(23),94(24),81(47), 80(43),79(27),74(100), 67(33),59(26),55(27), 43(44),41(42)		wahrscheinlich Δ 5 Doppelbindung

144	1587	$C_{12}H_{18}O_4$	$H_3COOC—C_8H_{12}—COOCH_3$	226	226(4%,M^+),195(10), 194(14),166(23), 163(29),162(44), 152(40),135(23), 134(24),107(18), 106(20),93(83),92(64), 91(46),79(83),77(38), 74(100),71(20),67(23), 59(47),55(17),53(18), 43(17),41(45),39(29)		Isomer zu 153
145*	1595	$C_{11}H_{14}O_4$		210	210(8%,M^+),192(3), 151(7),121(100),91(11), 78(10),77(11)	26,30,47	
146	1605	$C_{11}H_{16}O_5$	 ?	228	228(2%,M^+),210(32), 197(24),178(11), 177(8),168(25), 165(16),164(15), 155(89),151(40), 146(18),137(16), 136(24),123(100), 109(18),108(30), 95(90),81(82),74(71), 67(55),59(52),55(49), 43(39),41(56),39(27)		
147	1607		 ?		199(17%,M^+),168(5), 167(8),144(28),140(9), 131(17),112(18). 111(37),110(19), 90(43),83(100),68(12), 67(18),55(75)		

(Fortsetzung S. 296)

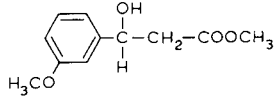
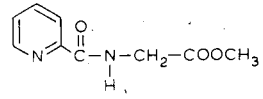
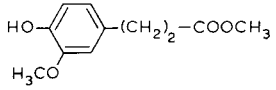
TABELLE I *(Fortsetzung)*

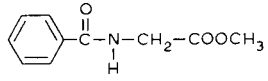
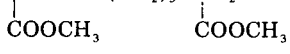
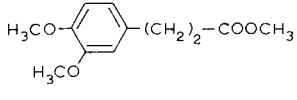
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
148	1610	$C_9H_{16}O_4$	$H_3COOC{-}C_5H_{10}{-}COOCH_3$	188?	157(12%), 128(43), 125(24), 109(10), 98(14), 97(23), 96(19), 81(30), 79(23), 75(100), 68(76), 67(43), 59(38), 57(36), 55(80), 43(60), 41(94)		
149	1610	$C_8H_{10}N_2O_4$ *	Methylimidazoldicarbonsäure	198	198(10%, M^+), 167(31), 166(100), 139(53), 135(18), 109(60), 107(30), 80(8), 51(24)		
150*	1612	$C_{12}H_{22}O_4$	$H_3COOC{-}(CH_2)_8{-}COOCH_3$	230	199(38%, M^+-31), 170(5), 166(23), 157(43), 139(18), 138(32), 125(58), 98(62), 97(44), 87(37), 84(50), 83(35), 74(100), 69(38), 59(47), 55(97), 43(58), 41(49)	48	
151*	1614	$C_{11}H_{13}NO_3$		207	207(2%, M^+), 148(34), 105(100), 77(35), 51(13)	3, 14, 38, 49–52	
152	1618				222(7%, M^+), 189(100), 188(52), 185(18), 174(11), 163(12), 160(42), 158(32), 146(18), 143(22), 129(31), 128(19), 103(35), 91(18), 88(18), 76(19), 74(16), 55(23)		Gemisch

153	1620	$C_{12}H_{18}O_4$		226	226(3%,M^+),210(11), 195(14),194(11), 179(13),178(10), 166(24),163(44), 162(38),152(35), 137(18),135(30), 134(32),121(40), 107(26),97(17),95(23), 94(36),93(62),92(46), 91(37),79(100),77(41), 74(80),67(25),65(20), 59(56),55(61),43(64), 41(40),39(33)	53
154	1622	$C_{12}H_{20}O_5$	$H_3COOC-C_8H_{14}O-COOCH_3$	244	213(12%,M^+−31), 212(14),195(10), 184(15),181(23), 180(19),171(67), 157(20),153(17), 152(32),139(50), 130(28),125(43), 115(23),111(44), 100(35),97(61),93(31), 85(49),74(86),59(90), 55(81),43(74),41(100)	
155	1625	$C_{12}H_{18}O_5$	$H_3COOC-C_8H_{12}O-COOCH_3$	242	242(5%,M^+),211(7), 210(17),193(9),182(9), 169(16),155(13), 150(17),137(24), 123(17),115(21), 109(23),108(27), 95(47),81(100),80(25), 74(30),67(32),59(38), 55(44),41(43)	

(Fortsetzung S. 298)

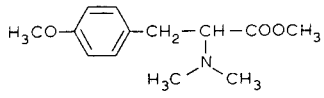
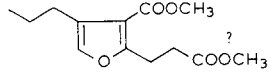
TABELLE I *(Fortsetzung)*

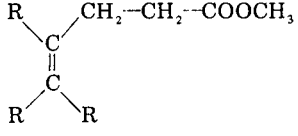
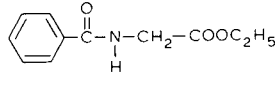
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
156	1634	$C_{11}H_{14}O_4$		210	210(24%,M^+),150(8), 137(48),109(100), 94(18),77(23)	8,9,54	
157*	1634	$C_9H_{10}N_2O_3$		194	194(7%,M^+),162(23), 135(86),106(64), 79(38),78(100),52(18), 51(28)		
158	1636				240(8%,M^+),210(5), 192(6),181(16), 180(17),167(21), 163(100),149(47), 107(39),77(19)		
159	1637	$C_{10}H_{11}NO_3$		193	193(12%,M^+),161(8), 151(13),150(9), 134(25),130(16), 127(20),105(100), 103(22),87(44),81(24), 77(43),74(18),71(25), 55(19),43(44)		Gemisch
160	1637				164(16%,M^+),149(7), 122(23),121(32), 102(100),91(3),82(4), 77(4),70(3),65(2), 42(31)		
161*	1644	$C_{11}H_{14}O_4$		210	210(25%,M^+),150(18), 137(100),122(6), 107(7),91(6),77(6), 65(5)	17	

162*	1650	$C_{10}H_{11}NO_3$		193	193(15%,M^+),162(5), 161(5),134(21), 105(100),77(55),51(22)	3,14,38, 49—52
163	1654	$C_{13}H_{20}O_5$	$H_3COOC-C_9H_{14}O-COOCH_3$	256	256(3%,M^+),224(8), 213(30),206(11), 193(14),180(12), 171(53),152(18), 139(55),112(32), 111(24),109(26), 101(33),98(45),97(42), 95(54),87(22),83(23), 81(26),74(100),69(81), 67(31),59(61),55(98), 43(58),41(86)	
164	1659	$C_{12}H_{20}O_4$		228?	197(19%,M^+−31), 196(24),169(31), 168(44),164(40), 137(43),136(70), 119(30),113(23), 108(29),100(60), 95(71),94(56),87(50), 81(82),74(66),69(72), 68(55),67(38),59(76), 55(100),41(90)	
165*	1676	$C_{12}H_{16}O_4$		224	224(34%,M^+),164(11), 151(100),149(11), 121(6),107(7),91(9), 77(10)	17
166	1680	$C_{10}H_{10}O_4$	Phthalester	194	194(9%,M^+),181(17), 163(100),149(63), 135(6),121(6),105(7), 104(7),92(9),77(17), 76(10)	

(Fortsetzung S. 300)

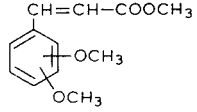
TABELLE I *(Fortsetzung)*

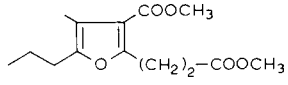
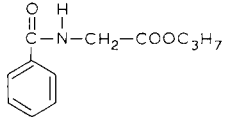
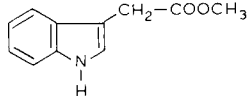
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
167*	1690	$C_{13}H_{19}NO_3$		237	178(15%,M^+–59), 163(3), 121(4), 116(100), 91(3), 89(5), 77(2), 56(6), 42(10)	3, 55	
168	1694		Lacton		181(3%), 153(4), 151(4), 141(5), 99(100), 88(10), 55(11), 43(28)		Gemisch
169	1697	$C_{13}H_{18}O_5$		254	254(16%,M^+), 223(15), 194(100), 181(10), 165(86), 99(36), 33(18)		
170	1698	$C_{13}H_{20}O_6$	$C_7H_{11}(COOCH_3)_3$?	272	241(12%,M^+–31), 209(13), 208(14), 185(100), 181(83), 153(66), 149(24), 125(37), 115(80), 109(38), 67(64), 59(55), 55(71)		
171	1703	$C_{12}H_{18}O_5$	$H_3COOC{-}C_8H_{12}O{-}COOCH_3$	242	242(2%,M^+), 241(4), 210(13), 185(23), 179(14), 178(24), 169(12), 153(25), 151(21), 150(23), 137(31), 136(23), 133(20), 110(33), 109(36), 108(25), 101(18), 95(46), 81(41), 74(85), 69(50), 67(70), 59(100), 55(92), 43(47), 41(90)		

172	1705				254(10%,M^+),210(12), 194(13),181(18), 178(24),168(9), 160(7),151(22), 150(24),137(28), 136(25),133(24), 121(61),110(33), 109(26),108(25), 101(16),95(41),87(30), 81(39),74(100),67(57), 59(90),55(84),43(46), 41(95)	Gemisch, enthält Verb. Nr. 171
173	1710	$C_{12}H_{16}O_4$ *		224	224(48%,M^+),193(12), 165(100),164(18), 151(21),135(8), 121(8),105(11), 91(10),79(8),77(10)	
174	1721	$C_{12}H_{18}O_7$	$C_6H_8(OH)(COOCH_3)_3$?	274	256(2%,M^+−18),242(3), 211(12),192(10), 182(7),169(51), 168(34),164(27), 157(31),137(100), 125(14),121(16), 109(36),102(22), 97(43),95(84),91(18), 81(58),79(42),74(58), 67(32),59(53),55(33), 43(30),41(58),39(37)	
175	1726	$C_{11}H_{13}NO_3$		207	207(7%,M^+),162(2), 161(3),134(21), 105(100),77(36),51(13)	siehe Nr. 162

(Fortsetzung S. 302)

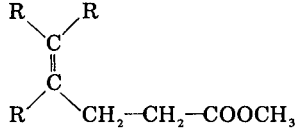
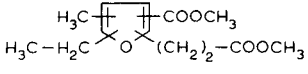
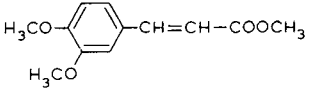
TABELLE I *(Fortsetzung)*

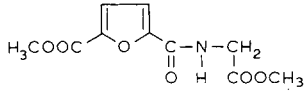
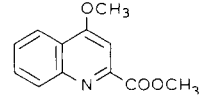
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
176	1728	$C_{12}H_{14}O_4$		222	222(100%,M^+),207(18), 191(47),179(7),164(8), 163(12),147(14), 133(8),119(12),91(13), 79(10),77(15),74(8), 59(11),51(17)		
177	1728	$C_{12}H_{14}O_4$	Hydroxycarbonsäure	222	187(14%),155(100), 109(32),95(12),90(10), 87(11),83(15),74(14), 67(30),59(22),57(18), 55(39),41(32)		
178	1741	$C_{12}H_{18}O_5$	R_1–CH(OH)–CH_2–$COOCH_3$ R_2–CH_2–CH(CH_3)–$COOCH_3$	242?	211(3%,M^+–31),210(2), 179(10),158(38), 115(12),103(42), 101(100),88(23),71(24), 69(20),61(13),55(28), 43(52),41(35)		Gemisch
179	1752	$C_{11}H_{16}O_5$	H_3COOC–$C_7H_9(OH)$–$COOCH_3$	228	197(7%,M^+–31),196(5), 183(6),165(12),164(7), 144(28),141(39), 103(66),101(31), 95(42),87(100),74(54), 71(45),69(27),61(24), 59(38),55(38),43(88), 41(44)		

180*	1758	$C_{14}H_{20}O_5$		268	268(12%,M^+),239(17), 237(16),222(10), 209(18),208(77), 207(13),195(51), 179(100),147(14), 135(8),121(9),93(11), 91(10),79(10),77(9), 74(8),71(11),59(17), 55(23)	
181*	1764	$C_{12}H_{15}NO_3$		221	221(3%,M^+),179(2), 162(5),135(13),134(30), 105(100),77(38),51(13), 43(17)	siehe Nr. 162
182*					identisch mit 183 und kleine Menge unbekannter Verbindung	
183*	1767	$C_{11}H_{11}NO_2$		189	189(23%,M^+),130(100), 103(7),102(5),77(16), 65(5),51(7)	26,33,39, 43
184	1770	$C_{13}H_{18}O_5$	$H_3COOC{-}C_9H_{11}(OH){-}COOCH_3$?	254?	254(11%,M^+),222(37), 194(19),191(53), 190(68),180(56), 162(31),149(47), 148(37),147(32), 135(17),134(19), 121(52),120(58), 107(77),106(78), 93(100),91(70),80(38), 79(95),77(34),67(36), 59(35),55(26)	

(Fortsetzung S. 304)

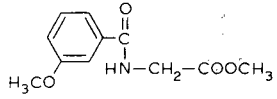
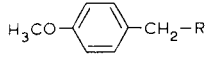
TABELLE I *(Fortsetzung)*

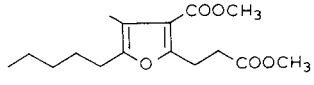
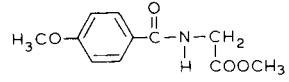
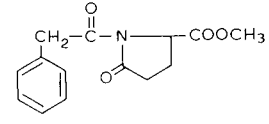
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
185★	1797	$C_8H_{10}N_4O_2$	Coffein	194	194(100%,M^+),165(7),137(8),109(61),82(27),67(37),55(44),42(18)	8,56	
186★	1803	$C_{16}H_{32}O_2$	CH_3—$(CH_2)_{13}$—$COOCH_3$	256	256(12%,M^+),225(5),213(7),143(12),87(69),74(100)	57	
187	1807	$C_{14}H_{16}O_3$		232	232(33%,M^+),172(30),171(16),159(100),145(4),128(4),116(7),115(8),84(5),71(4)		
188	1813	$C_{13}H_{18}O_5$		254	254(93%,M^+),239(18),223(11),195(10),194(7),181(100),179(74),151(18),137(11),136(9)		
189	1827	$C_{14}H_{22}O_7$		302?	211(4%),187(23),179(3),155(13),145(26),127(7),115(100),113(10),95(4),87(7),85(31),59(13),55(26)		
190★	1834	$C_{12}H_{14}O_4$		222	222(100%,M^+),207(18),191(50),179(8),164(11),163(14),147(15),133(9),119(12),91(15),77(14),51(19)	33	

191*	1845	$C_{10}H_{11}NO_6$		241	241(10%,M^+),210(4), 182(50),153(100), 146(8),105(7),77(6), 66(10)		
192	1861	$C_9H_{12}N_4O_3$	Tetramethylharnsäure	224	224(100%,M^+),209(18), 195(14),194(12), 181(17),111(11),83(45), 70(25),67(14)	56	
193	1877	$C_{13}H_{17}NO_2$		219	219(100%,M^+),204(23), 190(46),188(14), 176(16),173(12), 161(14),143(11), 133(30),90(12)		
194	1880			272?	241(5%),223(2),199(9), 181(3),167(4),143(100), 121(8),116(25), 111(60),101(13), 83(29),74(15),69(18), 59(19),55(40)		Strukturteil iso-Citronensäure
195	1887				241(4%),223(2),199(9), 181(2),172(3),167(4), 151(5),149(4), 143(100),121(9), 116(31),111(67), 101(14),83(28), 74(10),69(19),59(19), 55(40)		Isomer mit Verb. 194
196*	1889	$C_{12}H_{11}NO_3$ *		217	217(11%,M^+),187(3), 159(100),143(15), 130(12),115(16), 102(14)	58	

(Fortsetzung S. 306)

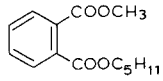
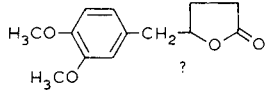
TABELLE I *(Fortsetzung)*

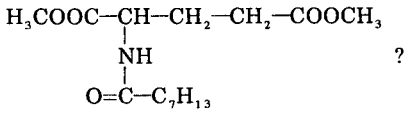
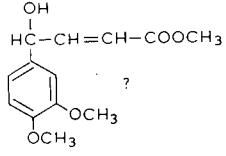
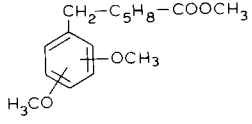
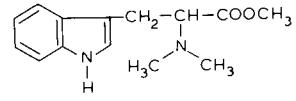
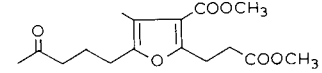
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
197	1893	$C_{11}H_{13}NO_4$		223	223(26%,M^+),191(3), 164(10),135(100), 107(22),92(12),77(14)	8,14	
198	1894	$C_{12}H_{24}O_2S$		232	232(100%),204(18), 200(78),172(17),144(8), 140(24),119(25), 114(16),112(36), 87(37),82(16),59(50), 55(20)		
199*	1910	$C_{17}H_{34}O_2$	$H_3C-(CH_2)_{14}-COOCH_3$	270?	270(9%,M^+),239(4), 227(6),143(11), 87(66),74(100)	8,14,59	
200	1917	$C_{13}H_{17}NO_5$		267	267(2%,M^+),236(3), 221(4),208(4),192(12), 176(5),161(5),121(100)		
201	1934	$C_9H_{12}N_4O_3$	Tetramethylharnsäure	224	224(100%,M^+),209(55), 167(10),152(8),139(9), 135(10),124(24), 109(11),83(89),82(25), 67(28)	56	
202	1937		Stearinsäure und unbekannte Verbindung	298?	145(70%),113(100), 87(25),74(64),68(40), 67(62),59(29),55(23)	57	

203★	1938	$C_{16}H_{24}O_5$★		296	296(18%,M^+),265(16), 239(30),236(71), 223(34),207(19), 179(100),166(8), 147(12),135(9), 121(7),105(5),91(7), 77(6),55(17)	8,60	siehe Nr. 180
204	1938				274(4%),246(100), 218(7),186(42), 154(3),128(10),126(5), 100(4),68(8),59(9), 42(12)		
205	1940	$C_{11}H_{13}NO_4$		223	223(14%,M^+),191(3), 164(5),135(100), 107(7),92(12),77(14)	8,38	
206	1944	$C_{15}H_{22}O_6$	$C_9H_{13}(COOCH_3)_3$	298	266(10%,M^+–32), 251(9),249(9),248(8), 234(30),207(23), 206(32),202(100), 191(43),175(39), 174(53),161(14), 147(69),146(54), 133(24),119(27), 105(47),91(89),65(31), 59(57),55(50)		
207	1971	$C_{12}H_{19}O_3$	Lacton	211?	85(100%),74(12), 55(22)		
208★	1988	$C_{14}H_{15}NO_4$		261	261(4%,M^+),202(2), 144(3),118(100), 110(2),91(24),90(10), 84(8),82(4),65(10)		

(Fortsetzung S. 308)

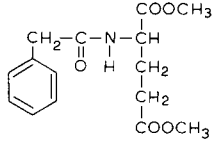
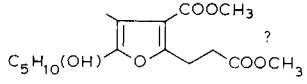
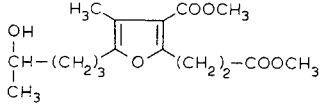
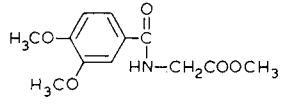
TABELLE I *(Fortsetzung)*

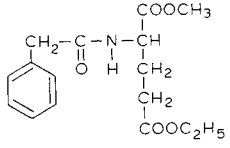
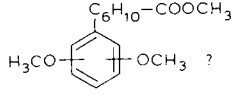
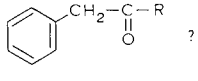
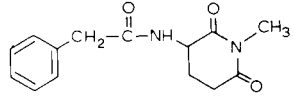
Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
209	1998	$C_{14}H_{18}O_4$		250	250(4%,M^+),218(3), 181(22),163(100), 149(45),135(8), 112(12),83(11), 77(14),70(32)		
210	2005	$C_{16}H_{24}O_5$?	$H_3COOC{-}C_{12}H_{17}(OH){-}COOCH_3$?	296	278(18%,M^+–18), 247(43),246(37), 237(28),214(81), 205(22),204(33), 173(55),169(43), 145(42),131(76), 130(57),129(40), 117(100),115(39), 105(38),91(61),74(38), 59(42)		
211	2007	$C_{13}H_{16}O_4$		236	236(18%,M^+),151(100), 85(18)		
212	2014	$C_{14}H_{20}O_7$?	$C_8H_{10}(OH)(COOCH_3)_3$	300	269(17%,M^+–31), 268(20),240(8), 236(48),209(37), 208(34),205(23), 204(48),177(31), 176(60),162(23), 154(24),149(25), 135(32),121(31), 107(42),93(54),81(68), 79(47),69(100),59(73), 55(52),41(71),15(98)		

213	2031	$C_{15}H_{25}NO_5$	 ?	299	299(4%,M^+),284(17), 270(29),176(14), 174(12),158(10), 144(50),142(27), 116(100),110(16), 98(18),84(58),55(95)	
214	2031	$C_{13}H_{16}O_5$ *	 ?	252	252(74%,M^+),220(17), 196(8),191(6),178(25), 167(100),164(43), 137(22),109(10), 81(11),73(14)	
215	2038	$C_{16}H_{22}O_4$?		278	278(63%,M^+),247(11), 246(13),218(12), 191(14),189(11), 151(100),150(65), 138(10),123(9), 122(11),91(18)	
216	2053				190(100%),162(38), 134(39),125(34), 112(13),91(17)	
217*	2056	$C_{14}H_{18}N_2O_2$		246	246(4%,M^+),187(10), 143(12),130(29), 116(100),111(8), 101(10),77(7)	55
218	2100	$C_{16}H_{22}O_6$		310	310(7%,M^+),281(8), 279(8),252(40), 237(10),221(14), 207(15),193(23), 192(100),179(57), 147(22),73(18), 55(24),43(31)	

(Fortsetzung S. 310)

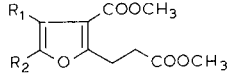
TABELLE I *(Fortsetzung)*

Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
219	2101	$C_{19}H_{36}O_2$	$H_3C-(CH_2)_7-CH$ $\|\|$ $H_3COOC-(CH_2)_7-CH$	296	296(3%,M^+),265(8), 264(21),232(4),180(8), 97(34),87(30),83(42), 74(46),69(69),55(100), 43(33),41(75)	63	
220*	2110	$C_{15}H_{19}NO_5$		293	293(5%,M^+),261(4), 234(17),202(16), 174(28),144(23), 142(43),118(19), 116(64),110(17), 98(16),91(100),84(31), 82(18),65(18)	3,8,38	
221	2110	$C_{16}H_{24}O_6$		312	312(18%,M^+),294(23), 281(20),265(24), 234(43),221(50), 207(48),206(29), 205(30),179(100), 166(22),147(33), 91(35),73(53),55(42)		
222	2119	$C_{16}H_{24}O_6$		312	312(12%,M^+),281(14), 252(30),239(15), 235(16),207(30), 194(25),192(64), 179(100),166(17), 147(25),115(21), 91(12),73(11),55(23)		
223*	2121	$C_{12}H_{15}NO_5$		253	253(23%,M^+),221(4), 165(100),137(8)		

224	2152	$C_{14}H_{14}O_4$?		246?	246(100%,M^+),231(7), 215(53),203(11), 187(17),172(10), 157(8),129(10), 115(9),101(11)		
225	2173	$C_{16}H_{21}NO_5$		307	307(4%,M^+),275(3), 261(2),248(11), 216(12),188(21), 144(31),142(40), 130(43),118(19), 110(17),91(100), 84(43),65(17)	3,8,38	
226	2183	$C_9H_{12}N_4O_3$	Tetramethylharnsäure	224	224(100%,M^+),167(28), 139(39),82(82),69(24), 67(52),42(22)	56	
227	2205	$C_{16}H_{22}O_4$		278	278(28%,M^+),151(100), 150(29)		Isomer mit Verb. 215
228	2208			350?	350(11%,M^+),318(2), 223(14),222(11), 118(62),98(12),92(34), 91(89),84(100),65(22)		Gemisch
229	2216	$C_{14}H_{16}N_2O_3$		260	260(17%,M^+),169(64), 141(19),118(74), 98(14),92(42),91(100), 84(96),65(27)		

(Fortsetzung S. 312)

TABELLE I *(Fortsetzung)*

Nr.	RI	Summenformel	Strukturformel	MG	Massenspektrum	Literatur	Bemerkungen
230	2229	$C_{19}H_{30}O$	Steroid als Sulfat vorliegend	274	274(24%, M^+), 259(43), 242(58), 236(55), 223(80), 210(20), 195(45), 149(51), 148(64), 107(62), 94(100), 93(79), 91(60), 81(49), 79(84), 77(40), 67(34), 55(41)		
231	2241	$C_{18}H_{28}O_6$		340	340(17%, M^+), 309(28), 280(64), 277(10), 267(17), 266(24), 248(9), 239(17), 236(45), 235(27), 223(51), 207(28), 195(30), 193(47), 179(100), 147(20), 101(14), 100(13), 91(15), 59(25), 55(32)		

*Struktur die durch Vergleichsmessung eindeutig abgesichert ist.

DISKUSSION

Mit den hier vorgeführten Aufarbeitungs- und Trennverfahren lassen sich etwa 500 saure Komponenten im Harn nachweisen. Etwa 2/5 davon konnten durch Aufnahme von Massenspektren charakterisiert werden. Sicher ist die Zahl der im Harn enthaltenen gaschromatographisch erfassbaren Verbindungen noch weit höher, wenn Trenn- und Nachweisverfahren noch verbessert werden.

Die Zahl der fassbaren Komponenten hängt auch sehr von der Art der Aufarbeitung und Derivatisierung ab. Beispielsweise wurden bei der hier angewandten Aufarbeitung auch Phenole und Aminosäuren gefunden, die bei anderen Verfahren abtrennbar sind, manchmal erfolgt bei der Derivatisierung nur eine teilweise Umsetzung oder eine, die verschiedene Produkte ergibt, so dass eine ursprünglich vorhandene Komponente durch zwei oder mehrere Peaks angezeigt wird (Beispiel: Harnsäure).

Umgekehrt werden bei der Derivatisierung aromatische Säuren, die als Phenole und Phenolmethylether auftreten können, nur in Form ihrer Phenolmethylether gefasst. Diese Nachteile der Derivatisierungsverfahren wurden bewusst in Kauf genommen, da die Massenspektren der unbekannten Stoffe als Methylderivate wesentlich leichter auswertbar sind als in Form anderer Derivate.

Im wesentlichen gehören die aufgefundenen Verbindungen den folgenden Verbindungsklassen an: Monocarbonsäuren; Dicarbonsäuren; Tricarbonsäuren; Hydroxysäuren; Lactone; Dihydroxysäuren; Ketosäuren; Aromatische Säuren; Heterocyclische Säuren, insbesondere Furancarbonsäuren; Aminosäuren; Konjugate von Aminosäuren; Phenole und verschiedenartige Verbindungen.

Als Harnbestandteile sind die gesättigten Monocarbonsäuren Pentadecansäure (Nr. 186), Palmitinsäure (Nr. 199) und die ungesättigte Ölsäure (Nr. 219) schon lange bekannt. Auch Stearinsäure wird häufig gefunden (Nr. 202).

Da das Aufarbeitungsverfahren vorzugsweise zur Erfassung schwer flüchtiger Verbindungen angelegt war, wurden von den gesättigten unverzweigten Dicarbonsäuren Oxalsäure und Malonsäure nicht erfasst. Gefunden wurden aber die schon als Harnbestandteile bekannten gesättigten Dicarbonsäuren von der Bernsteinsäure (Nr. 10) über die Glutarsäure (Nr. 26), Adipinsäure (Nr. 40), Pimelinsäure (Nr. 62), Korksäure (Nr. 86) und Azelainsäure (Nr. 125) bis zur Sebacinsäure (Nr. 150). Bekannt ist auch das Vorkommen von Dicarbonsäuren mit einer Verzweigung in Stellung 3. Nachweisbar waren die 3-Methylglutarsäure (Nr. 30), die 3-Methyladipinsäure (Nr. 50), die 3-Methylpimelinsäure (Nr. 72) und die 3-Methylkorksäure (Nr. 104).

Von zwei Methylcarbonsäuren war bisher nur die 2-Methylbernsteinsäure (Nr. 16) als Harnbestandteil beschrieben. Offenbar enthält Harn aber auch die höheren homologe 2-Methyladipinsäure (Nr. 47), sowie die 2,4-Dimethyladipinsäure (Nr. 52) und wahrscheinlich 2,4-Dimethylpimelinsäure (Nr. 79), die alle durch charakteristische Ionen der Masse 88 in ihren Massenspektren gekennzeichnet sind.

Darüber hinaus sind weitere gesättigte Dicarbonsäuren mit Seitenketten vorhanden (Nr. 11, 42, 48, 53, 67, 75, 82, 92, 95, 98, 109, 117, 137, 138, 148), von denen bisher nur Ethylmalonsäure (Nr. 11) eindeutig identifi-

ziert werden konnte. Die hohen Schlüsselionen der Masse 116 in den Spektren der gesättigten Dicarbonsäuren Nr. 75, 109 und 114 deuten auf das mögliche Vorliegen von Säuren mit einem C_3-Substituenten an C-2. Eine Bestätigung dieser Annahme durch Synthese und Aufnahme von Vergleichsspektren steht allerdings noch aus.

Neben den gesättigten Säuren enthält Harn in kleiner Menge etwa 30 ungesättigte Dicarbonsäuren, von denen bisher nur wenige bekannt waren. Die Strukturableitung der unbekannten Säuren ist nur durch Synthese- und Vergleichsmessungen möglich. Bisher steht lediglich fest, dass es sich nicht um einfache α, β-ungesättigte Dicarbonsäuren handelt. Auch α-Methyl-verzweigte ungesättigte Säuren wurden gefunden (Nr. 111, 139).

Als dreifach ungesättigte Verbindung wurde die Verbindung Nr. 153 identifiziert. Eine dazu isomere Verbindung Nr. 144 tritt ebenfalls auf.

Hydroxymonocarbonsäuren wie die β-Hydroxy-isovaleriansäure (Nr. 2) sind schon von früheren Untersuchungen als Harnausscheidungsprodukte bekannt, für einige weitere (Nr. 3 und 4) konnten hier Strukturvorschläge gemacht werden. Nachteilig wirkt sich aus, dass gesättigte β-Hydroxysäuren (Nr. 19, 45) so leicht im Massenspektrometer ein Schlüsselion der Masse 103 bilden, dass keine Molekülionen erkennbar sind. Eine starke Anreicherung dieser Säuren haben wir bei einer bisher nicht identifizierten Stoffwechselerkrankung gefunden [61].

Eine grosse Gruppe von Verbindungen stellen die Hydroxydicarbonsäuren dar, von denen bisher nur α-Methyläpfelsäure (Nr. 25) und β-Hydroxy-β-methyl-glutarsäure (Nr. 38) als Harnkomponenten bekannt waren. Die Strukturaufklärung einer Reihe höher alkylsubstituierter β-Alkyläpfelsäuren (Nr. 35, 48, 68) und β-Hydroxy-glutarsäuren (Nr. 76) wird an anderer Stelle beschrieben [64], gemeinsam mit der Strukturaufklärung einer Reihe höherer β-Hydroxytricarbonsäuren (Nr. 112, 115, 120), die verwandtschaftliche Beziehung zur Citronensäure (Nr. 91) und Isocitronensäure (Nr. 94) zeigen. Zur gleichen Gruppe von Verbindungen dürften auch die in Peak Nr. 100 enthaltenen Verunreinigungen mit den Schlüsselionen der Masse 189, 157 und 125 sowie die Säuren Nr. 105, 174, 189, 194, 195 und 202 gehören, von denen die Verbindungen Nr. 174 und 189 noch eine zusätzliche Doppelbindung enthalten dürften. Dicarbonsäuren mit unbekannter Stellung der Hydroxylgruppe und zusätzlichen Doppelbindungen dürften die Säuren Nr. 90, 101, 146, 154, 155, 163, 171, 174, 179, 184 und 210 sein.

Eine Dihydroxymonocarbonsäurestruktur könnte den Verbindungen zukommen, deren Spektren durch ein intensives Schlüsselion der Masse 75 gekennzeichnet sind (1, 28, 126, 131 und 148). Als Dihydroxydicarbonsäuren wurden D,L-Weinsäure (Nr. 36), ihr Methyl- (Nr. 43) und ihr Dimethylether (Nr. 44), die offenbar bei der Methylierung von Weinsäure durch Alkylierung der Hydroxylgruppen entstehen, identifiziert.

Eine weitere Gruppe von Verbindungen ist durch intensive Schlüsselionen der Masse 85 bzw. 99 gekennzeichnet (Nr. 21, 54, 60, 168, 207). Bei diesen sollte es sich um γ- bzw. δ-Lactone handeln, die möglicherweise aus entsprechenden Hydroxysäuren gebildet wurden. Dieser Verbindungsklasse gehört offenbar auch die Verbindung Nr. 211 an.

Die hier angewandte Methode der Aufarbeitung ist nicht zur Erfassung von

Ketosäuren geeignet. Daher konnte neben Lävulinsäure (Nr. 7) und der 2-Keto-glutarsäure in Form ihres Enol-methylesters (Nr. 56) nur eine weitere Säure erfasst werden, die wahrscheinlich eine Ketosäure darstellt (Nr. 89).

Eine umfangreiche Gruppe stellen die aromatischen und heteroaromatischen Verbindungen dar, die wohl grossteils mit der Nahrung in den Körper gelangen (Nr. 8, 17, 20, 24, 27, 32, 41, 63, 64, 70, 78, 83, 87, 88, 102, 106, 116, 124, 127, 128, 130, 133, 135, 136, 141, 142, 145, 149, 151, 156, 157, 161, 162, 165, 166, 167, 175, 176, 183, 190, 193, 196, 197, 200, 204, 205, 208, 209, 211, 214, 215, 217, 223, 224, 225, 227, 228, und 229).

Bisher waren nur wenige Furancarbonsäuren als Harnbestandteile bekannt, wie die Furan-2,5-dicarbonsäure (Nr. 71), die Furan-2-carbonsäure und deren Glycin-Konjugat, sowie die 5-Hydroxymethyl-furan-2-carbonsäure (Nr. 57). Im Zuge dieser Untersuchungen wurden weitere Furancarbonsäuren entdeckt, z.B. konnte das Auftreten der Furylessigsäure (Nr. 46) und der 5-Methyl-furan-2-carbonsäure (Nr. 18) und des 5-Carboxy-2-furyl-glycins (Nr. 191) nachgewiesen werden. Interesse verdient vor allem eine Klasse von tetrasubstituierten Furandicarbonsäuren, über deren Strukturaufklärung an anderer Stelle gesondert berichtet wird [65] (Nr. 180, 188, 203, 218, 221, 222, 231). Auch die Verbindungen Nr. 99 und 169 dürften Furanderivate sein.

Bei der Methylierung mit Diazomethan werden Aminosäuren nicht nur verestert, sondern auch an der Aminogruppe methyliert, so dass Mono- und Dimethylaminosäureester gebildet werden, wie z.B. beim Leucin (Nr. 12 und 23). Als Dimethylaminoverbindungen wurden auch Tyrosin (Nr. 167) und Tryptophan (Nr. 217) gefunden. Interessant ist das Vorliegen einer acetylierten Aminosäure, der wahrscheinlich die Struktur einer N-Acetyl-α-amino-octansäure (Nr. 134) zukommt. Auch die Verbindung Nr. 58 dürfte eine Aminosäure sein. Als aromatische Aminosäure wurde Anthranilsäure (Nr. 63) gefunden. Möglicherweise hat die Verbindung Nr. 65 eine ähnliche Struktur.

Eine Reihe von Säuren liegt in Form von Konjugaten mit Glycin vor, wie die Methylcrotonsäure (Nr. 80), die Hippursäure in Form des Methyl- (Nr. 162), des Ethyl- (Nr. 175) und des Propylesters (Nr. 181), ihr N-Methylderivat (Nr. 151), ein Artefakt, sowie ihr *m*- und *p*-Hydroxyderivat (die Hydroxylgruppe wird bei der Aufarbeitung ebenfalls methyliert, Nr. 197 und 205), und ihr 3,4-Dihydroxyderivat (Nr. 223), sowie die Furan-2-carbonsäure (Nr. 110) und die Furan-2,5-dicarbonsäure (Nr. 191). Unbekannt war bisher das Auftreten des Konjugates der α-Picolinsäure (Nr. 157).

Phenylessigsäure bildet mit verschiedenen Aminosäuren Konjugate, z.B. mit Glutaminsäure, das als Dimethyl- (Nr. 220) und Methylethylester (Nr. 225) gefunden wurde.

Bisher unbekannt geblieben scheint das Vorhandensein der Pyroglutaminsäure als Konjugate der Phenylessigsäure (Nr. 208). Glutaminsäure bildet auch ein Konjugat mit einer $C_7H_{13}COOH$-Säure (Nr. 213).

Ein Konjugat dürfte auch in der Verbindung Nr. 147 und 229 vorliegen.

ZUSAMMENFASSUNG

Die in der Säurefraktion des Harns enthaltenen Komponenten wurden nach

Veresterung mit Diazomethan an Dünnschichtplatten in acht Fraktionen getrennt. Jede Fraktion wurde in der Kombination Glaskapillargaschromatograph—Massenspektrometer untersucht. Auf diese Weise konnten etwa 500 Verbindungen nachgewiesen werden, etwa 200 liessen sich durch Aufnahme von Massenspektren charakterisieren. Die Retentionsindices und Schlüsselionen wurden tabelliert. Viele der aufgefundenen Verbindungen sind noch unbekannt.

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Biomedical Applications

CHROMBIO. 392

ÜBER DAS AUFTRETEN α-ALKYL-SUBSTITUIERTER ÄPFELSÄUREN UND β-HYDROXY-β-ALKYL-SUBSTITUIERTER DICARBON- UND TRICARBONSÄUREDERIVATE ALS NORMALE STOFFWECHSEL-PRODUKTE

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SUMMARY

Occurrence of α-alkyl-substituted malic acids, and β-hydroxy-β-alkyl-substituted dicarboxylic and tricarboxylic acid derivatives in normal urine

Urine contains a number of α-hydroxy acids so far unknown to occur in biological liquids. Besides the already as urine constituent known methylmalic acid, also the ethyl, isopropyl and butyl derivatives of malic acid were found. Further metabolites in urine are a β-propyl-substituted β-hydroxyglutaric acid, a β-hydroxy-β-[methyl-carbomethoxy]-adipinic acid and two isomeric α-methylcitric acids.

EINLEITUNG

Im Zuge eingehender Studien [1] über die in der sauren Fraktion menschlichen Harns enthaltenen Säuren mit Hilfe der Kombination Glaskapillargaschromatographie—Massenspektrometrie (GC—MS) stiessen wir auf eine Gruppe von Hydroxycarbonsäuren, von denen neben der Citronensäure bisher nur die α-Methyläpfelsäure und die β-Hydroxy-β-methylglutarsäure als normale Harninhaltsstoffe bekannt waren.

ISOLIERUNG UND GEWINNUNG

Durch präparative Dünnschichtchromatographie wurde die Gesamtsäurefraktion des Harns nach Veresterung mit Diazomethan in acht Zonen zerlegt [1]. Die Eluate dieser Zonen wurden mit der Kombination GC—MS untersucht. Die Hydroxysäuren waren — wie die Massenspektren zeigten — vorzugsweise in den Zonen DC 2, DC 3 und DC 4 (Ausschnitte der Glaskapillargaschromatogramme Fig. 1, 2 und 3) enthalten.

Die Eluate dieser Zonen wurden einer präparativen gaschromatographischen

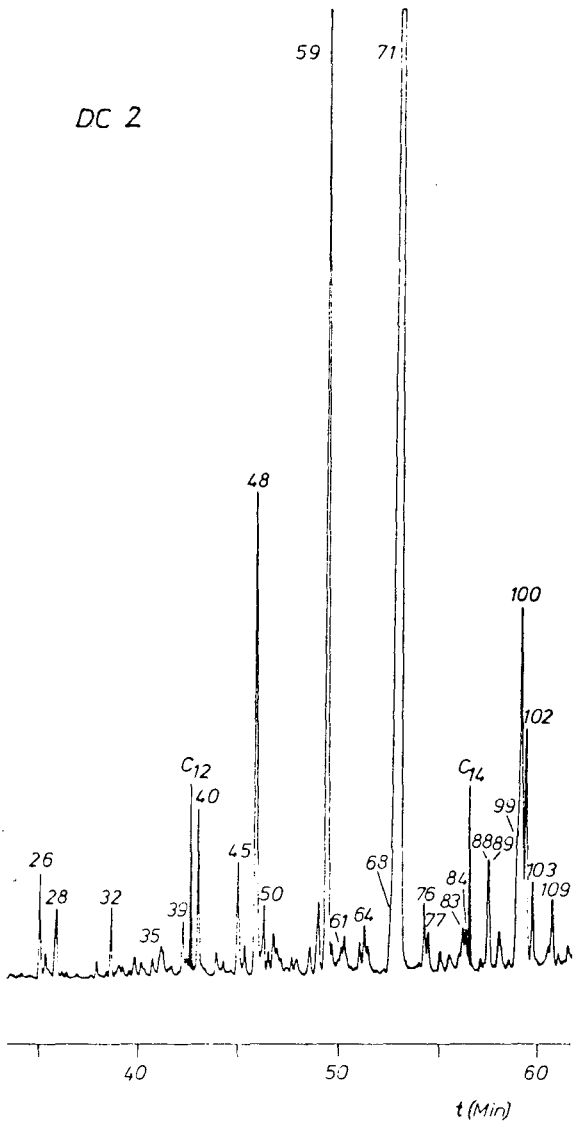


Fig. 1. Ausschnitt aus dem Gaschromatogramm der aus der Zone DC 2 isolierten Säuren, die durch Zahlen gekennzeichnet sind. Die zur Messung von Retentionsindices beigemengten Kohlenwasserstoffe sind als C_9, C_{10}, etc. markiert.

Trennung unterworfen. Die Anreicherung wurde durch Aufnahme von Glaskapillargaschromatogrammen überprüft. Die so erzielte weitgehende Anreicherung reichte aus, um von den wichtigsten Schlüsselbruchstücken durch die peak-matching Technik mit hochauflösender Massenspektrometrie Bruttoformeln zu bestimmen.

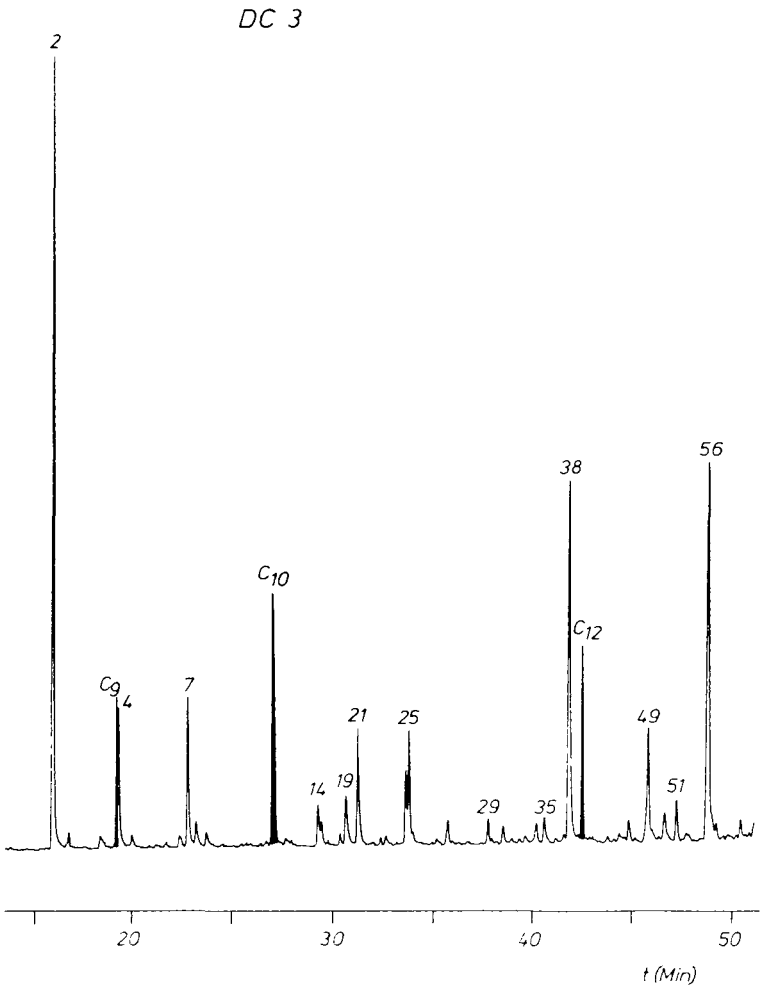


Fig. 2. Ausschnitt aus dem Gaschromatogramm der aus der Zone DC 3 isolierten Säuren.

DARSTELLUNG DER VERGLEICHSVERBINDUNGEN

2-Methyläpfelsäuredimethylester wurde aus Acetessigsäuredimethylester durch Cyanhydrinsynthese nach Barker [2] [F = 115° (Essigester)], 2-Ethyläpfelsäure aus Propionylessigsäuremethylester und NaCN nach Strassmann und Ceci [3] [F = 117° (Essigester)], Isopropyläpfelsäure aus Isobutylessigester und NaCN nach Yamashita [4] [F = 145—147°] und *n*-Butyläpfelsäure nach derselben Vorschrift dargestellt und ohne weitere Reinigung für die Vergleichsmessungen eingesetzt.

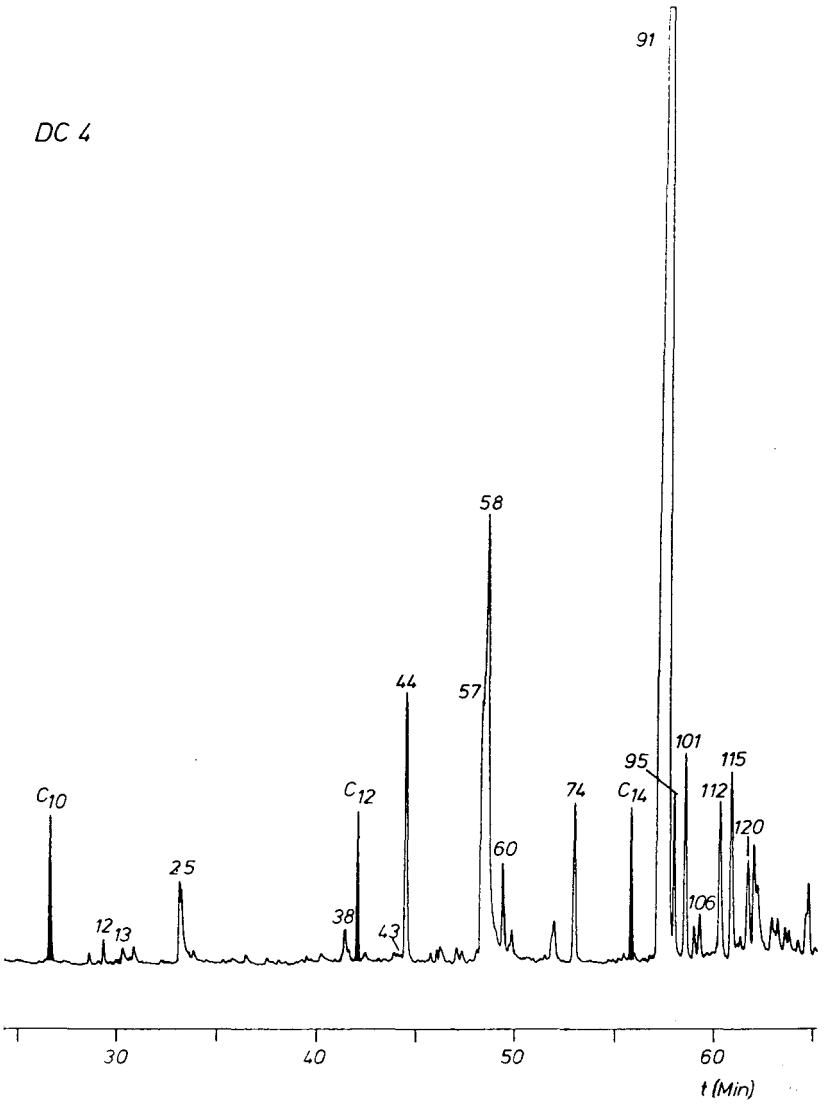


Fig. 3. Ausschnitt aus dem Gaschromatogramm der aus der Zone DC 4 isolierten Säuren.

AUFNAHMEBEDINGUNGEN DER MASSENSPEKTREN UND GASCHROMATOGRAMME

Massenspektrometer—Gaschromatograph

LKB-2091 Gerät mit getrennten Öldiffusionspumpen (150 l/sec Saugleistung) für Quelle und Einlass.

E.I.-Ionenquelle: 250° , Elektronenenergie 70 eV, Beschleunigungsspannung 3.5 kV, TIC-Signal bei 20 eV registriert. 20 m SE-30 Dünnfilmglaskapillarsäule mit Platinkapillarenanschluss. Injizierte Menge: 0.2—0.8 μl, Injektor-Tempera-

tur: 275°, Detektor-Temperatur: 275°, Ofen-Temperatur: 75°, 7 min isotherm, Temperaturprogramm: 2°/min bis 280°, Splitverhältnis: 1:20, Abschwächer (Attenuator): 32.

Separator: 2-stufiger Molekül-Jet-Separator (nach Becker-Ryhage) und "sliding valve" zur Trennung von GC- und MS-Teil, Temperatur: 250°.

Gaschromatograph: Pye-Unicam Ein-Säulengerät, Temperaturprogram: 2°/min. Säule: Dünnfilm-Glaskapillarsäule: 25 m, Trägergas: Helium (2 ml/min).

Datensystem: LKB 2130 Gaschromatograph—Massenspektrometer, PDP-11 Rechner (16-bit Memory) mit Disk-System der Fa. Digital Equipment Corporation, Sichtschirm Tektronix 4012 und Versatec-Plotter.

Präparativer Gaschromatograph

Carlo Erba Fractovap 2400 T. Säule: 1.5 m, 6 mm Durchmesser, gefüllt mit 3% SE-30 auf Supelcoport (80—100 mesh). Injizierte Menge: 30 µl, Injektor-Temperatur: 275°, Detektor-Temperatur: 275°, Ofen-Temperatur: 100°, Temperaturprogramm: 100°, 7 min isotherm 2°/min bis 280°, Splitverhältnis: 1:100. Abschwächer (Attenuator): 64.

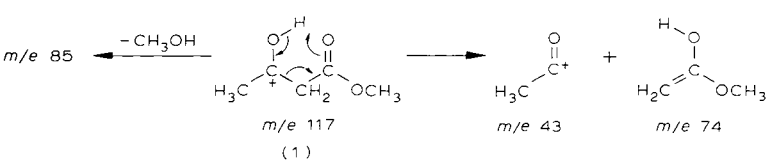
Gaschromatograph

Carlo Erba 2301 Doppelsäulengerät mit Flammenionisationsdetektor, Trägergas: Wasserstoff (2 ml/min).

ERGEBNISSE UND DISKUSSION

Das Massenspektrum der einfachsten Verbindung zeigt Schlüsselionen der Masse 15, 43, 85 und 117, die in den höheren Homologen (Verbindungen mit den Nummern von 35, 48 und 68) jeweils um 14, 28 bzw. 42 Masseneinheiten zu höheren Massen verschoben sind.

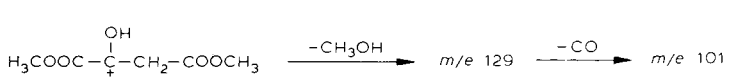
Das Schlüsselion der Masse 117 im Massenspektrum der mit Nr. 25 in dem Gaschromatogramm gekennzeichneten Verbindung (Fig. 4) hat die Summenformel $C_5H_9O_3$ (1) und wird wie folgt abgebaut:



(1)

Die höheren Homologen müssen anstelle des Methylrestes eine Ethylgruppe (Verbindung Nr. 35), bzw. einen C_3H_7- (Verbindung Nr. 48) oder einen C_4H_9-Rest (Verbindung Nr. 68) tragen (Fig. 5—7), da die Schlüsselionen um eine entsprechende Zahl von Masseneinheiten verschoben sind.

Die Ionen der Masse 161 (2) entstehen durch Abspaltung des Alkylrestes. Sie werden nach dem folgenden Schema weitergespalten:



(2)

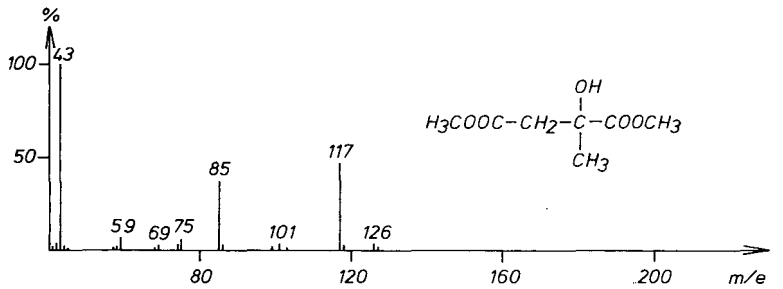


Fig. 4. Massenspektrum des Methyläpfelsäuredimethylesters.

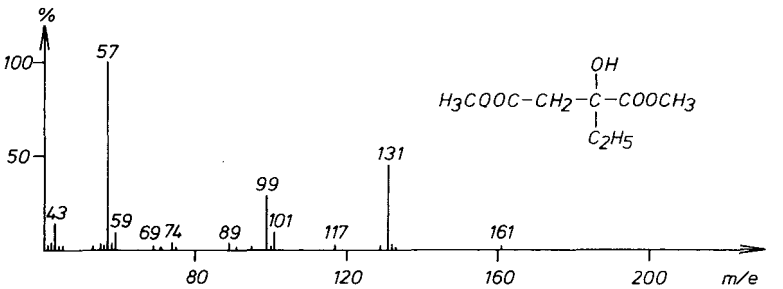


Fig. 5. Massenspektrum des Ethyläpfelsäuredimethylesters.

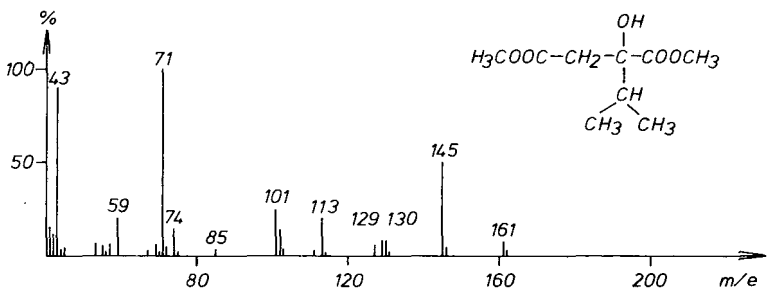


Fig. 6. Massenspektrum des Isopropyläpfelsäuredimethylesters.

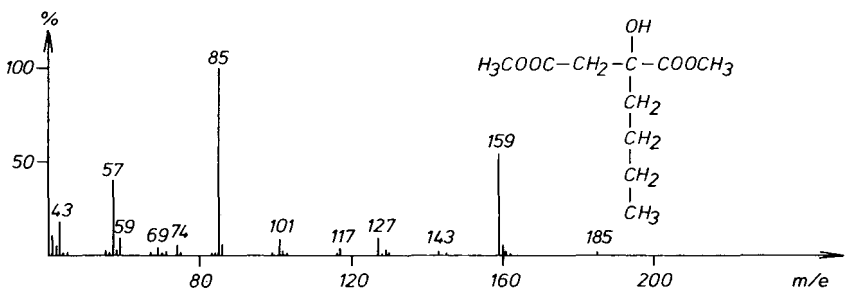


Fig. 7. Massenspektrum des Butyläpfelsäuredimethylesters.

Demnach ergibt sich für den einfachsten Vertreter der Reihe, für die Verbindung Nr. 25, die Struktur eines α-Methyläpfelsäuremethylesters [Retentionsindex (RI) 1075], für das nächst höhere Homologe die eines Ethyläpfelsäuremethylesters (RI 1181) und für die beiden anderen eines C_3H_7- bzw. C_4H_9-substituierten Äpfelsäuremethylesters (RI 1247, RI 1351).

Um die Struktur sicherzustellen, wurde α-Methyläpfelsäure nach Yamashita [4] aus Acetessigester und Acetylchlorid synthetisiert. Das Massenspektrum des Syntheseproduktes erwies sich identisch mit der aus Harn isolierten Verbindung. Auch die Retentionsindices von Natur- und Syntheseprodukten stimmen überein. Bei der Koinjektion beider Verbindungen in den Gaschromatographen erhielt man nur einen Peak, womit ihre Identität erwiesen ist.

In gleicher Weise wurde die Ethyläpfelsäure hergestellt [3], die sich mit dem Naturprodukt identisch erwies.

Das Massenspektrum der dritten homologen Verbindung Nr. 48, (Fig. 6) ist mit der Struktur einer Propyl- oder Isopropyläpfelsäure vereinbar. Im Vergleich zur Methyl- und Ethyläpfelsäure ist der Retentionsindex relativ erniedrigt, was bei Vorliegen einer Propyläpfelsäure nicht zu erwarten wäre. Der Verbindung sollte daher die Struktur einer Isopropyläpfelsäure zukommen. Die Synthese von Propyl- und Isopropyläpfelsäure [4] und nachfolgende Vergleichsmessungen von Massenspektren und Retentionsindices sowie die Koinjektionsprobe bestätigen diese Vermutung.

Das Spektrum der vierten Verbindung (Nr. 68) dieser Reihe entspricht dem *n*-Butyläpfelsäuredimethylester, wie durch Synthese und Vergleichsmessungen sichergestellt wurde.

Während bereits 1966 Dalgliesh et al. [5] über das Auftreten von Methyläpfelsäure im Harn berichtete, wurden homologe Verbindungen bisher nur in Mikroorganismen, Wein und Obst nachgewiesen [3, 6—18]. Das Auftreten der Isopropyläpfelsäure als Zwischenprodukt bei der Synthese von Leucin wurde von Strassmann und Mitarbeitern [19,20] postuliert, Isopropyläpfelsäure wurde unseres Wissens aber noch nicht in Körperflüssigkeiten nachgewiesen. Über Butyläpfelsäure als Naturprodukt liegen keinerlei Angaben vor.

Eine zweite Gruppe von Verbindungen (Nr. 38, 76) ist durch analoge Abbaureaktionen im Massenspektrometer wie der α-Alkyläpfelsäuren charakterisiert. Das Massenspektrum der ersten Verbindung dieser Reihe (Nr. 38, Fig. 8) ist wie das des Methyläpfelsäuremethylesters durch Schlüsselionen der Massen

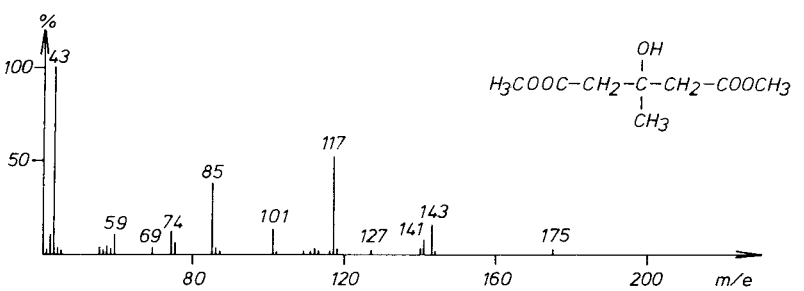
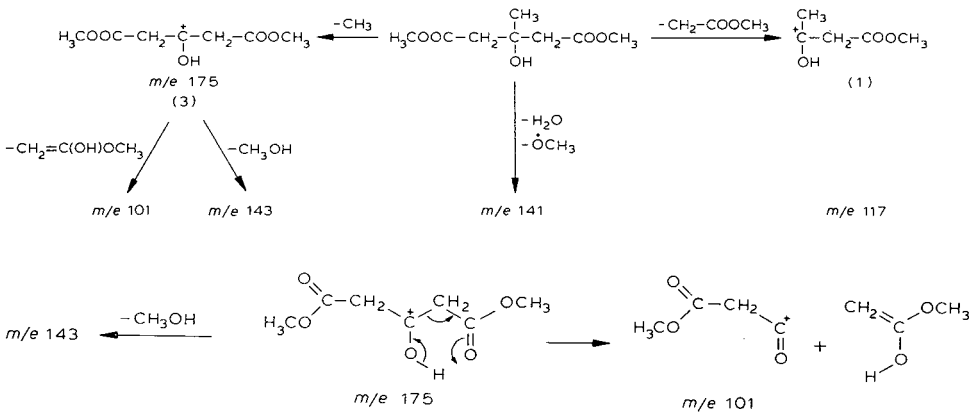


Fig. 8. Massenspektrum des β-Hydroxy-β-Methylglutarsäuredimethylesters.

117, 85 und 43 gekennzeichnet. Die Verbindung sollte demnach ebenfalls das Strukturelement (1) enthalten.



Aus dem Auftreten eines Schlüsselions der Masse 175, das offensichtlich dem Verlust einer Methylgruppe entspricht, lässt sich das Molekulargewicht 190 ermitteln. Der weitere Abbau des so charakterisierten β-Hydroxy-β-methyl-glutarsäuredimethylesters (RI 1191) geht aus den Spaltschemata hervor.

β-Hydroxy-β-methyl-glutarsäure wird bei der β-Hydroxy-methyl-glutarsäure-acidurie [21—26] in vermehrter Menge im Harn ausgeschieden, sie konnte aber auch von Dalgliesch et al. [5] und anderen [27, 28] im Urin Gesunder nachgewiesen werden.

Die zweite Verbindung dieser Klasse (Nr. 76, Fig. 9) hat in ihrem Massenspektrum ebenfalls ein Schlüsselion der Masse 175 (3). Aus dem Auftreten eines Ions der Masse 145 (4), das weiter zerfällt unter Verlust von Methanol

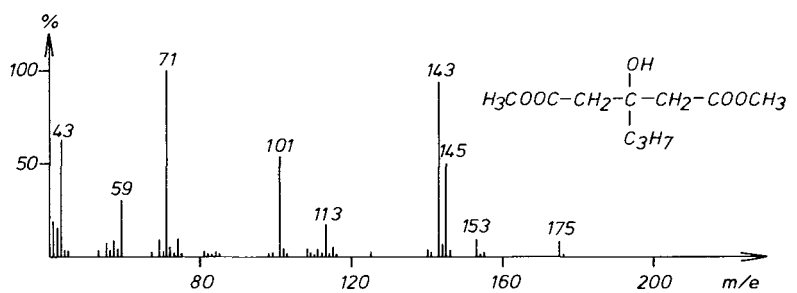
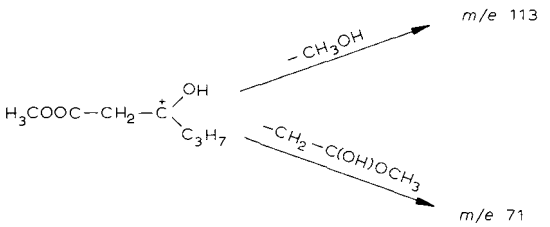
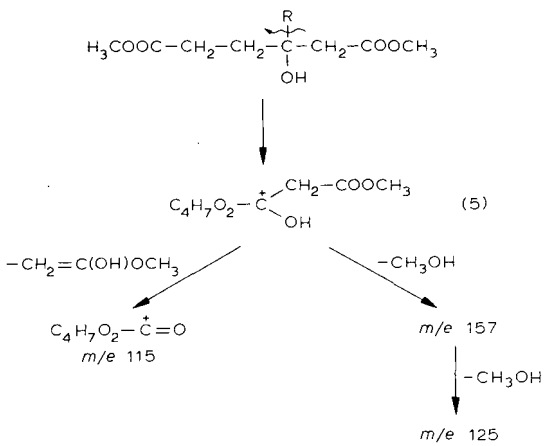


Fig. 9. Massenspektrum des β-Hydroxy-β-Propylglutarsäuredimethylesters.

und $CH_2=C(OH)OCH_3$ lässt sich schliessen, dass das Molekül das Molekulargewicht 145 + 73 (CH_2COOCH_3) = 218 hat, so dass es sich hierbei entweder um den β-Hydroxy-β-isopropyl-glutarsäuredimethylester oder β-Hydroxy-propyl-glutarsäuredimethylester handelt (RI 1367).



Bei der dritten Verbindung dieser Reihe (Nr. 112, Fig. 10) tritt als Schlüsselion im oberen Massenbereich das Fragment der Masse 189 der Bruttoformel $C_8H_{13}O_5$ (5) auf.



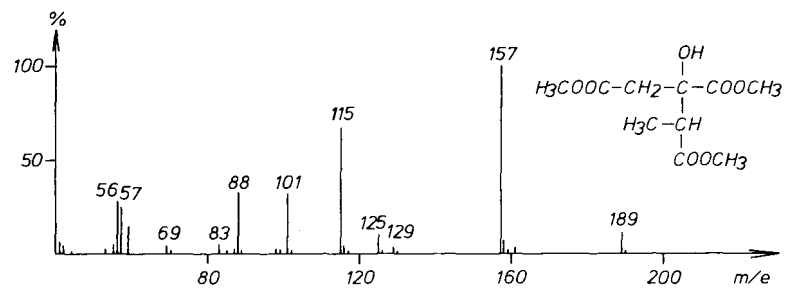
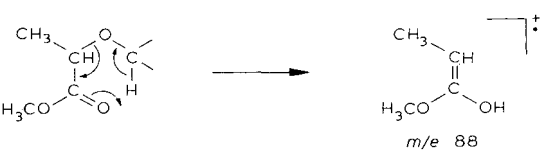


Fig. 10. Massenspektrum des α-Methyl-citronensäuretrimethylesters.

Der $C_4H_7O_2$-Teil ist durch das für α-Methylcarbonsäuremethylester typische Auftreten des Ions der Masse 88 erkennbar:



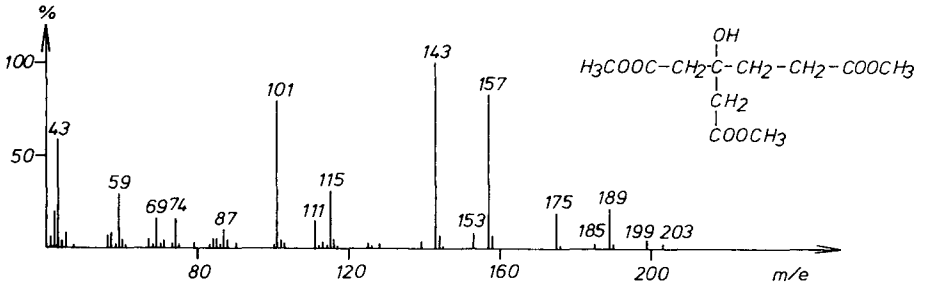


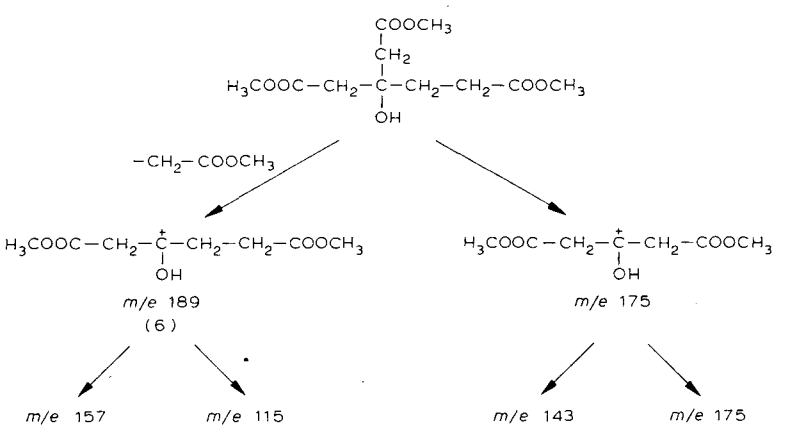
Fig. 11. Massenspektrum des β-Hydroxy-β-[carbomethoxy-methyl]-adipinsäuretrimethylesters.

Damit ergibt sich für die Verbindung die Struktur eines α-Methyl-citronensäuretrimethylesters (RI 1473).

Eine zweite Verbindung (Nr. 115), die ein völlig gleiches Massenspektrum, jedoch einen höheren Retentionsindex zeigt, muss eine zur Verbindung 112 diastereomere Struktur haben (RI 1482).

Eine Ausscheidung von α-Methylcitronensäure wurde bei Propionsäureacidurie beobachtet. Sie soll für diese Krankheit typisch sein. Als natürliches Stoffwechselprodukt konnte man α-Methylcitronensäure bisher nicht nachweisen [29, 30].

Eine weitere isomere Säure, deren Massenspektrum (Fig. 11) allerdings sehr unterschiedlich aussieht, ist die Verbindung Nr. 120. Aus dem Auftreten des Ions der Masse 189 und den dazugehörigen Abbauprodukten lässt sich das Vorliegen des Strukturelements (6) erkennen. Aus den übrigen Ionen lässt sich die Struktur eines β-Hydroxy-β-[carbomethoxy-methyl]-adipinsäuredimethylesters (RI 1496) ableiten:



ZUSAMMENFASSUNG

Harn enthält eine Reihe α-hydroxylierter Dicarbonsäuren, deren Vorkommen in biologischen Flüssigkeiten bisher nicht bekannt war. So finden sich

neben der als normales Stoffwechselprodukt bereits bekannten Methyläpfelsäure auch die Ethyl-, Isopropyl- und Butyläpfelsäure. Als weitere, offenbar bisher unbekannte Stoffwechselprodukte wurden eine β-propyl-substituierte β-Hydroxyglutarsäure, die β-Hydroxy-β-[methyl-carbomethoxy]-adipinsäure und zwei Isomere der α-Methylcitronensäure entdeckt.

DANK

Wir danken der Deutschen Forschungsgemeinschaft, dem Fonds der Chemischen Industrie und der Doktor Robert Pfleger-Stiftung für die Unterstützung durch Sachmittel. Herrn Dr. G. Remberg danken wir für die Messung der Hochauflösungswerte, Herrn Dr. J. Reiner für die Herstellung der Glaskapillarsäulen und Herrn W. Kern für seine tatkräftige Mitarbeit bei der experimentellen Arbeit.

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AUTOMATED QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF INTACT LIPIDS

II. ACCURACY, PRECISION AND REPRODUCIBILITY OF RESULTS

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SUMMARY

The effect of various factors on the precision and accuracy of the gas chromatographic determination of neutral lipids was studied in the concentration range where the correction factors are dependent on the amount analyzed. The mutual effect of individual components of the neutral lipid spectrum on the recovery was examined. A method is described which provides the stable recovery of the components present at low concentrations, using the addition of high-molecular-weight triglyceride (triarachidin) which does not interfere in the determination of the usual triglycerides. The validity of the correction factors measured with pure compounds was verified by hydrogenation of biological samples of various compositions. Hydrogenation of the sample also solves the problem of the determination of the triglyceride fraction of carbon number 46, which interferes under normal conditions with the determination of the cholesteryl ester fraction of carbon number 47. A method for the standardization of the gas chromatographic determination of neutral lipids is given, using pure compounds instead of lyophilized biological samples.

Long-term quality control was carried out using synthetic control samples. The results show sufficiently low values of the variation coefficients over the whole period. The values of the variation coefficients measured over an interval of 25 weeks are about 4% for the main components of the neutral lipid spectrum and 6.3% for the components present at concentrations up to 5%. The within-day variation for the most neutral lipid fractions and for lipid classes attains a value of 40—75% of the day-to-day variation. The most satisfactory values are obtained for the variation within a single series which amounts to less than 2% for all substances except for triglyceride fractions 48 and 54. The correlation of the determination of total cholesterol and triglycerides by gas chromatography and by enzymatic methods shows a very good agreement between the results obtained by the two methods. Using quality control, it is possible to follow the accuracy of the calibration and to demonstrate objectively the necessity for system recalibration.

INTRODUCTION

The precision and accuracy of the results of gas chromatographic analyses have been discussed in a number of papers [1—6]. However, in all cases the concentration of the compounds was in the region where the weight correction factor is independent of the amount injected. The possibility of using quantitative gas chromatography for the determination of amounts in the nanogram range was first discussed by Kuksis et al. [4]. The effect of a larger peak preceding a smaller one on the recovery of the smaller peak in triglyceride analysis was also discussed [4]. The conditions for quantitative analysis of neutral lipids in the concentration range where the weight correction factor is a function of the amount analysed were studied and discussed in the previous part of this study [7].

The precision, accuracy and reproducibility of the results were studied especially with respect to the accuracy and stability of the correction factors, to the relation of the recovery of individual components and their concentration ratios and to the possibility of using the weight correction factors measured using saturated compounds for the analysis of biological samples.

The accuracy of the results was checked using model samples prepared from pure compounds; a comparison of the results measured gas chromatographically and by enzymatic methods [8, 9] using commercial Boehringer-Mannheim sets was evaluated statistically. The time dependence of the analytical results due to changes of the correction factor was studied using internal quality control by means of the model samples (the same as those used for the accuracy control). The accuracy of the results obtained with individual fractions of the lipid profile was also studied with respect to the determination of some minor triglyceride components, which are not separated from cholesteryl esters under the conditions used.

MATERIALS

All standards, solvents, stationary phases and supports were the same as those described in the previous paper [7]. The hydrogenation catalyst, PtO_2, was obtained from Merck (Darmstadt, G.F.R.). Reagent sets for the enzymatic determination of total cholesterol and triglycerides were supplied by Boehringer, Mannheim, G.F.R. (cat. Nos. 172626 and 126012, respectively). Hexane, p.a., obtained from Lachema (Brno, Czechoslovakia), was distilled before use.

METHODS

Apparatus and operating conditions

In addition to the instruments described in the previous paper [7], a Perkin-Elmer F-17 gas chromatograph with a Perkin-Elmer Sigma 10 Laboratory Data System which was equipped with a Teletype 33 ASR-FR Terminal (Teleprint, Frankfurt, G.F.R.) was used for the chromatographic analyses. Samples were injected manually using an 85-N Hamilton microsyringe. The gas chromatograph was equipped with glass-lined stainless-steel columns (Supelco,

Bellefonte, Pa., U.S.A.), 0.6 m × 1.8 mm I.D. Other analytical conditions were the same as described previously [7].

Preparation of the control samples

Model mixtures were prepared from the stock solutions of the individual compounds used for calibration. The concentration of all stock solutions was 1 mg/ml in a solvent mixture of isooctane—chloroform (80:20, v/v). Such solutions are stable for several months when stored at 4°.

After all components had been pipetted the samples were dried, redissolved in a corresponding volume of the internal standard solution, divided into 1-ml aliquots and dried again. The control samples were stored at −20° and redissolved in the isooctane—chloroform solvent mixture before analysis. The biological samples were hydrogenated as described in the literature [10].

Mutual effect of components on the recovery of individual compounds of the neutral lipid spectrum

A 20-μl volume of the stock solution of the component being studied and 1000 μl of the internal standard solution were pipetted into a glass microvial. The sample was dried under nitrogen (temperature of the water-bath about 60°) and redissolved in 1000 μl of the isooctane—chloroform (80:20, v/v) mixture. After 1 μl of the sample containing 20 ng of the component under study and 200 ng of the internal standard had been injected into the gas chromatograph, 200 μl of a solution of the interfering component were added to the sample solution. The sample was dried, redissolved in the solvent mixture and analysed as described before. For the mathematical evaluation, the recovery of the component studied in the first analysis was calculated as 100%.

Recovery study of rac-glycerol-1,3-stearate-2-palmitate

Five samples containing 20 μl of the stock solution of the triglyceride and 1000 μl of the internal standard solution were dried under nitrogen, redissolved in the isooctane–chloroform (80:20, v/v) mixture and analysed. Then 200 μl of the triarachidin stock solution were added, the sample was dried and redissolved in the same way, and analysed again.

Quality control and statistical evaluation

The quality control was carried out using synthetic samples described before. In each run the first and the tenth samples were control samples. The reproducibility of the repeated analyses was calculated from six samples analysed in one run. Within-day and day-to-day variations were calculated on the basis of 28 duplicate control samples analysed over 14 weeks according to the formulae generally used [11]. Long-term reproducibility was evaluated from all the control samples analysed over a period of 25 weeks.

RESULTS AND DISCUSSION

The precision and accuracy of the results of neutral lipid analysis are dependent on the accurate and reproducible measurement of the weight correction factors (f_w) for individual components. The greatest reproducibility of

the correction factor measurement is required when f_w depends on the amount of the component analysed. It is known that especially in such cases the recovery of individual components is dependent on their concentration ratios [4]. Theoretically, the correction factors measured during calibration should be valid under the conditions of measurement. In practice, each measurement is made under different conditions, because of the different compositions of the biological samples. The selection of the optimum compounds and conditions for the calibration is very important. For the measurement of the correction factor of triglycerides, a number of different compounds, synthetic and natural, were used [3—5]. Natural compounds are chemically insufficiently stable; from an analytical point of view they are not exactly defined. However, calibration mixtures of natural origin are chemically more similar to the compounds being analysed in the biological samples. Using natural triglyceride mixtures it is possible to study neither the interactions between individual components of the sample and the chromatographic system, nor the dependence of the recovery of individual components on their concentration ratios. These relationships were studied using pure compounds — standards for the column calibration. It was found that a larger amount of one component increased the recovery of a second component present only in a low concentration, irrespective of the elution order. Some results are given in Fig. 1.

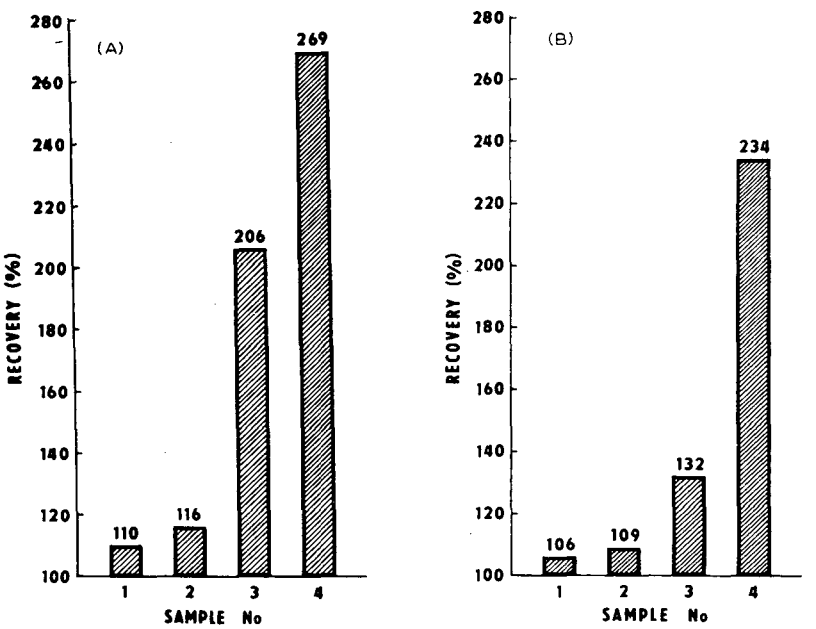


Fig. 1. Effect of some triglycerides on the recovery of tristearin (A) and *rac*-glycerol-1,3-stearate-2-palmitate (B). Composition of the samples injected: component studied (A) tristearin (20 ng), (B) *rac*-glycerol-1,3-stearate-2-palmitate (20 ng); internal standard, cholesteryl butyrate (200 ng); interfering components (200 ng). (A) tripalmitin (1), *rac*-glycerol-1,2-palmitate-3-stearate (2), *rac*-glycerol-1,3-stearate-2-palmitate (3), triarachidin (4); (B) tripalmitin (1), *rac*-glycerol-1,2-palmitate-3-stearate (2), tristearin (3), triarachidin (4). The recovery obtained with the sample without interfering components (20 ng of the component studied and 200 ng of the internal standard) was taken as 100%.

As shown in Fig. 1, the recovery is affected not only of the components eluted immediately one after another, but also of those with greater differences in elution times. The degree of such interference depends on the molecular weights of both components, also on their concentration ratio and on the amount of each injected. In the 20—200-ng range of the compound injected, the higher the molecular weights of both compounds (studied and interfering) and their concentration ratio, the greater is the effect on the recovery. This dependence decreases with increasing amounts of the test compound. For example, with amounts over 400 ng of tristearin or *rac*-glycerol-1,3-stearate 2-palmitate, the change in the recovery caused by any interfering compound did not exceed 5% at concentration ratios of the component studied to the interfering one of 1:10, 1:5, 1:2 and 1:1.

According to these experiments, the equilibrium state of the chromatographic system, which determines the recovery of the compounds, is influenced by the composition of the sample being analysed. The reason is probably a competitive saturation of the system by compounds with increasing molecular weight. The compound added in excess probably affects the physical and chemical properties of the stationary phase; the shift of the sorption equilibrium can lead to a higher recovery of the compound being analysed. This hypothesis was also supported by additional experiments in which the effect of triarachidin on the recovery of *rac*-glycerol-1,3-stearate-2-palmitate in repeated analyses was studied. The results are given in Fig. 2. Taking into account the results of further analyses of other neutral lipid components and their mutual influence, control samples of different compositions were prepared for the quality control. Using triarachidin, which is not usually present in biological samples, the mutual effect of individual cholesteryl esters and triglycerides on the recovery was practically eliminated. Under such conditions, the calibration data measured with pure compounds with constant concentration ratios are valid for the biological samples of variable composition.

A further problem studied was to check the usefulness of the correction factors measured with saturated compounds for the determination of biological

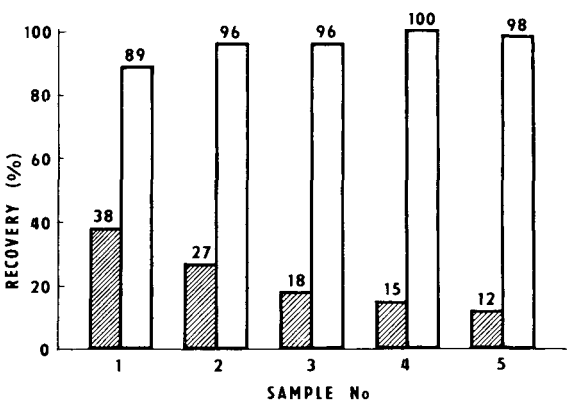


Fig. 2. Effect of triarachidin on the recovery of *rac*-glycerol-1,3-stearate-2-palmitate. Composition of the samples injected: ▨, *rac*-glycerol-1,3-stearate-2-palmitate, 20 ng, internal standard (cholesteryl butyrate), 200 ng; □, the same samples after the addition of 200 ng of triarachidin.

samples containing both saturated and unsaturated compounds. As known from the literature, the f_w values for compounds with an identical number of carbon atoms but with a different number of double bonds are different under various conditions [2, 12, 13]. Bezard and Bugaut [14] studied the dependence of f_w on the number of the double bonds and the amount analysed for the triglycerides with carbon number 54 (tristearin, triolein, trilinolein and trilinolenin). It was found that for all the triglycerides studied the f_w values increase with increasing number of double bonds and with decreasing amounts of the compound analysed. The course of the plot of f_w versus the amount analysed was similar for all triglycerides studied. In the case of the f_w values for tristearin and triolein, the smallest differences were observed in the whole range under investigation.

The f_w values are dependent not only on the chemical structure and amount of the compounds analysed, but also on the properties of the entire chromatographic system (especially on the column, construction of the injection port and the sampling technique). The validity of the correction factors calculated from the calibration data was checked over a wide range of plasma neutral lipid concentration using hydrogenation of the biological samples. The results obtained in the measurement of the correction factors for both saturated and unsaturated individual triglycerides and cholesteryl esters were also confirmed by the hydrogenation experiments.

The chromatograms of two typical samples with normal and elevated triglyceride levels before and after hydrogenation are shown in Fig. 3. The respective results are given in Table I.

As shown in Table I, there are no significant differences in the results when the correction factors measured with saturated compounds were used for the determination of unsaturated compounds. This finding is in agreement with the results of correction factor measurements for saturated and unsaturated compounds published by other authors [12—14]. The differences that appear with fractions of higher carbon number, especially 56 and 58, could be caused by the lower chemical stability of these compounds which contain a relatively high percentage of unsaturated fatty acids [15]. Part of the highly unsaturated triglycerides could be decomposed during the analysis [6]. However, the differences observed have only a small influence on the recovery of the total triglycerides. After hydrogenation, the individual fractions are more homogeneous, resulting in a narrower peak width and a better separation of the compounds. This can also influence the comparison of analyses of the same sample in hydrogenated and non-hydrogenated form. According to our results, columns with high efficiency (ΔC_{48-54} = 1.3—1.8) are suitable for the separation of non-hydrogenated samples [7]. When the column does not have sufficient efficiency, the result of integrating the non-hydrogenated sample can be different from that of the hydrogenated sample, because of insufficient separation. A final solution to the problem for triglycerides of carbon numbers 56 and 58 is possible only on the basis of analytical data measured with pure compounds. These measurements cannot be performed at present because these compounds are not commercially available. The experiences with the analysis of plasma neutral lipids cannot be generalized for the triglycerides with a high number of the double bonds without previous confirmation based on a detailed study using pure synthetic standards.

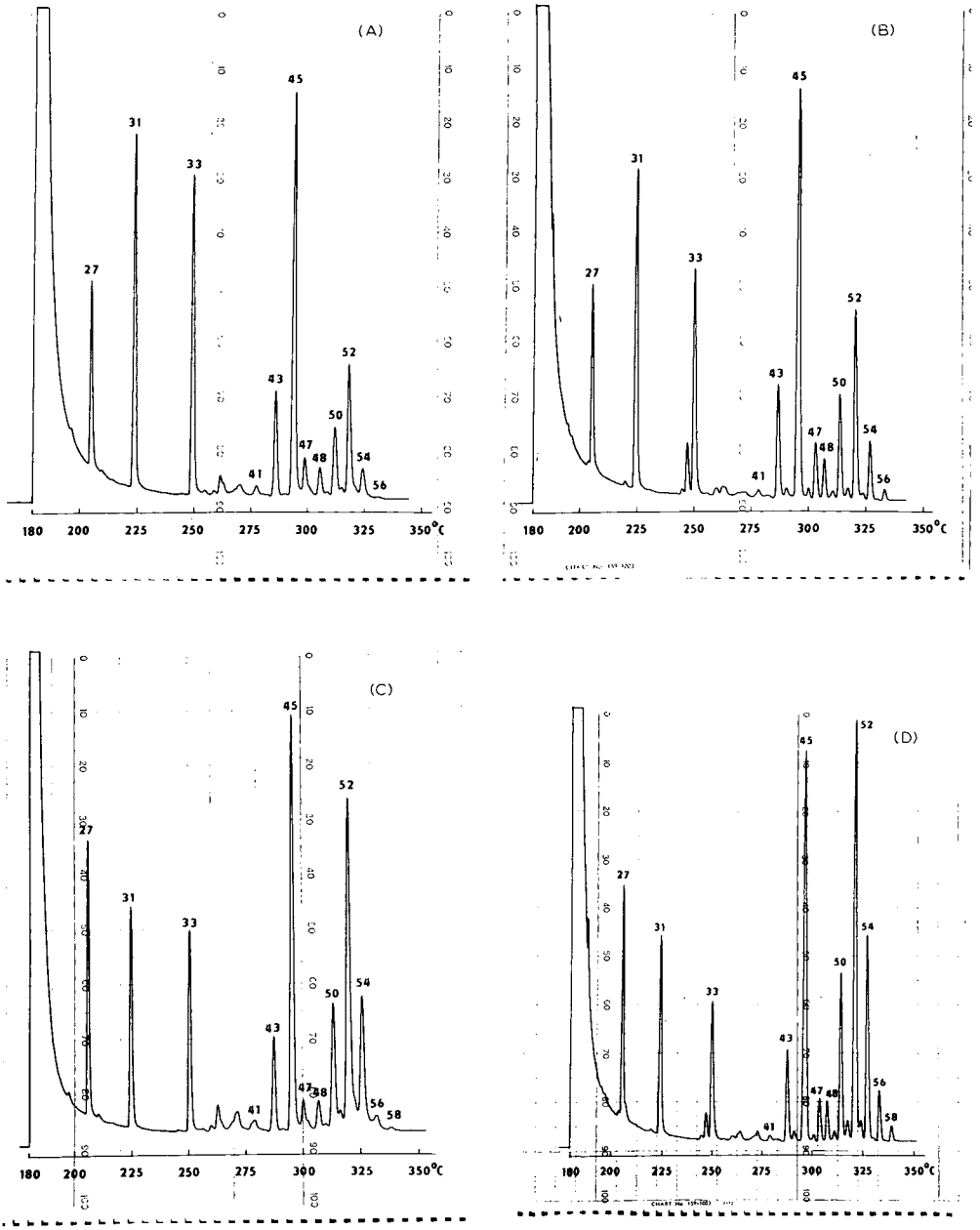


Fig. 3. Gas chromatograms of normal plasma before (A) and after (B) hydrogenation, and of hyperlipidemic plasma before (C) and after (D) hydrogenation. 27 = free cholesterol, 31 = cholesteryl butyrate (internal standard), 33 = cholesteryl benzoate (standard for laboratory control), 41—47 = cholesteryl esters, 48—58 = triglycerides. Column, 0.6 m × 1.8 mm I.D. glass-lined stainless-steel packed with 1% OV-1 on Gas-Chrom Q (100—120 mesh); carrier gas, helium 100 ml/min; temperatures, injector 300°, oven 180°, rate 5°/min, detector FID 350°, sample volume, 2 μl; solvent, isooctane—chloroform (80:20, v/v); sensitivity, 1/64; chart speed, 5 mm/min.

TABLE I

EFFECT OF HYDROGENATION ON THE ANALYTICAL RESULTS OF BIOLOGICAL SAMPLES OF VARIOUS COMPOSITIONS

All results represent a mean ± S.D. of five measurements obtained within two days. The statistical significance of the differences between the results measured with hydrogenated and non-hydrogenated samples was tested by the *t*-test ($P < 0.01$). Instrument: Perkin-Elmer F-17.

Compound* (carbon number)	Sample A			Sample B		
	Before hydrogenation (mg/dl)	After hydrogenation (mg/dl)	SS**	Before hydrogenation (mg/dl)	After hydrogenation (mg/dl)	SS**
41	4.91 ± 0.22	4.57 ± 0.27	NS	7.01 ± 0.36	6.32 ± 0.44	NS
43	36.93 ± 0.97	37.55 ± 0.78	NS	56.12 ± 1.52	53.24 ± 1.38	NS
45	146.78 ± 2.39	151.54 ± 2.24	NS	247.75 ± 4.55	252.01 ± 3.72	NS
47	24.95 ± 0.98	23.02 ± 0.91	NS	36.73 ± 1.81	37.07 ± 1.60	NS
48	18.34 ± 0.68	17.81 ± 0.50	NS	28.67 ± 1.11	29.76 ± 0.96	NS
50	40.32 ± 0.92	41.99 ± 0.73	NS	113.10 ± 1.64	115.85 ± 1.76	NS
52	76.96 ± 1.52	77.63 ± 1.27	NS	325.03 ± 4.65	325.50 ± 3.60	NS
54	28.98 ± 1.08	30.90 ± 0.85	NS	154.96 ± 4.78	160.71 ± 3.42	NS
56	7.70 ± 0.71	10.71 ± 1.19	S	40.84 ± 1.78	53.73 ± 1.46	S
58	—	—	—	17.69 ± 1.35	25.89 ± 1.05	S
FC	41.97 ± 1.80	43.12 ± 1.95	NS	93.41 ± 1.27	91.20 ± 1.08	NS
CE	213.44 ± 2.99	216.57 ± 2.43	NS	348.49 ± 6.86	348.57 ± 5.27	NS
TC	169.53 ± 3.01	172.60 ± 1.99	NS	301.07 ± 4.83	299.34 ± 3.52	NS
TG	172.12 ± 4.45	178.96 ± 3.86	NS	680.06 ± 13.75	711.28 ± 9.33	S

*FC = free cholesterol; CE = cholesteryl esters; TC = total cholesterol; TG = triglycerides.
**SS = statistical significance; S = significant; NS = not significant.

Analysis of some minor and interfering components of the neutral lipid spectrum

In parallel with the problem of the accuracy of the results in neutral lipid analysis by gas chromatography, the problem of the minor and interfering components was also studied. These components can be divided into two groups:

(1) Components of the neutral lipid spectrum that are present in minor concentrations but which do not interfere in the determination of other fractions. This group includes, in particular, triglycerides of carbon number 56, 58 and 60. The problems connected with the analysis of these compounds were discussed in our previous paper [7].

(2) Components that are present in minor concentrations but which do interfere in the determination of other lipid fractions. This group contains, in particular, triglycerides of carbon number 46 and 44, or lower, which interfere in the determination of cholesteryl esters with carbon numbers 47, 45, or 43. In most cases the concentration of these compounds is sufficiently low that the effect on the determination of cholesteryl esters is negligible. In samples with higher triglyceride levels or in those with an atypical triglyceride composition, the concentration of the fractions with carbon numbers 46, 44, or lower is increased. The effect of fraction 46 on the determination of cholesteryl ester of carbon number 47 can be significant, even if the effect on the determination of the total cholesteryl ester level is not significant, as shown in Table II. In most cases this problem can be solved by hydrogenating the sample, which enables the separation of fractions 46 and 47, as shown in Fig. 4.

The decision as to whether correction is necessary depends on the accuracy required for the determination of cholesteryl ester fraction 47. For orientation

the amount of interfering fraction 46 can be estimated from the ratio of fractions 48 and 50. With extremely high triglyceride levels or atypical compositions, separation of the triglycerides and two separate analyses of the cholesteryl esters and triglycerides are necessary.

The accuracy of the analytical results was evaluated using model samples prepared from pure compounds. The composition was chosen to be as close as possible to that of an average biological sample. The compositions of the model samples and the results of their analysis are surveyed in Table III, from which a very good agreement between the analytical values and the real composition

TABLE II

EFFECT OF THE TRIGLYCERIDE LEVEL AND COMPOSITION ON THE DETERMINATION OF CHOLESTERYL ESTER FRACTION 47, TOTAL CHOLESTEROL AND TRIGLYCERIDES

Each value represents a mean of five measurements obtained within one week. The statistical significance of the differences between the corrected and non-corrected values was checked by the *t*-test ($P<0.01$). Instrument: Perkin-Elmer F-17. For abbreviations see Table I.

Sample No.	46 (mg/dl)	47 (mg/dl)	47_{corr}* (mg/dl)	SS	TC (mg/dl)	TC_{corr}* (mg/dl)	SS	TG (mg/dl)	TG_{corr}* (mg/dl)	SS
948	17.1	42.9	29.6	S	303.5	290.6	S	373.1	390.2	S
1053	16.0	35.2	21.0	S	258.0	244.7	S	810.6	826.6	NS
949	14.5	20.6	14.9	S	251.2	245.3	NS	366.2	380.7	NS
977	8.7	21.3	14.7	S	171.8	164.4	NS	217.8	226.5	NS
967	7.6	40.3	36.4	S	270.9	266.9	NS	175.2	182.8	NS
980	3.9	17.3	15.2	S	149.1	147.2	NS	122.7	126.6	NS
975	2.4	27.4	26.3	NS	171.1	170.5	NS	301.7	304.1	NS
964	1.6	37.4	36.8	NS	313.4	312.6	NS	547.4	549.0	NS

*A correction was made according to the determination of the triglyceride fraction 46 after the isolation of triglycerides using column chromatography with Florisil. The purity of the fraction isolated was checked by thin-layer chromatography.

TABLE III

A SURVEY OF THE ANALYTICAL RESULTS OBTAINED WITH MODEL SYNTHETIC SAMPLES

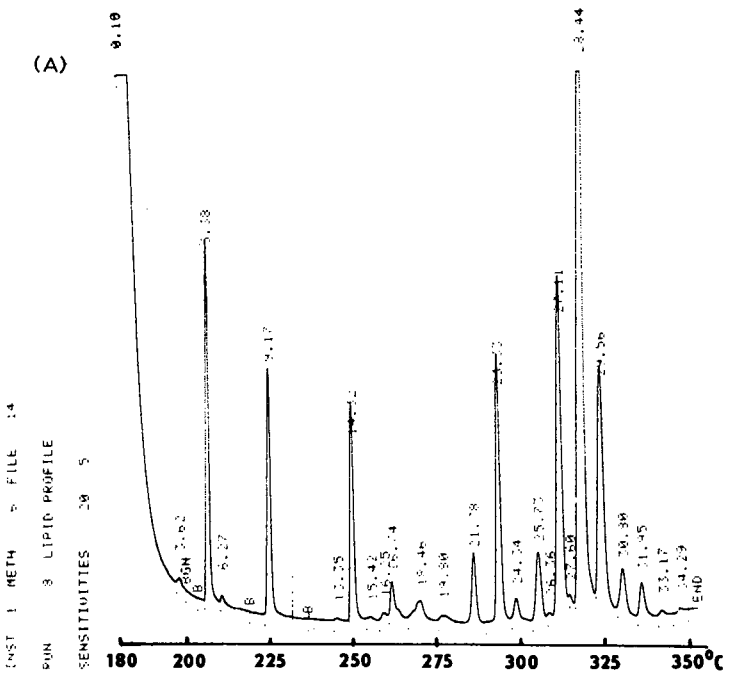
Volume injected = 2 μl. The results are expressed in mg/dl. Instrument: Perkin-Elmer F-30.

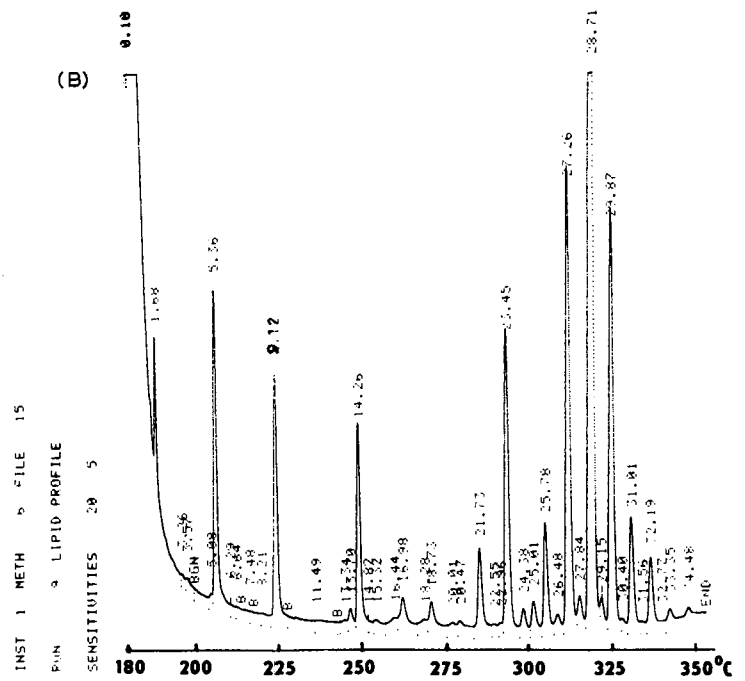
Compound* (carbon number)	Sample**					
	K-3A		K-3B		K-4B	
	Given	Found ± S.D.***	Given	Found ± S.D.***	Given	Found ± S.D.***
41	4.25	5.01 ± 0.32	5.50	5.82 ± 0.31	1.25	1.54 ± 0.13
43	40.98	41.75 ± 0.97	41.30	43.21 ± 1.13	12.50	13.28 ± 0.54
45	166.75	168.65 ± 3.78	165.00	166.28 ± 2.69	62.50	60.70 ± 1.73
47	25.00	26.40 ± 0.92	18.30	18.84 ± 0.76	8.75	9.20 ± 0.32
48	4.25	5.03 ± 0.28	5.50	5.50 ± 0.26	1.87	2.11 ± 0.19
50	33.25	32.30 ± 0.89	43.80	44.52 ± 0.65	4.52	4.48 ± 0.21
52	83.25	82.85 ± 1.75	82.50	84.99 ± 1.52	12.50	12.70 ± 0.44
54	41.75	42.02 ± 1.71	48.80	47.66 ± 1.53	6.25	6.48 ± 0.26
FC	62.50	63.40 ± 0.72	68.80	67.01 ± 1.01	21.00	21.88 ± 0.58
CE	237.75	241.03 ± 4.22	230.10	233.17 ± 4.17	85.00	84.72 ± 1.72
TC	204.65	207.48 ± 3.05	206.58	206.42 ± 2.77	71.62	72.50 ± 1.37
TG	162.50	162.18 ± 2.59	180.60	182.07 ± 2.47	24.75	25.76 ± 0.74

*For abbreviations see Table I.

**All samples contained triarachidin (200 ng/μl).

***Each value represents the mean ± S.D. of five measurements obtained within one week.


(A)
INST 1 METH 5 FILE 14
RUN 8 LIPID PROFILE
SENSITIVITIES 20 5
180
200
225
250
275
300
325
350°C


(B)
INST 1 METH 5 FILE 15
RUN 9 LIPID PROFILE
SENSITIVITIES 20 5
180
200
225
250
275
300
325
350°C

of the sample can be seen. The positive differences found with low carbon numbers, especially 41 and 48, are probably caused by the residual effect of the other components because these fractions were lowest in concentration. However, the differences did not exceed the limit of ±3 S.D. as shown by the recovery of the individual fractions and lipid classes.

Quality control

Model samples were also used for quality control. The results obtained over 25 weeks using one column are given in Table IV, and include within-day and day-to-day variation. As shown in the table, the coefficient of variation never

TABLE IV

STATISTICAL EVALUATION OF THE CONTROL SAMPLE K-3B

Instrument: Perkin—Elmer F-30.

Compound* (carbon number)	Actual content (mg/dl)	Variation**							
		Within series		Within-day		Day-to-day		Long-term	
		Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)	Mean (mg/dl)	C.V. (%)
41	5.50	5.82	1.24	5.70	3.58	5.70	5.21	5.73	6.13
43	41.30	43.21	1.46	43.37	2.22	43.37	2.79	43.41	3.92
45	165.00	166.28	1.33	166.93	1.61	166.93	4.02	167.19	4.25
47	18.30	18.84	1.64	18.62	2.29	18.62	3.47	18.71	4.00
48	5.50	5.50	2.05	5.54	3.68	5.54	4.95	5.60	6.31
50	43.80	44.52	0.83	44.35	1.90	44.35	2.10	44.22	2.79
52	82.50	84.99	1.05	85.14	1.80	85.14	2.74	84.94	2.95
54	48.80	47.66	2.72	47.96	2.74	47.96	4.06	48.09	4.29
FC	68.80	67.01	0.51	66.93	0.80	66.93	1.33	66.91	1.53
CE	230.10	233.17	1.31	233.56	1.61	233.56	2.75	232.13	3.68
TC	206.58	206.42	0.96	206.65	1.16	206.65	2.26	205.73	2.73
TG	180.60	182.07	0.89	183.11	1.88	183.11	2.73	183.44	3.54

*For abbreviations see Table I.

**The series variation was calculated on the basis of six analyses of the same sample in one run; the within-day and the day-to-day variations were calculated from 28 duplicate analyses of the control sample measured over a period of 14 weeks; the long-term variation was calculated from 75 analyses of the control sample measured over a period of 25 weeks. C.V. = coefficient of variation.

Fig. 4. Separation of triglyceride fraction 46 and cholesteryl ester fraction 47 using hydrogenation of the sample with an atypical triglyceride composition. Individual compounds are identified according to their elution times (min): (A) 5.38 = cholesterol; 9.17 = cholesteryl butyrate (internal standard); 14.32 = cholesteryl benzoate (standard for laboratory control); 15.42—18.46 = diglycerides and decomposition products of phospholipids; 19.80, 21.78, 23.33 = cholesteryl esters of carbon numbers 41, 43, 45, respectively; 24.34 = cholesteryl esters of carbon number 47 and triglycerides of carbon number 46 (not separated); 25.73, 26.36, 27.11, 27.60, 28.44, 29.56, 30.80, 31.95, 33.17, 34.29 = triglycerides of carbon numbers 48, 49, 50, 51, 52, 54, 56, 58, 60, 62, respectively. (B) 5.36 = cholesterol; 9.12 = cholesteryl butyrate (internal standard); 14.26 = cholesteryl benzoate (standard for laboratory control); 15.32—20.01 = diglycerides and decomposition products of phospholipids; 20.47, 21.73, 22.55, 23.45, 25.01 = cholesteryl esters of carbon numbers 41, 43, 44, 45, 47, respectively; 24.38, 25.78, 26.48, 27.26, 27.84, 28.71, 29.15, 29.87, 30.40, 31.01, 31.56, 32.19, 33.35, 34.48 = triglycerides of carbon numbers 46, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 62, respectively. Gas chromatograms were recorded by means of a Sigma 10 Data System. Vertical lines under the baseline are the marks of the start and end of the integration. Sample volume, 2 μl; chart speed, 5 mm/min; other analytical conditions are given in the text.

exceeded 6.5%. This finding confirms the possibility of discontinuous analyses and shows good reproducibility of the entire chromatographic system. The within-day variation for the majority of the compounds analysed was 40—75% of the day-to-day variation. Comparison of the serial variation with the within-day, day-to-day and long-term variations shows that the variability of the results is given by changes in the decisive parameters of the chromatographic system. The relatively high values of the variation coefficients in the day-to-day and long-term variation for cholesteryl esters with carbon numbers 43, 45 and 47 are probably caused by the method of integration of the individual peak areas measured by the Perkin-Elmer M-2 Integrator. This integrator is capable of retaining in its memory only four incompletely separated peaks; each further peak detected in a group of incompletely separated peaks results in a horizontal baseline approximation for the first peak measured. Even with a very good separating ability of the columns, cholesteryl esters and triglycerides represent a group of eight incompletely separated peaks. The integration of the cholesteryl ester peaks is affected by the baseline detection before the group of incompletely separated peaks and by the start of the integration of each peak detected with the horizontal baseline approximation. With triglyceride fraction 54, the higher values of the variation coefficients are caused probably by recovery changes during reversible changes in the saturation of the whole system with triglycerides of higher molecular weight. However, the values of the variation coefficients show a very good reproducibility in the analytical results for neutral lipids and their fractions by gas chromatography. The values of the variation coefficients for free cholesterol, cholesteryl esters, total cholesterol and triglycerides measured over the concentration range in which the weight correction factors are dependent on the amount analysed are in full agreement with those measured over the range in which the correction factors are independent of the amount analysed [6].

In addition to the quality control, the accuracy of the results was also checked using enzymatic determination of the total cholesterol and triglyceride levels. The results obtained with 35 duplicate samples are compared in Table V. The values of the correlation coefficients show a very good agreement between the two methods.

TABLE V

COMPARISON OF THE TOTAL CHOLESTEROL AND TRIGLYCERIDE DETERMINATIONS BY GAS CHROMATOGRAPHY AND ENZYMATIC METHODS

Determination	r*	Reference method	Correlation equation	C.V.** (%)
Total cholesterol	0.986	Enzymatic	$y = 13.05 + 0.95x$	3.46
		Chromatographic	$x = 4.28 + 1.02y$	1.06
Triglycerides	0.998	Enzymatic	$y = -1.86 + 0.98x$	2.53
		Chromatographic	$x = -0.80 + 1.01y$	2.16

*The correlation coefficient.

**The coefficients of variation were calculated on the basis of duplicate analyses of identical samples measured by both methods.

The results of a few thousands analyses measured over a period of five years demonstrate that gas chromatography allows the determination of neutral lipids, even in a low concentration range, when the values of the correction factors are dependent on the amount analysed. The column packing should have a low percentage of loading with the stationary phase. The demands on the quality of the whole chromatographic system including the column are much higher for this type of analysis than for analyses in higher concentration ranges, when the correction factors are not dependent on the amount analysed. On the basis of the study of interactions between the compounds analysed and the chromatographic system, a method which enables reproducible results to be obtained was evaluated. The quality control system permits standardization of the gas chromatographic determination of neutral lipids. The quality control made with pure compounds eliminates the problems connected with the standardization by means of lyophilized biological samples. The method is sufficiently sensitive and enables precise measurement of the lipid levels especially in the lipoprotein classes.

The next part of this study will be devoted to the problem of sample preparation, isolation of neutral lipids and clinical applications of the method described.

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ROUTINE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MEASUREMENT OF URINARY VANILLYLMANDELIC ACID IN PATIENTS WITH NEURAL CREST TUMORS

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SUMMARY

A rapid and simple reversed-phase high-performance liquid chromatographic procedure for the determination of vanillylmandelic acid (VMA) is described. This method was applied in the determination of the VMA content in urine from normal subjects and patients with neural crest lesions. Sample preparation is minimal and the analysis is short (20 min) and reproducible. The sensitivity of the UV detection is in the ng range. By this technique, fourteen adult control subjects were found to excrete a mean of 2.86 μg VMA per mg creatinine, whereas twelve patients with pheochromocytoma excreted a mean of 15.7 μg VMA per mg creatinine.

INTRODUCTION

The urinary analysis of catecholamine metabolites including vanillylmandelic acid (VMA) has given the clinician the ability to separate those patients with neural crest lesions from the vast majority of hypertensive subjects whose

hypertension is of different etiology [1—3]. The discovery of the metabolic pathways of catecholamines by Armstrong et al. [4] in 1957 revolutionized the approach for diagnosis of these lesions. The more stable O-methylated and/or amine-oxidized metabolites are excreted in much larger quantities than their precursors and thus are more easily quantified. In addition, since characteristic excretion patterns are found for different tumor types [5, 6], it is possible to diagnose and differentiate them by determining the levels of urinary VMA content.

Although a variety of techniques have been applied to the assay of VMA. their complexity, inadequate sensitivity, poor reproducibility or non-specificity have seriously compromised their value in the diagnosis of pheochromocytoma. Among the analytical methods for measurement of VMA are spectrophotometry [7, 8], electrophoretic techniques [9], isotope dilution [10], paper chromatography [4], thin-layer chromatography (TLC) [11], gas–liquid chromatography (GLC) alone [12] and coupled with mass spectrometry [13], and more recently high-performance liquid chromatography (HPLC) [14—18]. Although GLC methods offer specificity and sensitivity, the procedures are too complex for routine use by the clinical laboratory. Paper chromatographic and TLC procedures, on the other hand, lack precision and require procedures too time consuming for routine use. Unfortunately, the alternative methods, largely colorimetric, are so non-specific as to almost totally lack clinical usefulness [1].

Recent advances in HPLC suggested the usefulness of this technique for the rapid analysis of urinary VMA. The known separating power of HPLC and its ability to analyze directly non-volatile, polar or thermally labile compounds, make it ideally suited for the analysis of urinary acids. Recently developed microparticulate, chemically-bonded reversed-phase packings for HPLC are slowly displacing the ion-exchange mode because of the simplicity of operation of the reversed-phase columns, their longer life times and the ability to analyze simultaneously both nonpolar and polar compounds. In addition this methodology appeared to offer the requisite sensitivity and specifity to detect and quantify VMA. A method utilizing reversed-phase HPLC coupled with UV spectrophotometric detection was developed for analysis of VMA content in urine from control subjects and those with neural crest tumors.

EXPERIMENTAL

Methods and materials

Random urine samples [1] were obtained from 15 control subjects, 11 patients with pheochromocytoma, 1 patient with ganglioneuroma and 1 with neuroblastoma. Fourteen of the control subjects were adults with uncomplicated essential hypertension and one was a normal 7-month-old baby. All samples were acidified with 6 *N* hydrochloric acid (to pH less than 1) and refrigerated until assayed. A 5-ml specimen was extracted with three 5-ml portions of ethyl acetate. The pooled extracts were then evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 0.5 ml of distilled water in preparation for assay by HPLC or two-dimensional paper chromatography as subsequently discussed.

A Model 6000 A Solvent Delivery System, Model U6K Universal Injector

and Model 660 Solvent Programmer, all from Waters Assoc. (Milford, Mass., U.S.A.) were used in all determinations. The detection system consisted of two Model SF 770 Spectroflow Monitors (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.) connected in series. Urine constituents were simultaneously monitored at two wavelengths, 254 nm and 280 nm. One UV monitor was also equipped with a Scanning Drive and MM 700 Memory Module used for obtaining stopped-flow UV spectra.

Areas of chromatographic peaks were electronically integrated using a Hewlett-Packard Model 3380 A Electronic Integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Stainless-steel columns (30 cm × 4.6 mm I.D.) were prepacked at the factory with 10 μm totally porous silica support, utilizing an octadecyl (C_{18}) chemically-bonded stationary phase (Waters Assoc.).

All reagents used were of highest purity (A.C.S. Certified grade): VMA was purchased from Sigma (St. Louis, Mo., U.S.A.), and potassium dihydrogen phosphate from Mallinkcrodt (St. Louis, Mo., U.S.A.). Solutions of the reference compound were prepared in distilled–deionized water and kept refrigerated when not in use.

Methanol, distilled in glass, was purchased from Burdick & Jackson (Muskegon, Mich., U.S.A.) and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, N.J., U.S.A.). All solvents and chemicals for two-dimensional paper chromatography were obtained from Fisher Scientific.

Chromatographic conditions

For the determination of VMA in urine samples, a gradient elution mode of the reversed-phase HPLC was employed. The low concentration eluent was 0.1 *M* KH_2PO_4 (pH 2.50) and the high-strength eluent was a 3:2 (v/v) mixture of anhydrous methanol and distilled–deionized water. The low-strength eluent was filtered through a Millipore membrane filter (Millipore Corp., Bedford, Mass., U.S.A.), pore size 0.22 μm, and the secondary eluent was degassed under vacuum. A 20-minute linear gradient from 0 to 100% of the high-strength eluent was used. The flow-rate was 1.2 ml/min, and the temperature was ambient in all cases. Chromatographic peaks were quantified by measuring absorbance at 280 nm.

Peak identification

Initial peak identification of VMA was performed on the basis of retention times and comparison with the reference compound, and an increase in the area of the suspected peak was taken as further proof of peak identity. In addition, since the peaks in HPLC effluents were simultaneously monitored at 254 nm and 280 nm, ratios of absorbances were computed for the VMA reference compound and compared with those of the peaks in the urine extracts.

Finally, peak identity and purity were also confirmed using stopped-flow UV spectra. In order to obtain these spectra, a blank gradient was run and the flow stopped at the point where VMA elutes. The UV spectrum was then scanned in the region between 220 and 320 nm and the scan automatically stored in the memory module. This permits elimination of the spectral background arising from the changing spectral properties of the solvents, flow cells and the monochromator. Next, samples were chromatographed and the spectrum of the peak

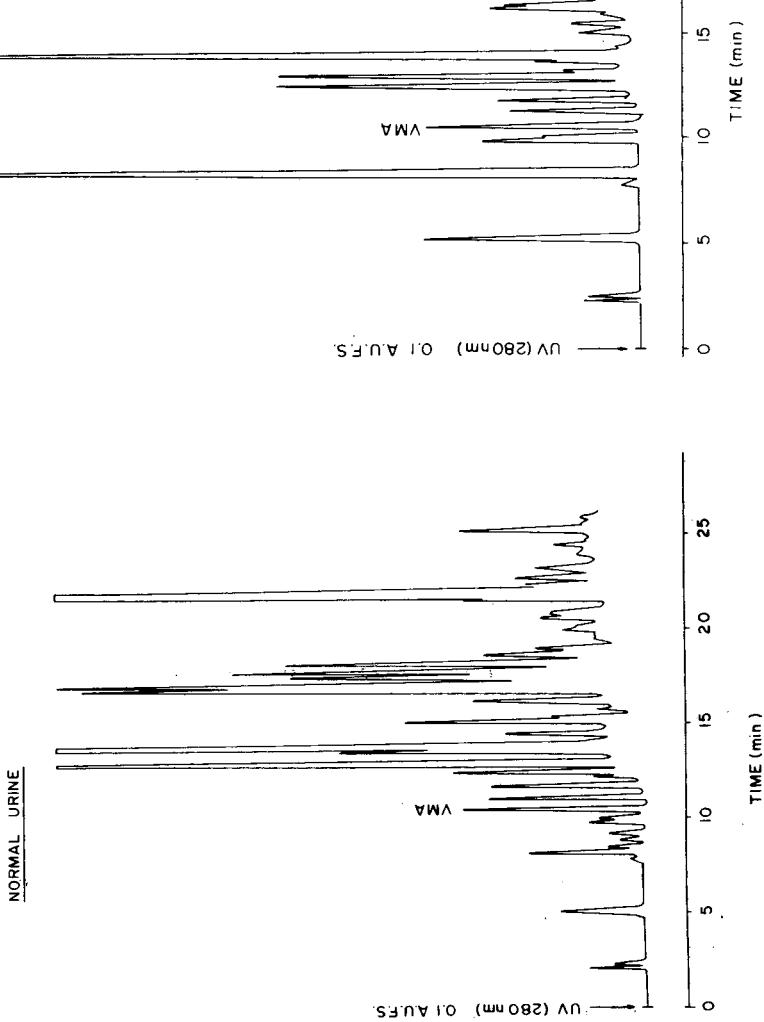

Fig. 1. Chromatogram of the ethyl acetate extract of a urine sample from a control adult subject. Volume injected: 20 µl. Chromatographic conditions: column, µBondapak C_{18} (10 µm particle size); low-strength eluent, 0.1 *M* KH_2PO_4 (pH 2.50); high-strength eluent, methanol—water (3:2, v/v); gradient, linear from 0 to 100% of the high-strength eluent in 20 min; flow-rate, 1.2 ml/min; temperature, ambient.

Fig. 2. Chromatogram of the ethyl acetate extract of urine from a baby. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1.

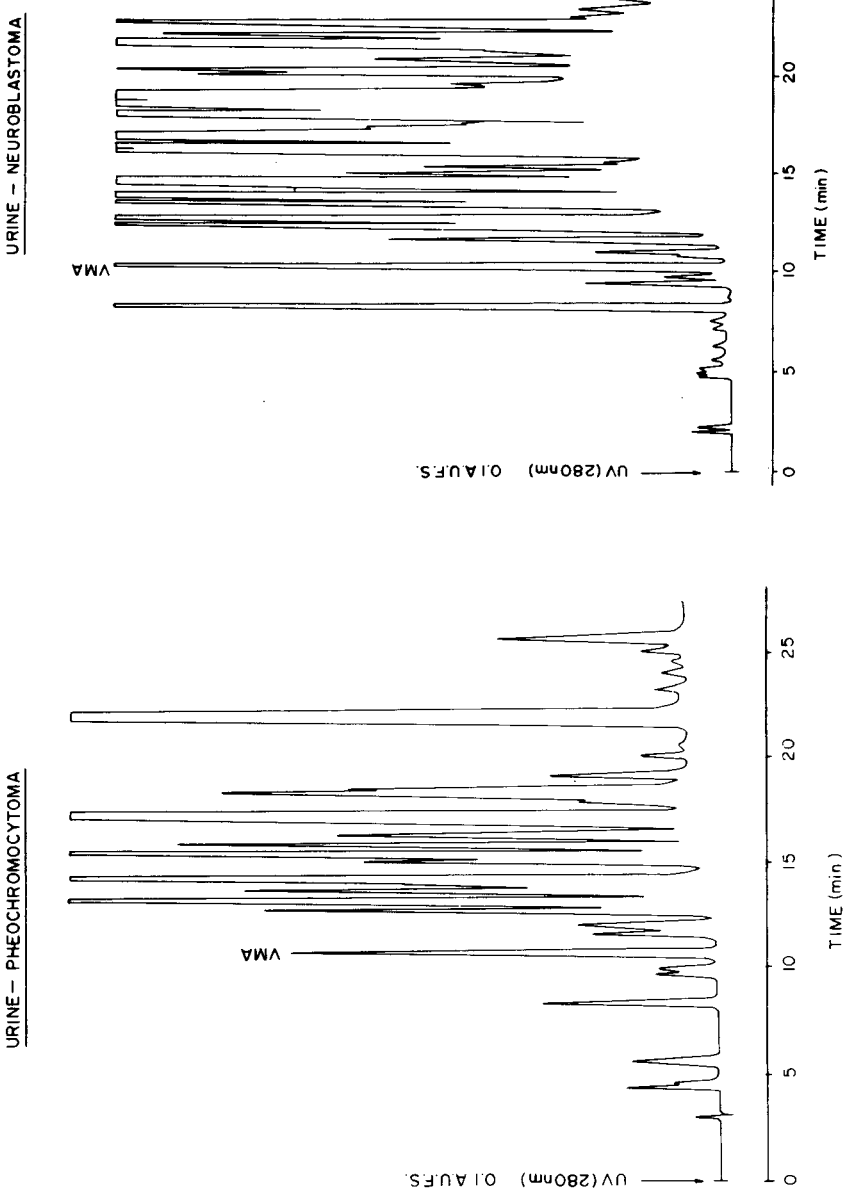

Fig. 3. Chromatogram of the ethyl acetate extract of a urine specimen from a patient with pheochromocytoma. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1.

Fig. 4. Chromatogram of the ethyl acetate extract of a urine specimen from a patient with neuroblastoma. Volume injected: 20 µl. Chromatographic conditions as in Fig. 1.

under study scanned over the same wavelength range. Those spectra were then compared with the spectrum of the VMA reference solution.

Linearity of detector response and detection limits

Linearity of detector response was determined by plotting peak heights or areas versus the amounts of VMA injected. The response was found to be linear over the entire working range. In addition, the lower limit of detection was found to be approximately 10 ng.

Reproducibility of retention times and peak areas

Reproducibility of retention times and peak areas was determined by repetitive injections of the urine extracts. The percentage standard deviations obtained from ten chromatograms of the same extract gave 1.0% for retention time and 1.2% for peak areas.

Interferences

This reversed-phase HPLC separation of the UV-absorbing constituents in ethyl acetate extracts of urine was found to be interference-free for all the samples analyzed. The purity of the VMA peak was constantly checked by determining the ratios at 280 nm and 254 nm, and periodically by obtaining the stopped-flow UV spectra. This helped to ensure that the correct peaks were quantified.

Two-dimensional paper chromatography

Urine specimens were concomitantly assayed for VMA content by the HLPC technique noted above and the standard two-dimensional chromatography procedure routinely in use at the Catecholamine Research Laboratory [1, 19].

Creatinine determination

Creatinine content of urine specimens was determined by the method of Jaffé [20].

RESULTS

Experiments were conducted in order to obtain the best analytical conditions for the separation of VMA from other UV-absorbing urine constituents. The reversed-phase gradient elution mode of HPLC was tested in the analysis of urine samples from control subjects and patients with neuroblastoma, pheochromocytoma and ganglioneuroma. Typical chromatograms of urine samples from a control adult subject and a 7-month-old child are shown in Figs. 1 and 2, respectively. The chromatograms of the urine extracts from patients with pheochromocytoma and neuroblastoma are presented in Figs. 3 and 4, respectively.

Chromatographic peaks were identified on the basis of evidence accumulated from retention characteristics, co-chromatography with the reference compound, absorbance ratios and stopped-flow UV scanning. The comparison of the UV spectra of the peak in the urine extract from a patient with pheochromocytoma and the VMA reference compound is shown in Fig. 5. A

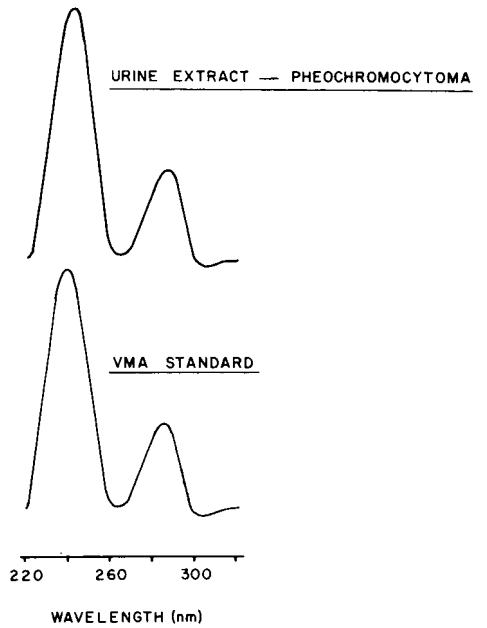

Fig. 5. UV spectra obtained by stopped-flow UV scanning (rate = 100 nm/min).

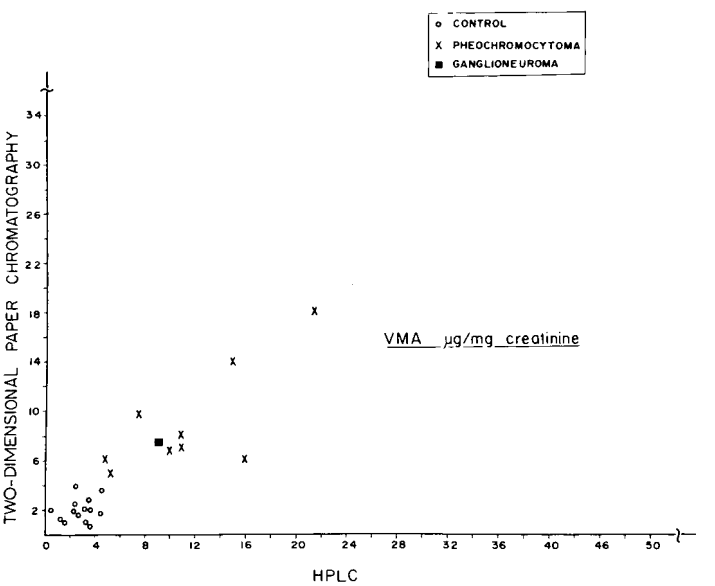

Fig. 6. Correlation of quantitative data obtained by two-dimensional paper chromatography and HPLC (r = 0.96).

scattergram, Fig. 6, depicts the correlation between the techniques. Table I lists the quantitative VMA assay results obtained with each procedure for control subjects and those with pheochromocytoma.

TABLE I

COMPARISON OF HPLC AND TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC QUANTIFICATION OF THE VMA CONTENT IN URINE SAMPLES FROM NORMAL SUBJECTS AND PATIENTS WITH PHEOCHROMOCYTOMA

All values are expressed in μg VMA per mg creatinine.

	n	HPLC		Paper chromatography	
		Mean	Range	Mean	Range
Normal subjects	14	2.86	0.5—4.8	2.25	1—4
Patients with pheochromocytoma	12	15.7	4.5—50	12.5	5—50

DISCUSSION

There is more than ample evidence [1, 21] that the clinical detection and diagnosis of pheochromocytoma can be accomplished but rarely, and that the majority of these life-threatening tumors would remain undetected in the absence of reliable biochemical techniques for determination of their catecholamine by-products. Although assays for VMA have been available to the clinician for over 20 years, many physicians may still be misled by unreliable results [22]. The majority of clinical laboratories in the United States are still utilizing non-specific colorimetric VMA assays. Since by-products of foodstuffs result in falsely elevated values, the normal range of VMA excretion has been set artificially high for such techniques. Such a loss in specificity results in a serious loss in credibility for the practicing physician. In an area where the diagnosis of a highly lethal tumor depends upon factors other than clinical appearance, the absolute reliability of laboratory procedures is essential. In this era of automated clinical laboratories, GLC [12], isotope dilution [10] or two-dimensional paper chromatography [19] have not been welcomed by routine laboratory facilities. It was the above circumstances that led to the development of the present procedure since HPLC is a technique which could ultimately be adapted to automation.

The use of reversed-phase HPLC possesses special advantages: a short analysis time (20 minutes elution time for VMA) and the stability of the microparticulate bonded phases. An average of 400—500 assays can be performed with a single column without deterioration of the plate count or peak distortion. This technique can be used with several on-line methods of peak identification such as absorbance ratios and stopped-flow UV scanning. This is crucial in achieving reliable quantification since in highly populated chromatograms of complex biological matrices such as urine, identification by retention time alone is not sufficient. The electronic integration of peak areas and the high resolving power of HPLC gives highly reproducible results.

Although differences may exist between HPLC and the two-dimensional paper chromatography both techniques can be used for the differentiation of control subjects from those with neural crest lesions. Two-dimensional paper chromatography, while yielding values in close agreement with such reliable

techniques as GLC, must be recognized as being semi-quantitative in nature. If the HPLC method had been compared with the GLC procedure, a more precise correlation might have been anticipated. However, the complexity and difficulty of the assay discouraged its use as a control technique in the present study.

No effort was made to perform a definitive study of the variables which might conceivably influence the present assay procedure. However, as with other specific techniques such as two-dimensional paper chromatography, isotope dilution and gas chromatography, dietary variations do not influence this procedure.

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DETERMINATION OF PHENYLALANINE IN SERUM USING REVERSED-PHASE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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SUMMARY

A liquid chromatography procedure is reported for determining phenylalanine in small volumes of serum. A 10-μl volume of serum was deproteinized with ethanol and an aliquot was derivatized with dansyl chloride reagent. The dansylated phenylalanine and the norleucine internal standard were separated using reversed-phase chromatography and measured with a fluorescence detector. Linearity was excellent over the range 50—800 mg/l. Within-run precision was better than 4%. Total analysis time including chromatography was approximately 40 min. As little as 300 pg of dansylated phenylalanine was detected.

INTRODUCTION

Phenylketonuria, an inborn error of metabolism, is characterized by incomplete hydroxylation of phenylalanine to tyrosine giving rise to abnormally high serum concentrations of phenylalanine. It is associated with varying degrees of mental retardation. The positive determination of serum phenylalanine concentrations is required as early as possible in the neonate in order to assess the need for proper nutritional therapy.

Several techniques have been utilized for determining serum phenylalanine concentrations. Classical ion-exchange column chromatography [1,2] has been used but generally requires large volumes of serum, which does not lend itself well to neonate screening. Primary screening procedures based on colorimetry [3,4], fluorimetry [5], and microbiological [6] techniques also require large volumes of serum and are not entirely specific for phenylalanine. Gas chromatography has also been used successfully for determining amino acid concentrations in physiological fluids [7]. Adams [8] reported a procedure using gas chromatography for determining phenylalanine in serum by forming the *n*-propyl N-acetyl derivatives. Gas chromatographic procedures that require the formation of a double derivative of the amino acids frequently entail lengthy pretreatment.

Modern liquid chromatography with fluorescence detection has more recent-

ly been used for determining amino acids [9—12] after chemical modification. These procedures have provided detection capability in the picogram range. The dansyl derivatives have been separated using both reversed-phase and normal-phase chromatography [12]. A procedure using these derivatives has been published very recently for determining the amino acid content of several protein hydrolysates [13]. These derivatives are particularly useful in that they may be prepared quickly and render the amino acids relatively non-polar, making reversed-phase chromatography with aqueous based mobile phases well-suited for their separation. We have recently published a procedure using these derivatives for determining ϵ-aminocaproic acid in small volumes of serum [14].

The procedure which we report here requires only minimal sample pretreatment and may be used with very small volumes of serum. The dansyl derivatives, which may be prepared in only 5 min, appear to be quite stable and may be measured very sensitively using a fluorescence detector.

MATERIALS AND METHODS

Apparatus

We used a Perkin-Elmer Model Series 2/2 liquid chromatograph equipped with a Model 100 column oven, a Rheodyne 7120 injection valve with a 20-μl injection loop and a Model 204A fluorescence spectrophotometer equipped with an LC flow cell (part No. 010-0456). An RP-8 reversed-phase column (part No. 258-1484, 0.46 × 25 cm, particle size 10 μm) was used. All chromatograms were recorded using a Perkin-Elmer Model 56 recorder. Special glassware included 5-ml conical centrifuge tubes, 16 × 100 mm PTFE-lined screw-capped tubes, 2-ml PTFE-lined capped sample vials and 10-μl, 20-μl, 50-μl and 1-ml disposable pipets. A bench-top centrifuge, an evaporation manifold, with a source of dry air, a heating block and a vortex-type mixer were also used. A Model W-185 sonifier, from Branson Sonic Power Co. (Danbury, Conn., U.S.A.) was used for degassing the mobile phase solutions.

Reagents and standards

Methanol and acetone, distilled in glass, were obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The acetronitrile, distilled in glass, UV grade, was also from Burdick and Jackson. Phenylalanine and norleucine standards were obtained from Sigma (St. Louis, Mo., U.S.A.). Dansyl chloride, 100 g/l in acetone, was obtained from Pierce (Rockford, Ill., U.S.A.); store at 4°. Sodium bicarbonate "analyzed reagent" was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Absolute ethanol was obtained from U.S. Industrial Chemicals Co. (New York, N.Y., U.S.A.). Sodium hydroxide, reagent grade, was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Individual standard stock solutions were prepared for each amino acid by dissolving the amino acid in 0.01 *N* HCl to give a concentration of 1 mg/ml. The solutions were stored at 4°. The internal standard solution was prepared by diluting 1 ml of the norleucine stock solution with 4 ml deionized water to yield a concentration of 0.2 mg/ml. The solution was stored at 4°.

Dansyl chloride working solution was prepared by diluting 0.1 ml of dansyl chloride stock solution to a volume of 8 ml with acetone. This reagent was

prepared freshly each day. The buffer was prepared by adjusting the pH of a 0.1 mol/l sodium bicarbonate solution to 10.5 with 0.5 *N* NaOH. The buffer was filtered through a 0.45-μm filter before use.

The mobile phase solvents were prepared as follows. Pump A contained 50% acetonitrile in 0.1% phosphoric acid. Pump B contained 25% acetonitrile in 0.1% phosphoric acid. The deionized water used for preparing the mobile phase solvents was filtered before use through a Millipore 0.45-μm filter (Perkin-Elmer part No. 089-0839). Mobile phase mixtures were degassed using sonification at 50 W for 5 min.

Procedure

Add 10 μl of serum, 10 μl of internal standard working solution and 40 μl of ethanol to the bottom of a 5-ml conical centrifuge tube. Mix vigorously for 15 sec on a vortex-type mixer and centrifuge for 1 min at 2000 rpm (710 *g*). Transfer 20 μl of the supernatant to the bottom of a 2-ml sample vial and evaporate to dryness with a gentle current of air. Add 10 μl of pH 10.5 buffer followed by 50 μl of dansyl chloride working solution. Cap the vial tightly and mix vigorously for 15 sec. Heat the vial at 100° for 5 min in a block heater. Remove the vial and allow to cool. Inject 20 μl into the liquid chromatograph.

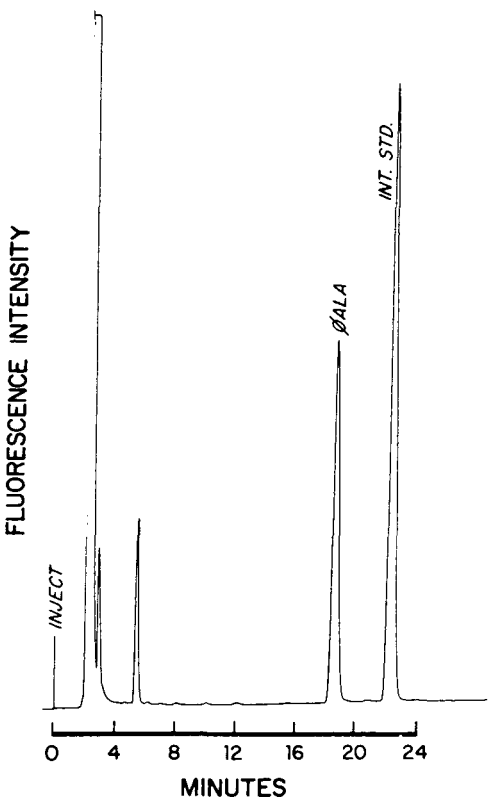

Fig. 1. Chromatogram showing the separation of dansylated phenylalanine (ϕALA) from the norleucine internal standard.

Chromatography

The mobile phase gradient was run from Pump B, containing 25% acetonitrile, to Pump A, containing 50% acetonitrile, at a rate of change of 3.6%/min. The mobile phase flow-rate was 1.5 ml/min and the column temperature was maintained at 60°. The fluorescence detector was set to an excitation wavelength of 320 nm and an emission wavelength of 522 nm. The bandwidths of both monochromators was set at 10 nm. Fig. 1 shows a chromatogram obtained from the injection of a 20-μl aliquot of a reaction mixture containing 1 μg each of phenylalanine and norleucine, which corresponds to approximately 300 ng of each dansylated amino acid on column. The retention times for the dansylated phenylalanine and norleucine were 18.7 and 22.5 min, respectively. It is important to run standards at the beginning of each working day to ensure that the chromatography conditions are satisfactory and to evaluate any long-term change in column efficiency.

Calibration

Calibration was accomplished by using the procedure on prepared serum standards to which varying concentrations of phenylalanine were added. Since serum pools contain amino acids at normal physiological concentrations, dialyzed serum was used to prepare the serum standards. To prepare the dialyzed serum, we took 20 ml of a serum pool and sealed it in a length of 7/8-in. dialysis tubing. The dialysis bag was placed in a beaker containing 2 l of deionized water and the beaker was put in the refrigerator. The water was replaced after 6 h with fresh deionized water and was refrigerated for an additional 16 h. At this time the amino acid concentration in the serum was insignificant.

From this dialyzed serum pool the serum standards containing phenylalanine were prepared over the concentration range of interest. Useful concentrations are 100, 250 and 500 mg/l. Before analyzing patients' sera, the serum standards were analyzed and the peak areas of the phenylalanine and norleucine peaks were measured. For each serum standard the peak area of the phenylalanine was divided by the peak area of the norleucine to obtain the peak area ratio. A working curve was prepared by plotting the peak area ratio of the phenylalanine against the concentration. The patients' sera were analyzed and the peak area ratios were calculated. The phenylalanine concentration in each serum was determined from the working curve.

RESULTS

Recoveries

We studied the recovery of phenylalanine from serum by adding the amino acid to dialyzed serum to yield a concentration of 250 mg/l. Nine samples were analyzed by the procedure and the area of the phenylalanine peaks were calculated and compared with the peak area of pure dansylated phenylalanine solutions. The average recovery was 98%. Recoveries approaching 100% are to be expected since the procedure does not require an extraction where losses might occur.

Linearity

The linearity of the procedure over the concentration range of interest was determined by adding known amounts of phenylalanine to dialyzed serum to give concentrations of 50, 100, 200 400 and 800 mg/l. Aliquots at each concentration were analyzed and the peak area ratios of the phenylalanine to the norleucine internal standard were calculated. A linear relationship of the peak area ratios existed over this concentration range.

Detection

We determined the detection limit for the dansylated phenylalanine by injecting known quantities of aqueous phenylalanine standards which had been previously dansylated. An injection aliquot containing 250 pg of the dansylated amino acid could just be detected using the criterion that the detection limit is equal to a signal that is twice the noise level. Normal physiological concentrations of serum phenylalanine are in the range of 3—22 mg/l. At a serum phenylalanine concentration of 3 mg/l, the lower limit of the normal concentration range, the amount of phenylalanine injected on column after processing a 10-μl serum sample according to the procedure, would be approximately 3 ng.

Precision

Within-run precision was estimated by analyzing nine 10-μl aliquots of a dialyzed serum standard containing added phenylalanine at a concentration of 250 mg/l. The results indicated that a precision of about 4% is obtained at this concentration.

Patient sera

Fig. 2A shows the chromatogram obtained from the analysis of a dialyzed serum standard containing added phenylalanine at a concentration of 300 mg/l. Fig. 2B shows the amino acid pattern of a normal serum sample. The concentration of phenylalanine was calculated to be 37 mg/l.

We analyzed about a dozen sera by this procedure from suspected phenylketonuric (PKU) patients. Fig. 3 shows the results obtained from two such sera. Chromatogram A illustrates an abnormal serum having an elevated phenylalanine concentration calculated to be 270 mg/l. Chromatogram B, likewise, is illustrative of another PKU serum, the phenylalanine concentration of which is 355 mg/l.

Interferences

We dansylated and chromatographed several other naturally occurring amino acids in order to assess them as possibly interfering with this analysis. Those compounds studied are shown in Table I. Leucine and cystine elute closest to the norleucine internal standard, but are well resolved and do not cause any interference. No amino acid tested eluted sufficiently close to the phenylalanine to be an interference. Previous work in our laboratory indicated that the other protein amino acids had retention times sufficiently different from either the phenylalanine or norleucine so as not to interfere.

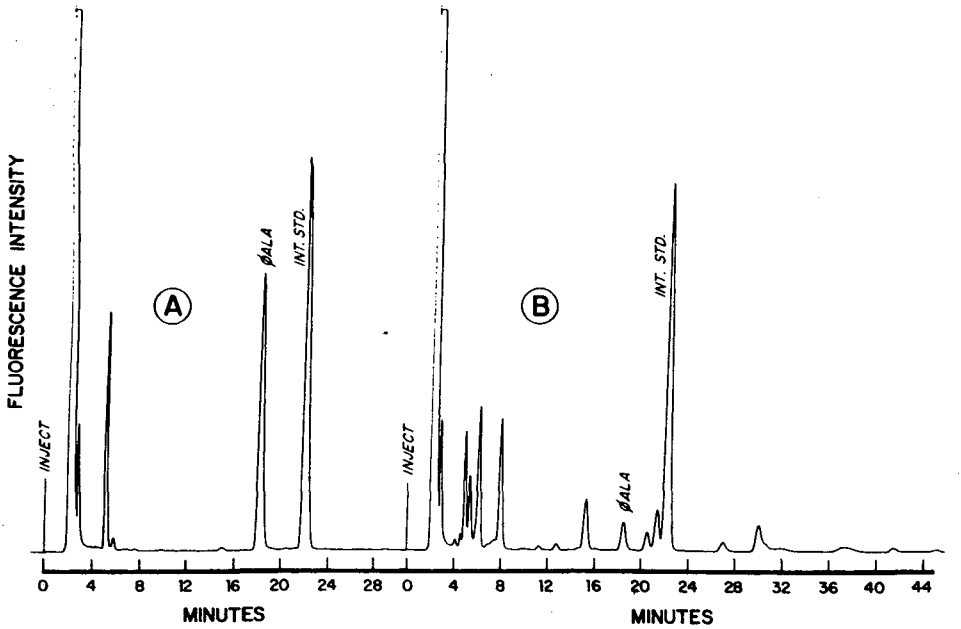

Fig. 2. (A) Chromatogram obtained from a serum standard containing added phenylalanine (ϕALA) at a concentration of 300 mg/l. (B) Chromatogram from a 10-μl aliquot of a normal serum sample.

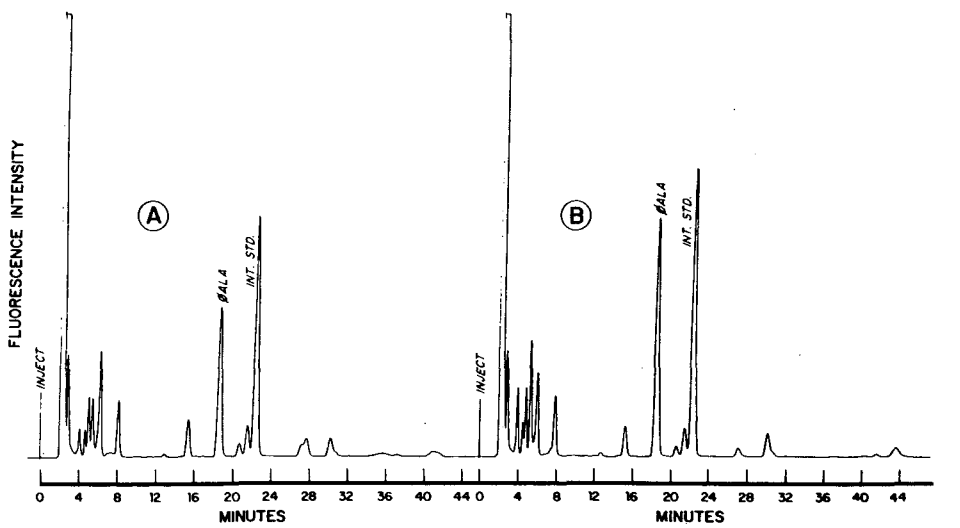

Fig. 3. Chromatograms of sera from patients with phenylketonuria. Chromatogram A shows a serum sample containing an elevated phenylalanine (ϕALA) concentration of 270 mg/l. Chromatogram B is of a patient serum containing phenylalanine at a concentration of 355 mg/l.

TABLE I

INTERFERENCE DATA

Compound	Retention time	Relative retention*
Methionine	14.9	0.636
Valine	15.3	0.655
Phenylalanine	18.7	0.820
Isoleucine	21.0	0.932
Leucine	21.4	0.951
Norleucine	22.4	1.000
Cystine	23.7	1.063
Tyrosine	42.3	1.966

*Norleucine = 1.00.

DISCUSSION

Liquid chromatography coupled with fluorescence detection is becoming an increasingly valuable technique for analyzing endogenous compounds at low concentrations. For those compounds which fluoresce weakly or not at all, derivatization to a suitable fluorophore may often be accomplished. Derivatization to the dansyl derivative is accomplished quickly and easily, and allows detection of picogram quantities.

We found the dansyl derivatives to be stable for at least two weeks when stored at 4°. The dansyl chloride reagent working solution was prepared fresh each day.

The dansylation of amino acids is most generally carried out at a reaction pH of about 9.5. However, we have used a pH of 10.5 in order to also derivatize phenolic hydroxyls, such as tyrosine. It is important to monitor the tyrosine concentration for this analysis because abnormal phenylalanine concentrations accompanied by high tyrosine concentrations may not be indicative of phenylketonuria but may rather reveal varying amino acid metabolism rates after birth. We did not observe any problem at the higher reaction pH due to increased hydrolysis of the dansyl chloride reagent.

The procedure provides very low detection limits for the dansylated amino acids which permits the quantitation of phenylalanine in 10-μl volumes of serum, an important consideration when testing for phenylketonuria in the neonate. Also, the chromatography conditions have been set to allow the separation of other amino acids as well. Thus, the procedure may also prove useful for screening other metabolic dysfunctions.

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Biomedical Applications

CHROMBIO. 399

AMNIOTIC FLUID URIC ACID LEVELS DETERMINED BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH SPECTROPHOTOMETRIC AND ELECTROCHEMICAL DETECTION

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SUMMARY

A rapid and precise, reversed-phase high-performance liquid chromatographic method for amniotic fluid uric acid is described. Detection of uric acid and other naturally occurring constituents is based on UV absorption at a wavelength of 280 nm and direct electrochemical oxidation at a potential of +0.800 V. The total analysis time is short (20 min) and the assay requires only filtration of the samples.

Uric acid levels were determined in 14 samples of amniotic fluid obtained during the 15th to 24th week of gestation. Results ranged from 0.897 to 4.39 mg per 100 ml of amniotic fluid.

INTRODUCTION

Amniotic fluid, a transudate from maternal serum across the placenta, fetal membrane and umbilical cord, is a dynamic system. While its composition is similar to maternal serum early in pregnancy, after the 14th week when the fetal kidneys begin to function it resembles neonatal urine.

The determination of age and maturity of the fetus by analyzing amniotic

fluid constituents is a subject of considerable interest. The knowledge of the exact composition of amniotic fluid is useful in elucidating the nature of solute transfer between the amniotic fluid, fetus, and mother, and in establishing the influence of fetal urinary excretion on the amniotic fluid composition.

A systematic study of the metabolic profile of organic acids in amniotic fluid was also reported at different gestational ages [1]. Marked similarities have been found between the organic acid profiles of amniotic fluid and urinary levels in newborns.

Moreover, a number of fetal abnormalities have been reflected by biochemically determined abnormalities in amniotic fluid [2]. Chemical study of amniotic fluid may also even reflect stress upon the fetus [3].

In the search for an indicator of fetal maturity, creatinine has been studied by several investigators [4–6]. Its levels have been found to parallel gestational age up to the 36th week [5]. Several attempts have also been made to correlate the levels of uric acid, the end-product of purine metabolism, with gestational age. This is still a subject of considerable controversy since several investigators report widely scattered levels [7], while others believe that uric acid is useful and possesses a predictive accuracy of up to 79% [8].

Clinical analysis of uric acid is currently being performed either on the basis of its reducing ability or by enzymatic degradation. The most common method is based on the reduction of phosphotungstic acid and subsequent spectrophotometric measurement of "tungsten blue" monitored at 700 nm. Due to interference from other reducing compounds, this assay is nonspecific and usually gives rise to falsely elevated results [9, 10].

Enzymatic degradation of uric acid with uricase with direct monitoring [11] or coupled with peroxidase [12] and/or catalase [13] gives improved specificity. However, the use of this method is severely compromised by enzyme inhibition and the need for highly pure substances. Alternatively, uric acid can also be analyzed by electrochemical measurement of the oxygen consumed during enzymatic degradation [14].

In an effort to develop a definitive method which would be reliable, selective and rapid for clinical testing of uric acid, high-performance liquid chromatographic (HPLC) analyses coupled with spectrophotometric [15] or electrochemical detection have been developed [16, 17]. The widespread use of this technique has been catalyzed by recent development of reversed-phase, microparticulate, chemically-bonded packings. These packing materials offer several advantages over ion-exchange in terms of column life, simplicity of operation and ability to analyze simultaneously compounds of a wide polarity range.

The inherently higher sensitivity of electrochemical detection compared to spectrophotometric detection has been the subject of several publications [16, 17]. It should be noted that the presence of high concentrations of oxidizable compounds in biological fluids such as urine and serum makes electrochemical detection less selective than UV absorption. This problem is usually overcome by means of simple extraction procedures which allow separation of certain classes of compounds.

Since the levels of uric acid in amniotic fluid are relatively high compared to other naturally occurring compounds, electrochemical and spectrophotometric detection are equally successful. On the other hand, electrochemical detection

is considerably more sensitive and permits use of appreciably smaller quantities of biological substrate.

Reported in this paper is the determination of uric acid in samples of amniotic fluid analyzed by reversed-phase liquid chromatography coupled with spectrophotometric, fluorometric, and electrochemical detection.

EXPERIMENTAL

Apparatus

A Model 6000 A Solvent Delivery System, Model U6K Universal Injector and Model 660 Solvent Programmer, from Waters Assoc. (Milford, Mass., U.S.A.) were used in all determinations. A Model SF 770 Spectroflow Monitor (Kratos Inc., Schoeffel Instrument Division, Westwood, N.J., U.S.A.). with a deuterium lamp and a 8-μl cell volume was used for monitoring column effluents. This detector was also equipped with a 339A Wavelength Drive and MM 700 Memory Module which were used for obtaining stopped-flow UV spectra.

In addition, a Metrohm/Brinkmann Voltametric/Amperometric Detector Model E 611 with an EA 1096 Detector Cell (Brinkmann, Westbury, N.Y. U.S.A.), operating on a three-electrode potentiostatic system, was used in line with the spectrophotometric and fluorometric detectors. The electrochemical detector employs a glassy carbon electrode as the indicating electrode and an Ag/AgCl electrode as reference.

Stainless-steel columns (30 cm $\times$ 4.6 mm I.D.) were prepacked at the factory with 10-μm, totally-porous silica support, utilizing an octadecyl (C_{18}) chemically-bonded stationary phase (Waters Assoc.).

Reagents and materials

All reference compounds, purchased from Sigma (St. Louis, Mo., U.S.A.), were of the highest purity (ACS Certified). Uricase (E.C. 1.7.3.3) was also obtained from Sigma. Potassium dihydrogen phosphate was purchased from Mallinckrodt (St. Louis, Mo., U.S.A.) and acetonitrile, distilled in glass, from Burdick & Jackson (Muskegon, Mich., U.S.A.).

Solutions of reference compounds were prepared in distilled–deionized water and were kept frozen when not in use.

Chromatographic conditions

For the analysis of amniotic fluid constituents, a gradient elution mode of reversed-phase liquid chromatography was used. The low-strength eluent was 0.1 *M* KH_2PO_4 (pH 2.50) and the high-strength eluent was a 60:40 (v/v) mixture of acetonitrile and distilled water. The low-concentration eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, Mass., U.S.A.), type HA, pore size 0.22 μm. The high-strength eluent was degassed prior to use.

A 30-min linear gradient from 0 to 100% of the high-strength eluent was used. The flow-rate was 1.2 ml/min, and the temperature was ambient in all cases. Chromatographic peaks were monitored spectrophotometrically at 280 nm, fluorometrically with an excitation wavelength of 285 nm and emission

cut-off filter of 320 nm, and electrochemically at a potential of +0.800 V.

Samples

Amniotic fluid samples were obtained by transabdominal amniocentesis from subjects who were undergoing testing for chromosomal abnormalities and fetal neural tube defects in a genetic testing center. The samples proved to represent a group of women each of whom later gave birth to normal children. Samples were obtained from the 16th to 24th week of pregnancy and they were kept frozen at −20° until analyzed.

Prior to chromatography, samples were filtered through Millipore Membrane Filters, Type HA, pore size 0.22 μm, to remove the particulate matter.

Peak identification

Peak identity was confirmed using several identification methods. Tentative identification was performed on the basis of retention behavior and co-chromatography with the reference compound. In addition, stopped-flow UV spectra were obtained for the reference solution and the peaks in amniotic fluid. This technique has proven to be a simple and elegant method of peak identification [18]. Furthermore, the identity of the uric acid peak was also tested using an enzyme peak-shift reaction with uricase which reacts according to the reaction:

$$\text{uric acid} + 2\,H_2O + O_2 \xrightarrow[\text{pH } 9.5]{\text{uricase}} \text{allantoin} + CO_2 + H_2O$$

In carrying out the enzymatic reaction, an aliquot of the sample was first buffered to a required pH and then incubated with the enzyme. Since the reaction product, allantoin, does not absorb at 280 nm, the disappearance of the substrate peak was taken as an indication of the peak identity.

In addition, the electrochemical oxidation reaction which presumably involves a loss of $2e^-$ and $2H^+$ to form a bis-imine is specific enough to be a confirmatory test when used in combination with other methods.

Linearity of detector response and limits of detection

Detector responses were found to increase linearly with concentration over the entire working range. For the spectrochemical and electrochemical detectors, the limits of detection were in the nanogram and picogram range, respectively.

Quantitation

Chromatographic peaks were quantitated on the basis of peak heights and comparison with the reference compound response. Triplicate injections gave relative standard deviation of peak heights and retention times of 1.0% and 0.5%, respectively.

RESULTS AND DISCUSSION

The conditions for the gradient elution mode of reversed-phase HPLC were optimized in order to obtain the best analytical conditions for a rapid analysis of uric acid, while maintaining good resolution of other amniotic fluid

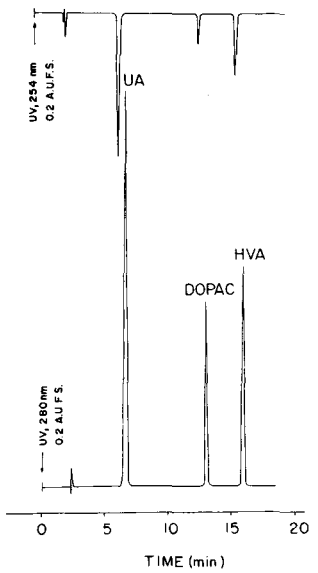

Fig. 1. Separation of a synthetic mixture of uric acid (UA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), detected at 280 nm and 254 nm. Chromatographic conditions: column, μBondapak C_{18} (10 μm average particle size); eluents, low strength 0.1 *M* KH_2PO_4 (pH 2.50), high strength acetonitrile—water (60:40, v/v); gradient, linear from 0 to 100% of the high-strength eluent in 30 min, flow-rate, 1.2 ml/min; temperature, ambient.

constituents. The separation of a synthetic mixture of some reference compounds detected at 280 nm and 254 nm is shown in Fig. 1. Simultaneous monitoring at two wavelengths aids in identification since the ratios of absorbance can be used to characterize solutes [19]. Furthermore, the use of stopped-flow UV scanning permits examination of the entire spectrum which, although lacking in fine structure, is nevertheless a powerful fingerprint of the chromophore. Fig. 2 illustrates the corrected stopped-flow UV spectra of the reference compounds.

Selective monitoring of the physiological levels of compounds in amniotic fluid was achieved by a combination of detection devices. In order to obtain

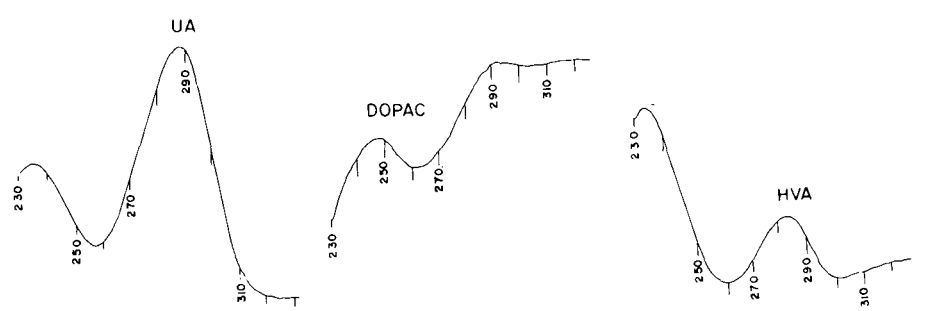

Fig. 2. Corrected stopped-flow UV spectra of the reference compounds shown in Fig. 1. Scanning rate: 100 nm/min.

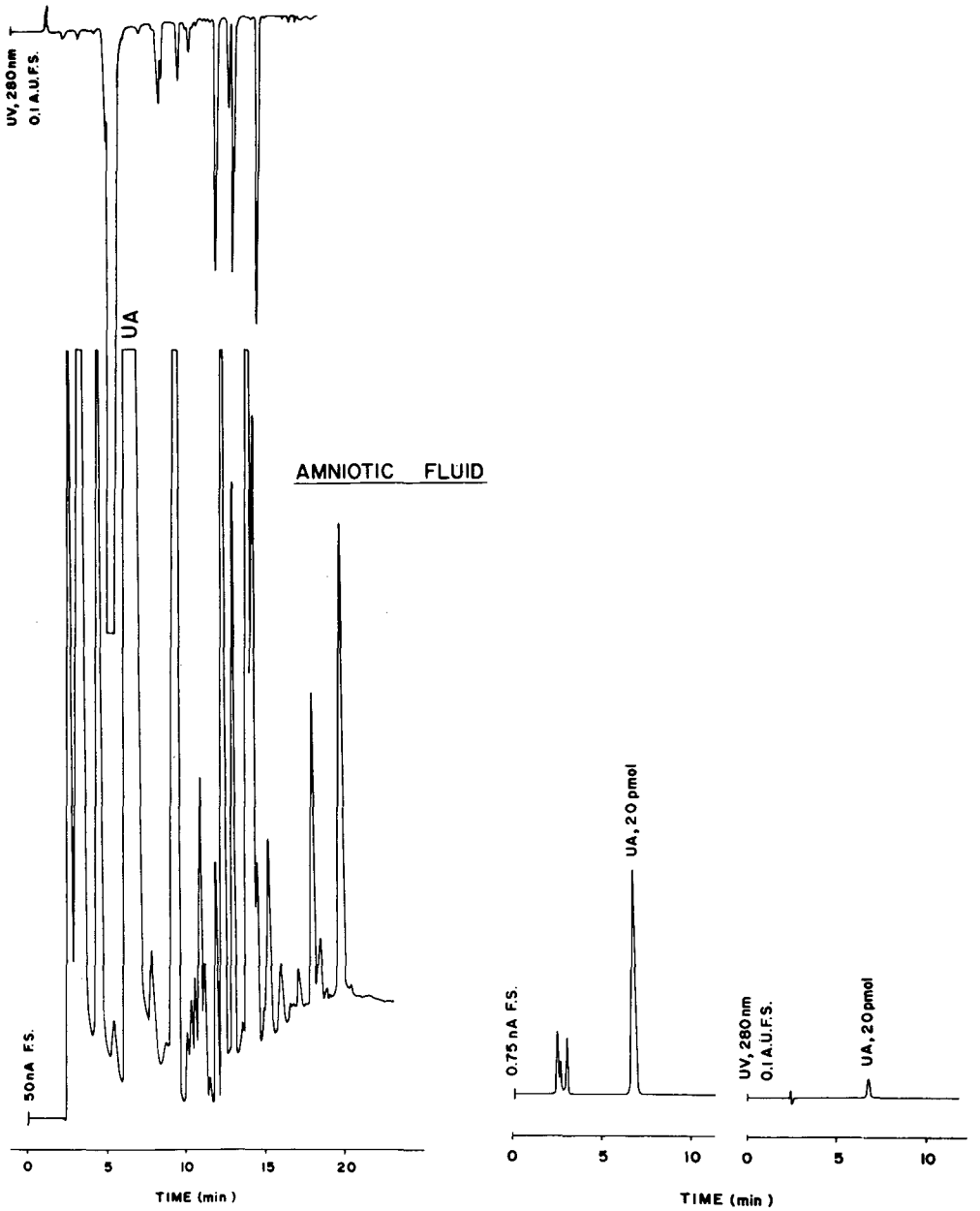

Fig. 3. Chromatogram of a sample of amniotic fluid detected amperometrically at +0.800 V and spectrophotometrically at 280 nm. Chromatographic conditions same as in Fig. 1. Volume of sample injected: 80 μl.

Fig. 4. Twenty picomoles of uric acid (UA) detected (left) amperometrically at +0.800 V, and (right) spectrophotometrically at 280 nm. Chromatographic conditions same as in Fig. 1.

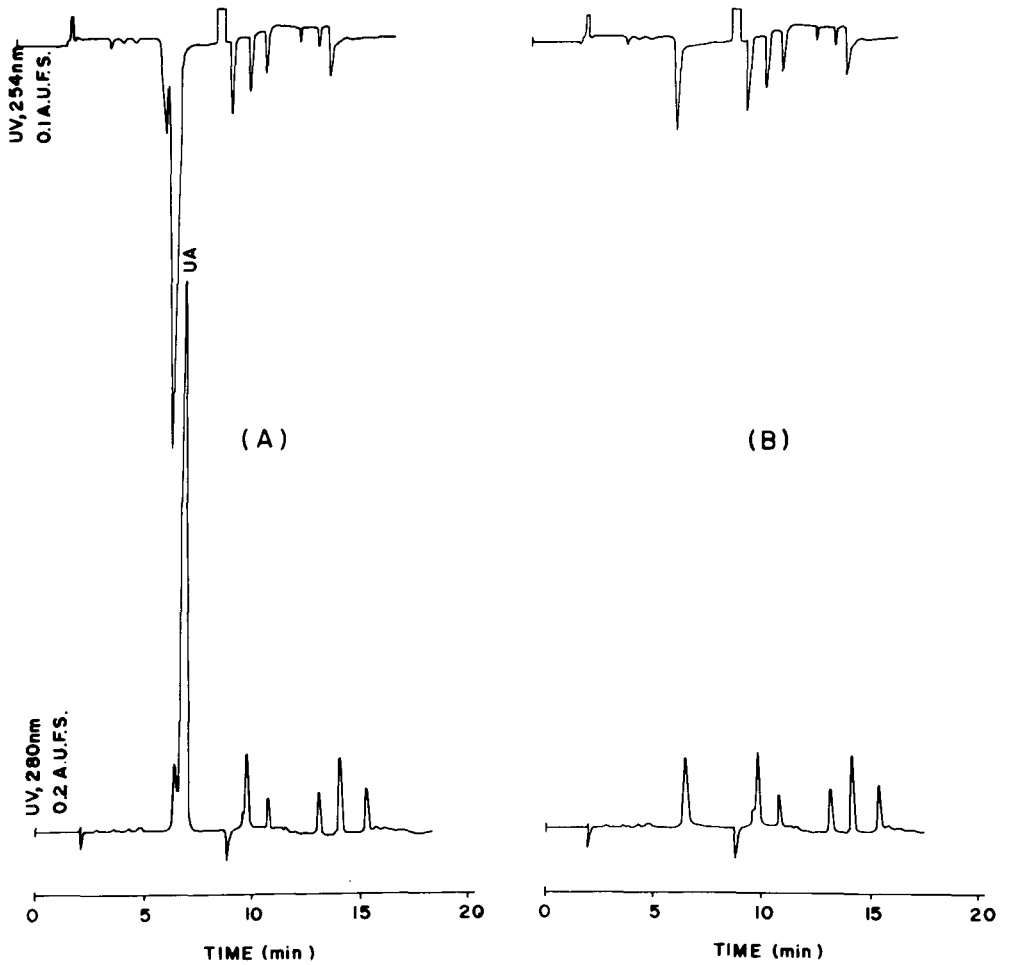

Fig. 5. (A) Chromatogram of a sample of amniotic fluid monitored spectrophotometrically at 280 nm and 254 nm. (B) Chromatogram of the same sample, incubated with uricase. Chromatographic conditions same as in Fig. 1. Volume of sample injected: 15 μl.

the sensitivity necessary for the analysis of trace amounts of catecholamine metabolites, currently under study in our laboratories, HPLC effluents were monitored amperometrically at a potential of +0.800 V against the standard Ag/AgCl electrode, and spectrophotometrically at 280 nm. While this enhanced sensitivity is not crucial in the analysis of uric acid, it is mandatory in determining certain compounds for which spectrophotometric detection is not adequately sensitive. Fig. 3 shows a chromatogram of a sample of amniotic fluid monitored amperometrically and spectrophotometrically. The inherently higher sensitivity afforded by electrochemical detection is illustrated in Fig. 4.

The specificity of fluorometric detection is well known and has been discussed in literature [19, 20]. Monitoring native fluorescence of compounds in complex biological mixtures matrices is particularly advantageous since high sensitivity can be achieved and interference eliminated or reduced.

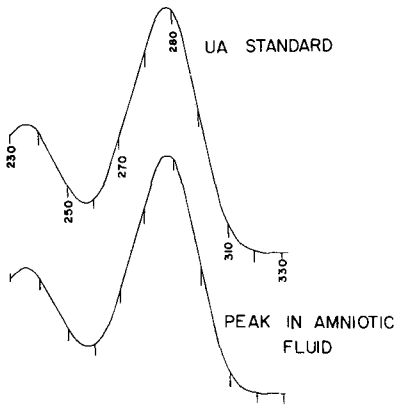

Fig. 6. Corrected stopped-flow UV spectra of uric acid (UA) reference solution and the peak in amniotic fluid with the same retention time. Scanning conditions same as in Fig. 2.

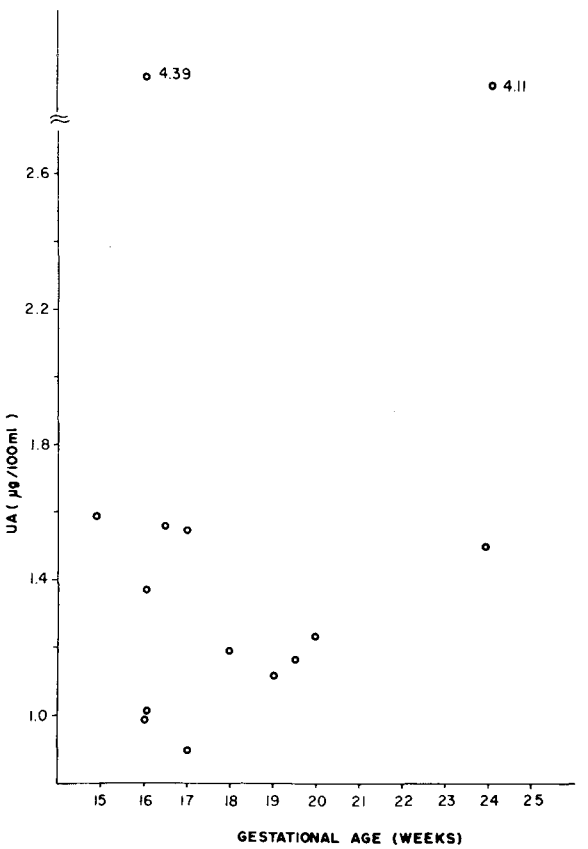

Fig. 7. Uric acid (UA) concentrations in amniotic fluid versus gestational age.

TABLE I

QUANTITATION OF URIC ACID IN AMNIOTIC FLUID SAMPLES

No. of samples	Gestational age (weeks)	Uric acid (mg per 100 ml)
2	24	4.110, 1.495
1	20	1.298
1	19.5	1.350
1	19	1.120
1	18	1.390
2	17	1.560, 0.897
1	16.5	1.580
4	16	1.378, 1.020, 4.390, 0.950
1	15	1.490

The described analytical conditions were then tested in the analysis of uric acid in amniotic fluid. Fig. 5A illustrates a typical chromatogram of a sample of amniotic fluid, monitored at 280 nm and 254 nm.

The identity of the peak with retention time of uric acid was first confirmed by an enzymatic peak-shift reaction with uricase. The chromatogram of the incubated mixture is shown in Fig. 5B. The disappearance of the peak under study confirms its identity. It should be pointed out that enzymatic peak-shift reactions also serve to "unmask" the chromatogram to prove that impurities are hidden under the major peaks [19, 21]. Furthermore, stopped-flow UV spectra were obtained for the uric acid reference compound and the peaks in amniotic fluid with the same retention time. Fig. 6 illustrates the agreement between the two spectra.

In an effort to establish the relationship between uric acid levels in amniotic fluid and the gestational age, 14 samples were analyzed using the described liquid chromatographic method. Quantitative results are given in Table I.

A scattergram (Fig. 7) depicts graphically uric acid levels as a function of gestational age. No apparent relationship exists between the uric acid content and fetal maturity for the samples analyzed. Whether or not this is due to the relatively narrow spread of gestational ages necessitates further follow-up. Current effort is being directed at identification and quantitation of other amniotic fluid constituents in a larger sample population to determine if any other compounds could be used as indicators of fetal maturity [22].

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DETERMINATION OF AMOXICILLIN IN BODY FLUIDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY COUPLED WITH A POST-COLUMN DERIVATIZATION PROCEDURE

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SUMMARY

Quantitative methods for determination of amoxicillin in body fluids are described. They comprise separation by reversed-phase chromatography (LiChrosorb RP-8, 5 μm) of the aqueous supernatants obtained from plasma or urine after purification steps involving protein precipitation followed by extraction in the case of plasma, or a double extraction procedure in the case of urine, post-column derivatization with air segmentation, and finally measurement of the UV absorbance at 310 nm. The derivatization involves formation of the mercuric mercaptide of penicillenic acid and is specific for compounds with an intact penicillanic acid ring system.

Detection limits achieved on injecting 200 μl of plasma and 20 μl of urine are about 25 ng/ml and 200 ng/ml, respectively, but it is possible to improve the sensitivity further by injecting larger volumes. Precisions (s_{rel}) obtained for determination of 0.10 and 0.45 μg/ml in plasma were 3.72 and 1.40%, respectively.

Some problems regarding column stability originating from the injection of biological samples are discussed.

INTRODUCTION

The analysis of ampicillin and mecillinam in biological material using high-performance liquid chromatography combined with post-column derivatization has previously been described [1]. The derivatives are penicillenic acid mercuric mercaptides formed by reaction of the penicillins with imidazole and mercuric chloride according to the method of Bundgaard and Ilver [2]. The same method is also, in principle, applicable to amoxicillin, but since this is a very polar compound which elutes early in reversed-phase liquid chromatographic systems, there are some problems regarding the appearance of the

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blank chromatograms. The disturbances were especially serious for urine samples. It was therefore found necessary to include cleaning-up extraction steps before the chromatographic step.

An analytical method for the determination of amoxicillin in plasma, urine and saliva based on reversed-phase liquid chromatography and direct UV absorption measurements has been published [3]. The limit of determination is about 0.5 μg/ml and the method can consequently not be applied for rigorous pharmacokinetic studies. Some methods based on fluorimetric assays of amoxicillin in plasma have also been published [4, 5].

EXPERIMENTAL

Apparatus

In the liquid chromatography experiments, the following components were used: Constametric II pump for the mobile phase, Haskel AO 15 pump for column packing, and the peristaltic pump Gilson Minipuls II for the reagent solution; LDC Spectromonitor II photometric detector; W. & W. 1100 recorder; Rheodyne Model 7120 injecting valve; columns of precision-bore stainless steel from Handy & Harman (length 100 mm, I.D. 4.0 mm, O.D. 1/4 in.) with modified zero dead volume, Swagelok® connections as column end-fittings.

Coils, mixing tees, debubblers, feed lines (polyethylene) and heating bath for the post-column derivatization were of Technicon quality. The eluent is mixed with the reagent in a mixing tee (PT2 116-B 000) connected to the column with a Teflon tube (3 cm long, I.D. 0.5 mm), and the outlet of the mixing tee, at an angle of 90° to the reagent and eluent streams, is connected to the injection fitting (116-0492-01C), where the air bubbles are introduced, by another Teflon tube (6—8 cm long, I.D. 0.9 mm, O.D. 1.3 mm) provided with a short (1 cm) and wide (I.D. 1.2 mm, O.D. 1.9 mm) Teflon tube to make a better fit into the injection fitting. The sequented stream then passes a small mixing coil (1 × 7 turns, I.D. 1.6 mm) before entering the reaction coil (6 cm long, I.D. 1.6 mm; 105-1128-01) that is kept at 40° in a heating bath. Into the debubbler (116-0203-01) a Teflon tube (10 cm long, I.D. 0.5 mm, O.D. 1.0 mm) is inserted in order to minimize the dispersion. At the end of this thin tube a wider Teflon tube is provided (I.D. 1 mm, O.D. 1.6 mm) for connection to the detector inlet stainless-steel capillary (I.D. 0.5 mm). At the outlet the detector is connected by a Teflon tube (I.D. 0.5 mm) to the peristaltic pump which sucks the liquid stream through the detector.

Pump tubes: reagent and air — orange—green (116-0549 PO 4), 0.32 ml/min; from detector — orange—white (116-0549 PO 6), 0.68 ml/min. Pump speed: 22 rpm.

Photometric measurements were performed on a Zeiss DMR 21 spectrophotometer with 10-mm quartz cells. The samples were mixed on a Fisons Whirlimixer and centrifuged in a Wifug X-1. The pH measurements were made with an Orion Digital Ionalyzer Model 801 A.

An ultrasonic bath (Bransonic 220) was used for homogenization and degassing of solvents.

Reagents and chemicals

LiChrosorb RP-8 (5 μm) (Merck, Darmstadt, G.F.R.) was used as chromatographic support.

Amoxicillin and ampicillin were obtained from the Department of Antibacterial Chemotherapy at Astra Läkemedel. Imidazole puriss p.a. (Fluka, Buchs, Switzerland) was recrystallized from toluene (375 g in 1.5 l), the precipitate being filtered and washed with diethyl ether (800 ml) and dried under vacuum in a desiccator overnight. The absorbance of an 8% aqueous solution at 310 nm is then about 0.06 (1-cm cell).

Mercuric chloride "pronalys" (May & Baker, Dagenham, Great Britain), methanol "zur Analyse", phosphoric acid p.a., sodium dihydrogen phosphate p.a. and disodium hydrogen phosphate p.a. from Merck were all used as received.

For protein precipitation and cleaning-up extractions the following reagents were used: tetrapropylammonium hydrogen sulphate (Labkemi, Gothenburg, Sweden), perchloric acid "zur Analyse", 70% (Merck), 1,2-dichloroethane, certified (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and *n*-amyl alcohol, certified (Fisher).

The water was deionized. The phosphate buffer had an ionic strength of 0.1.

Analytical methods

Sample preparation

Plasma. To 1.00 ml of plasma, 1.0 ml of dichloroethane is added followed by 20 μl of perchloric acid (70%), and mixing is carried out on a whirlimixer for about 30 sec. After centrifugation at 1600 *g* for 3 min, the aqueous (upper) phase is filtered through a piece of cotton wool applied at the tip of a Pasteur pipette, and 200 μl of the clear solution are injected without delay on to the chromatographic column.

Urine. The special reagents used were: reagent A, 0.05 *M* tetrapropylammonium hydrogen sulphate in a mixture of *n*-amyl alcohol—dichloroethane (2:1, v/v), saturated with water; and reagent B, a mixture of *n*-amyl alcohol—dichloroethane (3:4, v/v), saturated with water. A 1.00-ml volume of urine is extracted with 4.0 ml of reagent A by mixing for about 20 sec on a whirlimixer. After centrifuging at 350—800 *g* for 2 min, the organic (upper) phase is discarded by aspiration and 4.0 ml of reagent B are added. After extracting by mixing on the whirlimixer for another 20 sec, the mixture is centrifuged at 350—800 *g* for about 2 min and 10—20 μl of the aqueous (upper) layer are injected without delay on to the chromatographic column.

Chromatographic system

The column dimensions were 100 × 4 mm I.D. The mobile phase was phosphate buffer (pH 8, ionic strength 0.1)—methanol (92:8, v/v) at a flow-rate of 1.0 ml/min.

Post-column derivatization

The derivatization reagent was an aqueous solution of imidazole (33%) and

mercuric chloride (0.11%) adjusted to pH 7.2 by hydrochloric acid and containing Brij 35 (0.12%). The flow-rate was 0.3 ml/min, the air bubble rate 1 sec^{-1}. The absorbance was measured at 310 nm.

Quantitation

Quantitations were performed from standard curves constructed by adding small volumes ($\leqslant 10$ μl) of solutions of amoxicillin in citrate buffer (pH 5) to pooled plasma or urine, and plotting peak heights against added concentrations.

RESULTS AND DISCUSSION

Sample preparation

Considerable caution must be exercised in the analysis of penicillins owing to their comparative instability in aqueous environments (*cf.* ref. 1). Plasma and urine samples are therefore stored at $-70°$ before analysis, and the analytical procedures are performed in a highly reproducible fashion for each step.

The analysis of ampicillin and mecillinam [1] is performed by the direct injection of plasma/whole blood (after protein precipitation) or urine. Owing to the polar character of amoxicillin use of the optimal chromatographic mobile phase for this compound results in interference in the chromatograms due to polar endogenous compounds, which necessitates the introduction of purification steps. Precipitation of proteins by the addition of trichloroacetic acid did not prove to be possible with amoxicillin because unexpected deterioration of the chromatographic performance occurred on injecting such samples. Instead, perchloric acid was used, but the addition of an organic solvent, dichloroethane, was also necessary to achieve complete precipitation of the proteins. Obviously, some endogenous compounds will also be extracted into the organic phase in this procedure providing further purification of the plasma sample. In choosing the organic solvent its solubility in the aqueous phase must be considered, due to the risk of the appearance of serious interference in the chromatogram, with dichloromethane, for example.

Interference by endogenous compounds in blank chromatograms from urine samples was far more serious than in those from plasma samples, and a double extraction procedure was found necessary. Low solubility in the aqueous phase of the organic solvents used is essential, see discussion above, and mixtures of a higher alcohol, *n*-amyl alcohol, together with dichloroethane were chosen. The proportions of the solvents in the mixtures used were chosen so that their densities were such that the aqueous phase was the heavier one in the first step, where the organic phase is discarded, but the lighter one in the last step, after which the sample is injected on to the chromatograph. This obviously facilitates sample handling and increases the analytical capacity.

The presence of the salt of the quaternary ammonium compound in the first step results in an acidic solution (pH about 1), and since the salt is distributed in the aqueous phase and will be present in the injected sample, an increase in the retention volume occurs (about 15%) probably by an ion-pair effect. The achievement of the longer retention time is essential in order to eliminate the influence of interfering peaks.

Chromatography and post-column derivatization

The chromatographic performance of several amphoteric penicillins has been studied [1], and it was found that they are retained more in their charged forms. The selectivity for different penicillins using phosphate buffer (pH 8)—methanol (7:3, v/v) showed that amoxicillin was the least retained of the compounds investigated. As an example the selectivity factors for ampicillin/amoxicillin and carbenicillin/amoxicillin are 8.10 and 1.30, respectively under these conditions. A further illustration is given in Fig. 1, which shows the

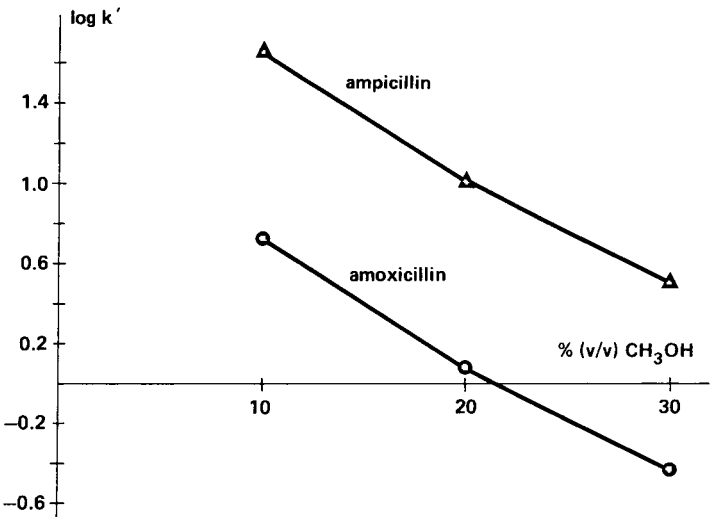

Fig. 1. Dependence of capacity ratios (k') on amount of methanol in the mobile phase. Support: LiChrosorb RP-8 (5 μm). Mobile phase: phosphate buffer (pH 8, ionic strength = 0.1)—methanol. •, Amoxicillin; ▲, ampicillin.

dependence of the capacity factors on the methanol content in the mobile phase for ampicillin and amoxicillin. The selectivity relative to the most important metabolite, the penicilloic acid which is even more polar than the parent compound, can also be expected to be very high in analogy with ampicillin and ampicilloate where the selectivity factor with 10% of methanol in the mobile phase and pH 8 is about 13.

The penicilloates will furthermore not react, or at least only to a very limited extent, in the derivatization procedure [2], which does not yield a quantitative reaction for amoxicillin under these conditions (*cf.* ref 1). A compromise between reaction time and completeness of the reaction is essential because of the band broadening that occurs in the reactor part [1].

Quantitative determinations

Representative blank and sample chromatograms obtained from plasma and urine are shown in Figs. 2 and 3, respectively. Endogenous peaks elute before the penicillin with only one exception in the case of plasma. Detection limits, defined as that concentration which gives a signal corresponding to twice the

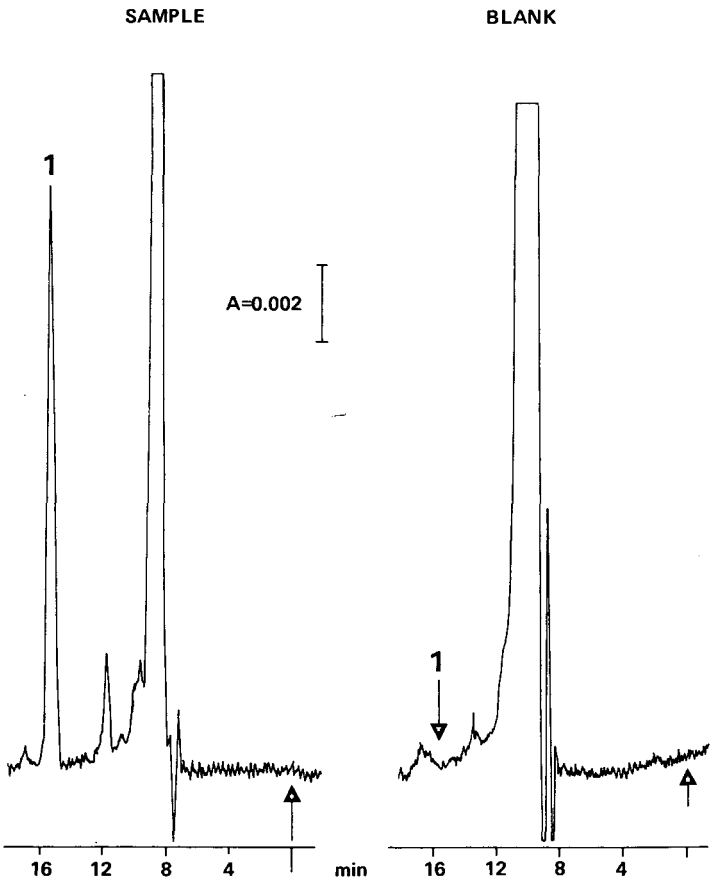

Fig. 2. Blank and sample chromatograms from plasma. Column: LiChrosorb RP-8 (5 μm) in 100 × 4 mm I.D. column. Mobile phase: phosphate buffer (pH 8)—methanol (92:8, v/v). 1 = amoxicillin.

baseline noise, are for plasma about 25 ng/ml and for urine about 200 ng/ml. These figures are valid for the injection of 200 μl plasma and 20 μl urine, but because of the trace enrichment effect [1, 6] that operates in chromatographic systems of this kind it is probably possible to improve the detection limits considerably by injecting larger volumes, as already demonstrated in the case of ampicillin and mecillinam [1]. To illustrate the performance of the analytical methods some data are presented on within-run recoveries and precision for the determination of low concentrations in plasma (Table I) and a standard curve obtained in a urine analysis, covering a rather broad concentration range and with the 99.9% confidence limits marked out (Fig. 4). Some examples of confidence limits (95%) by inverted predictions at different concentration levels (4.98—6.31, 35.7—36.9, 59.2—60.5, and 99.1—100.6 μg/ml) demonstrate the performance of this special standard curve.

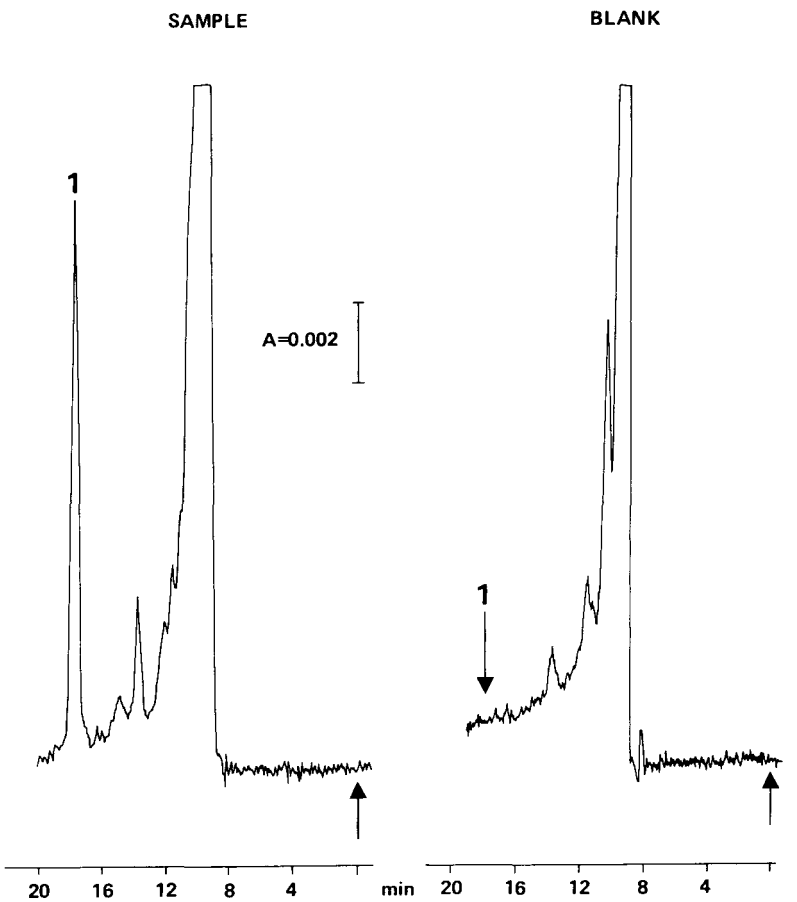

Fig. 3. Blank and sample chromatograms from urine. The same chromatographic conditions as in Fig. 2. 1 = amoxicillin.

TABLE I

QUANTITATIVE DETERMINATION OF AMOXICILLIN IN PLASMA

Standard curve with six standards in the range 85—1015 ng/ml.

Added (ng/ml)	Found (%)	s_{rel} (%)	n
100	101.7	3.72	6
454	99.6	1.40	5

Routine use

The method presented as well as the methods for ampicillin and mecillinam [1] have been used routinely in this laboratory for about 1.5 years. Performance has been excellent except for one point regarding column stability; namely, in order to maintain the column performance it has been found necessary to frequently (after about every fifteen injections of biological

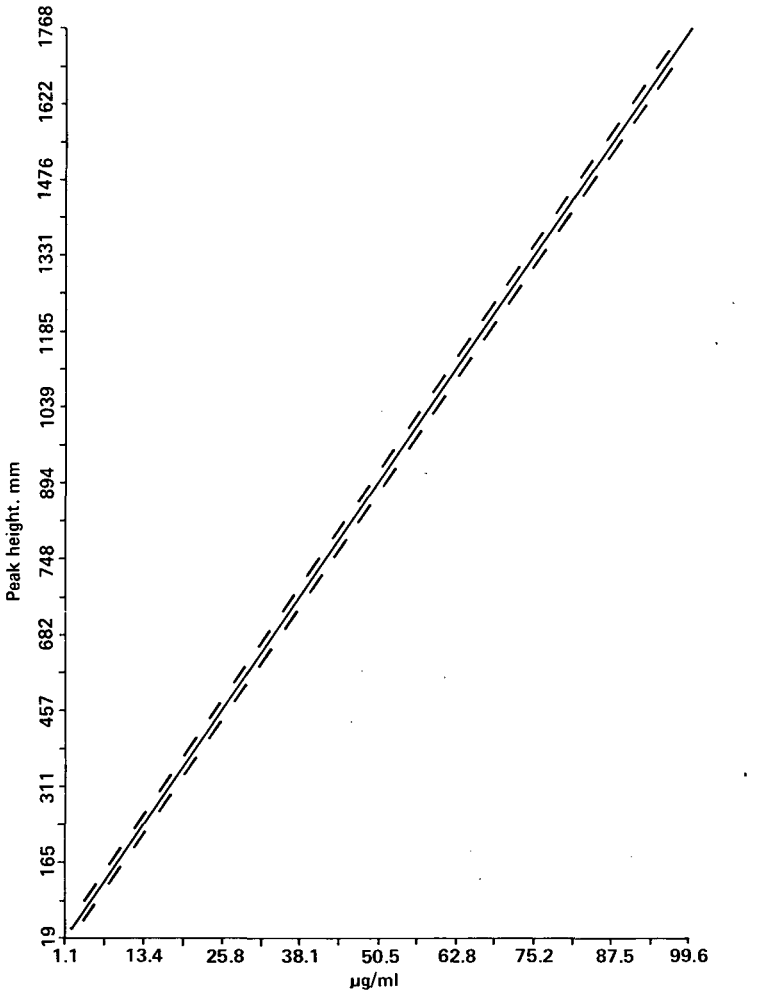

Fig. 4. Determination of amoxicillin in urine — standard curve. Analysis performed as described in "Analytical methods". Regression equation: $y = 2.13 + 17.69\,x$. Coefficient of correlation: 0.99989. 99.9% confidence limits are marked out. At 95% confidence limits: $17.57 < \text{slope} < 17.81$, $-0.68 < \text{intercept} < 4.94$.

samples) replace some millimeters of the support at the top of the column with new material. This procedure restores the chromatographic performance almost to its original condition and for runs carried out on the same day excellent standard curves are usually obtained. Columns treated in this way have been in use for more than six months when both routine and development work were carried out alternately. The cause of the column degradation is probably partly dependent on the absorption of non-polar endogenous compounds, such as fatty acids and lipids, that remain at the top of the column. However, this does not seem to be the only reason for the deterioration since it also occurs on injecting aqueous buffer samples, although in such cases it is then only discernible after a larger number of injections.

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Note

Simple inexpensive apparatus for the rapid DEAE-cellulose chromatography of small quantities of glycoproteins

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Previously [1,2] we obtained apparently homogenous epithelial glycoproteins from extracts of fresh, human or rat, colonic epithelial cells by fractionation with a combination of agarose (A15 M) gel and DEAE-cellulose chromatography. These conventional methods were, however, unsatisfactory for the fractionation of small quantities of glycoproteins such as those we have isolated recently [3] from formalin fixed tissues. In this paper we describe an apparatus designed for the fractionation of small quantities of acidic glycoproteins by stepwise, centrifugal elution from micro-columns of DEAE-cellulose. The apparatus was constructed from commercially available inexpensive materials which can be used in a standard benchtop centrifuge. It was based upon a design used previously for the desalting of small volumes of solutions [4] and for micro step exclusion chromatography [5]. The procedure, therefore, provides the DEAE-cellulose counterpart of such gel chromatographic systems.

MATERIALS AND METHODS

Glycoproteins were obtained, as previously described, from fresh rat colonic epithelial cells [1,2] and from formalin fixed specimens of normal and diseased human large and small intestine.

Analytical techniques

Ketosidically linked sialic acids were measured, after saponification of the glycoprotein, with a miniaturized version of the procedure of Culling et al. [6]. Cellulose acetate electrophoresis was performed for 35 min at 300 V on strips (6 × 1 in.) of Sephraphore III (Gelman, Ann Arbor, Mich., U.S.A.) using Tris—barbital—sodium barbital buffer (pH 8.8, i=0.05) and a current of 1.5—2.5 per strip. Electropherograms were stained with alcian blue [7] and were scanned at 605 nm in the gel scanner attachment of a Beckmann 25 spectrophotometer.

Construction of micro column assembly

The apparatus, shown in Fig. 1, was constructed from a Gilson, P1000, pipette tip (7.2 × 0.95 cm) suspended in a 7.0 × 1.2 cm Falcon plastic test tube by means of a sleeve consisting of the upper 2.5 cm of a conical 1.5-ml Eppendorf centrifuge tube (O.D. top 1 cm). A glass wool plug was inserted into the constricted end of the pipette tip and the column of DEAE-cellulose (Whatman DE 22-fibrous) was packed as a slurry in 0.02 *M* pyridine hydrochloride pH 5.5. The DEAE-cellulose had an initial height of 3.0—3.2 cm and, after centrifugation at 1000 *g* for 5 min, a packed volume of 800 μl.

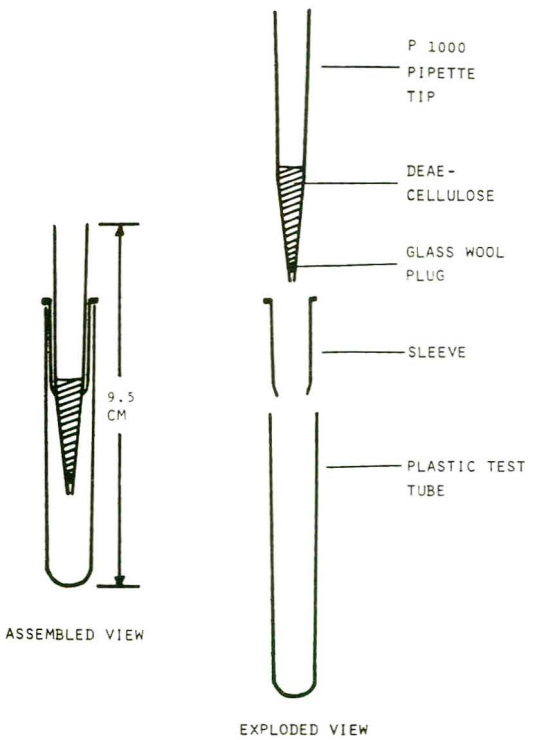

Fig. 1. Diagrams of exploded and assembled views of the apparatus for micro DEAE-cellulose chromatography.

Fractionation procedure

Glycoprotein extracts (100—200 μl containing up to 540 μg sialic acid) were mixed with an equal volume of 0.04 *M* pyridine hydrochloride and were then added to the column. The column was eluted with an aliquot (500 μl) of 0.02 *M* pyridine hydrochloride buffer pH 5.5 followed by aliquots (500 μl) of the buffer containing increasing concentrations of sodium chloride. Elution was carried out by centrifugation at 1000 *g* for 5 min. Following fractionation the column was regenerated by washing three times with aliquots (500 μl) of pyridine hydrochloride.

RESULTS

In previous studies [1,2] we were able to separate mixtures containing rat colonic epithelial glycoprotein and DNA by gradient elution, from conventional DEAE-cellulose columns with sodium chloride in 0.02 *M* pyridine hydrochloride pH 5.5. Since these columns could not be used with small quantities, preliminary studies were carried out with micro-columns of DEAE-cellulose. These demonstrated that such quantities could be separated by consecutive stepwise elution with 0.02 *M* pyridine hydrochloride pH 5.5 containing sodium chloride, the concentration of the sodium chloride being increased by 0.1 *M* between each elution. The micro-columns were then used to fractionate extracts of formalin fixed human colonic tissues. The columns were eluted with 500-μl aliquots of 0.02 *M* pyridine hydrochloride buffer pH 5.5 (once) and buffer containing the following concentrations of sodium chloride: (a) 0.02 *M* (twice); (b) 0.3 *M* (three times); (c) 0.35 *M* (once); (d) 0.5 *M* (twice) and (e) 2.0 *M* (twice). The eluents were combined to yield five fractions: 1 consisting of the buffer alone plus the 0.2 *M* eluent and fractions 2—5 consisting of the 0.3 *M*, 0.35 *M*, 0.5 *M* and 2.0 *M* eluents, respectively. Fig. 2 shows the

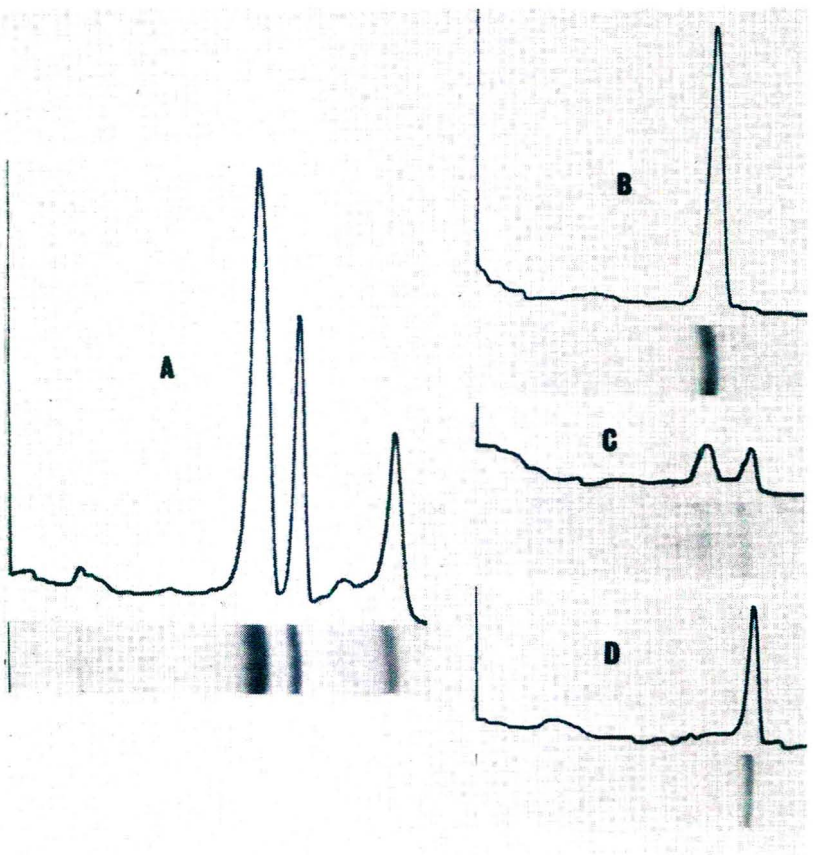

Fig. 2. Electropherograms and scans obtained from a representative fractionation. A = Unfractionated extract; B = fraction 2; C = fraction 3; D = fraction 4.

electropherograms and scans of fractions obtained from the DEAE-cellulose chromatography of a representative glycoprotein extract. Fractions 2 and 4 contained a single, apparently homogenous component while fraction 3 contained trace quantities of fractions 2 and 4. Typically fraction 5 (not shown in Fig. 2) contained one or two components with mobilities similar to DNA. Subsequent analysis showed that fraction 2 was essentially the only sialic acid containing component. The identity of the other fractions has not been established but presumably they contain one or more proteoglycans and possibly DNA.

This fractionation technique has been successfully applied to extracts of histologically normal resection margins and tumours from cases of carcinoma of the colon, specimens of colon from cases of ulcerative colitis and Crohn's disease and specimens of normal small intestine and small intestine from cases of Crohn's disease. It should be noted that, on occasion, sialoglycoprotein was eluted with 0.2 *M* sodium chloride and appeared in fraction 1.

DISCUSSION

This procedure provides a simple rapid method for the DEAE-cellulose chromatography of small samples of acidic glycoproteins which can be readily adapted for the routine, simultaneous, processing of multiple samples. In previous chemical and histochemical studies [8,9] we have shown that in many tumours of the colon there is a marked reduction in the degree of O-acetylation of the epithelial glycoprotein sialic acids; use of this method will facilitate the further analysis of such glycoproteins. Although the procedure has been applied primarily to extracts of fixed tissues it should be applicable to the separation of any mixture of acidic glycoproteins which can be fractionated by conventional DEAE-cellulose chromatography and therefore should be of value in many biochemical and biomedical investigations.

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Biomedical Applications

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Note

Quantitation of amphetamine in plasma and cerebrospinal fluid by gas chromatography—mass spectrometry—selected ion monitoring, using β-methylphenethylamine as an internal standard

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Quantitative determination of amphetamine in biological samples using the selected ion monitoring (SIM) technique was reported earlier by Cho et al. [1]. They used the N-trifluoroacetyl derivative for the quantitation and d_3 (γ-C^2H_3)-amphetamine as an internal standard. The base peak at *m/e* 140 of N-trifluoroacetylamphetamine and *m/e* 143 of the internal standard were monitored for this gas chromatographic—mass spectrometric (GC—MS) quantitation. More recently, Gal [2] reported the synthesis of d_5 (ring-d_5)-amphetamine for use as an internal standard, where the fragment ions at *m/e* 91 and 96 of the N-trifluoroacetyl derivative of amphetamine and the internal standard are monitored for GC—MS analysis by electron impact ionization. Matin et al. [3] reported the use of N-pentafluorobenzoyl-S-(—)prolyl (PFBP) derivative and chemical ionization mass spectrometry to determine the d and l isomers of amphetamine.

During the last two decades street use of large quantities of amphetamines has resulted in a disproportionate number of psychotic episodes which are virtually indistinguishable from paranoid schizophrenia [4,5]. This syndrome has been termed amphetamine psychosis. We have been studying the effects of chronic administration of d-amphetamine on selected members of a primate social colony to develop an animal model psychosis [6,7]. In the course of this work on the behavioral and biochemical correlates of the effect of hallucinogens on monkey colonies, we needed to determine amphetamine levels in plasma and cerebrospinal fluid (CSF) samples in order to correlate these with

changes in the levels of dopamine metabolites in CSF. For this purpose we used the isothiocyanate derivative (NCS) of amphetamine [8,9] for the GC—MS—SIM quantitation in the electron impact mode and monitored the molecular ion at *m/e* 177. In previous work [10] we discussed the use of non-biological isomeric compounds as internal standards. In the present study we used an isomer of amphetamine, β-methylphenethylamine, as an internal standard, and compared the results with those obtained using d_5-amphetamine as another internal standard.

MATERIALS AND METHODS

d-Amphetamine sulfate, d_5-(ring) amphetamine sulfate and β-methylphenethylamine hydrochloride were used as standards. Ethyl acetate (pesticide grade) and freshly distilled carbon disulfide (AR) were used for extraction and derivatization respectively. Stock solutions of amphetamine sulfate and β-methylphenethylamine hydrochloride in water were prepared to contain 1 mg/ml each of the base. Standard solutions to contain 0.1 μg/μl of the base were prepared.

Preparation of standards

Aqueous stock solutions of amphetamine sulfate (100 μl), d_5-amphetamine sulfate (100 μl) and β-methylphenethylamine hydrochloride (100 μl) were alkalinized with 0.1 ml of 2 *N* sodium hydroxide (pH > 11) and extracted into ethyl acetate (5 ml). The ethyl acetate extracts were treated with carbon disulfide (0.5 ml), and after shaking for 15 min, they were set aside for 2 h. The organic solvent was removed under vacuum or by a current of nitrogen gas. The residue was redissolved in ethyl acetate (1 ml) to give a solution containing 0.1 μg/μl. The solutions can be stored indefinitely without any deterioration. To avoid concentration by evaporation the standards were stored in a refrigerator. Dilute solutions of the mixture of the two NCS derivatives were made from the stock solution by suitable dilution.

Standard calibration curve

To two sets of 4 tubes (duplicate) containing 2 ml each of control human plasma, 1 μg of β-phenethylamine (10 μl of standard solution) and 1 μg of d_5-amphetamine were added. To each of these tubes varying quantities 100, 200, 400 and 600 ng of standard amphetamine solution were added. The samples were made alkaline with 0.5 ml of 2 *N* sodium hydroxide, salinized with 1 g sodium chloride and extracted twice with 5 ml of ethyl acetate by shaking on a mechanical shaker for 10 min with centrifuging for 5 min at 9000 *g*. The organic extracts were pooled and reextracted into 1 ml of 0.5 *N* hydrochloric acid. After centrifuging for 10 min the organic layer was aspirated and discarded. The aqueous layer was made alkaline with 0.5 ml of 2 *N* sodium hydroxide and reextracted into ethyl acetate. As much of ethyl acetate as possible was transferred from the top layer into a dry 15-ml centrifuge tube, mixed with 0.5 ml of carbodisulfide, shaken for 15 min and set aside for 2 h. Ethyl acetate was then removed under reduced pressure, the residue redissolved in 40 μl of ethyl acetate and an aliquot of 2 μl injected into the GC—MS system.

Sample preparation

Plasma or cerebrospinal fluid. To 2 ml of plasma sample or 1 ml of CSF sample in a 50-ml glass stoppered centrifuge tube, 1 μg of β-methylphenethylamine (internal standard) was added, the sample mixed on a Vortex mixer and 0.5 ml of 2 *N* sodium hydroxide and 1 g of sodium chloride were added to the mixture. The sample was processed as described above to obtain the NCS derivative. A 2-μl aliquot of 40 μl of the final sample was used for GC—MS determination.

Red cells. Two milliliters of red cells were mixed with an equal volume of distilled water, shaken for 15 min and the internal standard (1 μg) was added and extracted in the same manner as described for the plasma sample.

Brain tissue. The tissue was homogenized in 6 volumes of 0.1 *N* methanolic hydrochloric acid, and 1 μg of the internal standard was added to the homogenate which was then centrifuged. The supernatant was separated, methanol removed under reduced pressure and the residue alkalinized with 0.5 ml of 2 *N* sodium hydroxide and extracted as described earlier.

Gas chromatography

Three different columns (180 cm long) packed with 3% SE-30 on Supelcoport, 3% OV-17 on Chromosorb W HP or 3% OV-225 on Chromosorb W HP were used for the GC studies. Carrier gas (helium) flow-rate was 30 ml/min. Column temperatures for the three columns were 170°, 180° and 190°, respectively. For routine analysis of biological samples the 3% OV-225 on Chromosorb W HP was used at 190° isothermally.

Gas chromatography—mass spectrometry

A Varian CH7 mass spectrometer interfaced with a Varian gas chromatograph 2740 through a Watson Biemann separator was used. Mass spectral conditions were: separator temperature 280°, source temperature 280° and helium flow-rate 30 ml/min with an ionizing potential of 70 eV. We have also used a Finnigan 4000 GC—MS data system for some of the assays reported here.

Animal studies

In two separate studies [6,7] individual members of a social colony of juvenile and adult stumptail macaque monkeys were administered d-amphetamine chronically. The juvenile monkeys were administered nasogastrically 0.5 mg/kg of d-amphetamine for 25 days, and the adult monkeys received 1.6 mg/kg in time release form every 12 h for 12 consecutive days. Two baseline samples of blood (heparinized tubes) and lumbar CSF samples (2.5 ml) were drawn from the experimental monkeys. Similarly, during drug treatment daily blood samples were collected in heparinized tubes at 12 noon, 3 h after the administration of the drug. Lumbar CSF samples (2.5 ml) were collected from each of the experimental adult animals on days 3, 5 and 11. We also obtained 20 control blood samples and 10 CSF samples from individual monkeys of the social colony which were free from the drug. Plasma and CSF samples were stored frozen (—40°) until they were analyzed.

We also studied the effect of d-amphetamine treatment on dopamine metab-

olites in the caudate and whole brain of the rat. Male Sprague Dawley rats (200—250 g) were administered intraperitoneally (i.p.) d-amphetamine sulfate at a dose level of 5 mg/kg. The animals were killed by decapitation at various time intervals, the brains were removed, the caudate dissected from the brain. The caudate and the rest of the brain were stored at -40° until analyzed separately for amphetamine levels.

RESULTS AND DISCUSSION

The NCS derivatives of amphetamine and β-methylphenethylamine separate on the three columns used in this study (Table I) and give a fairly abundant molecular ion (*m/e* 177) (Fig. 1), with adequate sensitivity for quantitation at the nanogram level. Fig. 2 shows (a) a SIM of d-amphetamine and the two internal standards from an aqueous extract, (b) extract of two internal standards alone from plasma and (c) extract of all three from human plasma samples. As can be seen from Fig. 2b, there was no interference from any endogenous compounds in the plasma sample. The human or monkey plasma, the rat brain tissue and monkey CSF blanks do not show any interfering peaks at *m/e* 177, the ion that is used for quantitation. In 20 samples of plasma and CSF from monkeys, the baseline of amphetamine was zero. Fig. 3a is a SIM recording of a control (drug-free) plasma extract using only one step basic extraction. Two peaks with *m/e* 177 were seen with lower retention times than the amphetamine derivative. These peaks were not seen when the two-step extraction (second acid extraction) was carried out as described under the experimental procedure. Fig. 3c is a SIM recording of an extract of a sample of plasma of a monkey treated chronically with d-amphetamine using both internal standards and one-stage extraction. Plasma and CSF levels of amphetamine in experimental monkeys in our behavioral and biochemical studies are given in Tables II and III. The monkeys received amphetamine orally in the form of time release capsules, each dose being equivalent to 1.6 mg/kg/12 h. Whole brain and caudate levels of amphetamine in rats administered 5 mg/kg of amphetamine are shown in Table IV. The level of amphetamine calculated from peak areas was 110 ng/ml with d_5-amphetamine as an internal standard and 115 ng/ml with β-methylphenethylamine as an internal standard. We have used 2 ml of plasma in five

TABLE I

GC SEPARATION OF NCS DERIVATIVES OF AMPHETAMINE AND β-METHYLPHENETHYLAMINE

RT = retention time (min); RRT = relative retention time; column length = 180 mm.

		3% SE-30 on Supelcoport 170°	3% OV-17 on Chromosorb W HP 180°	3% OV-225 on Chromosorb W HP 190°
Amphetamine	RT	1.40	1.28	1.60
	RRT	1.0	1.0	1.0
β-Methylphenethylamine	RT	1.70	1.60	1.91
	RRT	1.21	1.25	1.20

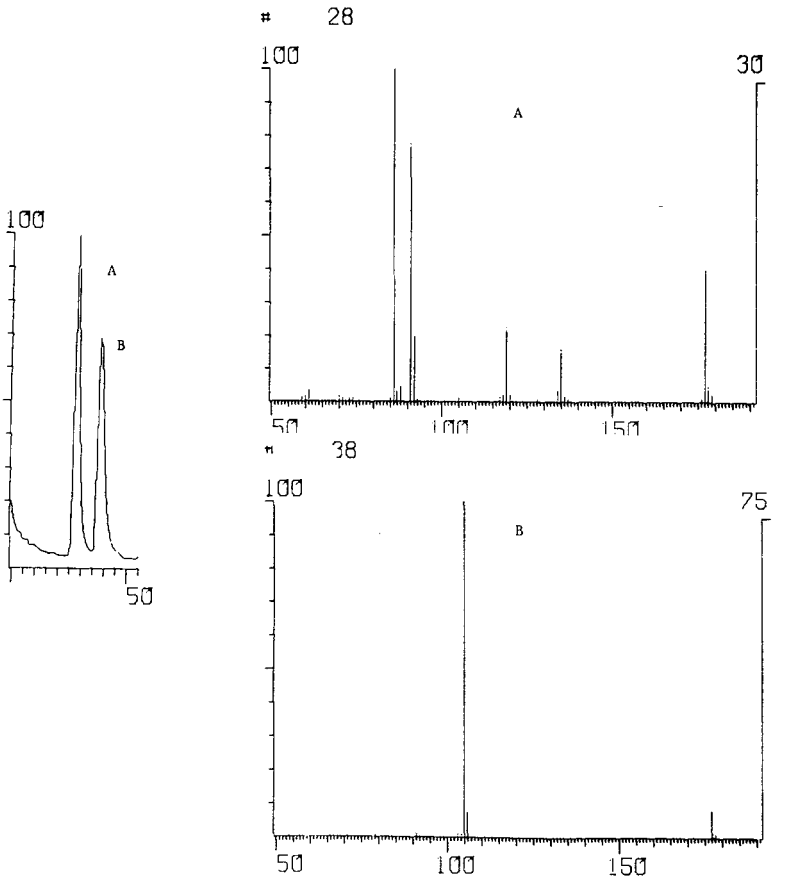

Fig. 1. GC separation of NCS derivatives of (A) amphetamine and (B) β-methylphenethylamine on 3% OV-17, and their mass spectra.

determinations where both internal standards were used. The results were within experimental error and showed a correlation coefficient of 0.95.

The isothiocyanate derivative provides a simple and elegant method for the quantitation of amphetamine in biological samples. The isomer β-methylphenethylamine, which is not of biological origin, serves as an internal standard, providing a common molecular ion for SIM, and because of its similarity in structure, serves as a carrier both in extraction procedures and on GC columns. The advantages of the NCS derivatives for the analysis of primary amines have been emphasized in earlier reports [8,9] and their specific biological application is an illustration of the general applicability of the method. Though the molecular ion *m/e* 177 is not the base peak, it provides enough sensitivity for the assay of biological samples, 1 ng in an injected sample giving a satisfactory signal-to-noise ratio of 3:1.

We have used 2 ml of plasma and 1 ml of CSF for routine analysis, and in some cases where adequate samples were not available, we have worked with 1 ml of plasma and 0.5 ml of CSF for the quantitation of amphetamine. The two-step extraction procedure and derivatization with carbon disulfide provides

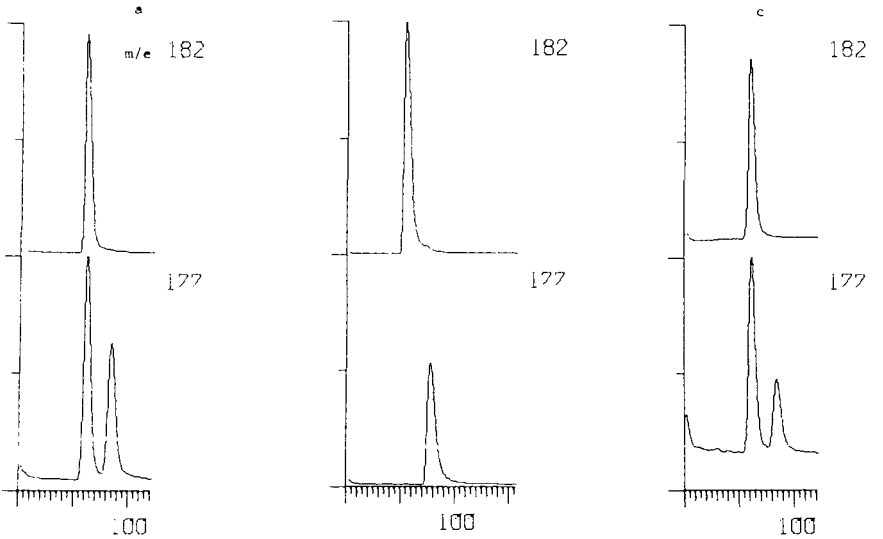

Fig. 2. (a) SIM recording of standard solution of amphetamine-NCS, β-methylphenethylamine-NCS (*m/e* 177) and d_5-amphetamine-NCS (*m/e* 182). (b) SIM recording of two internal standards added to human plasma sample. (c) SIM recording of 3 standards recovered from human plasma sample. x-Axis denotes scan number and y-axis denotes ion current intensity.

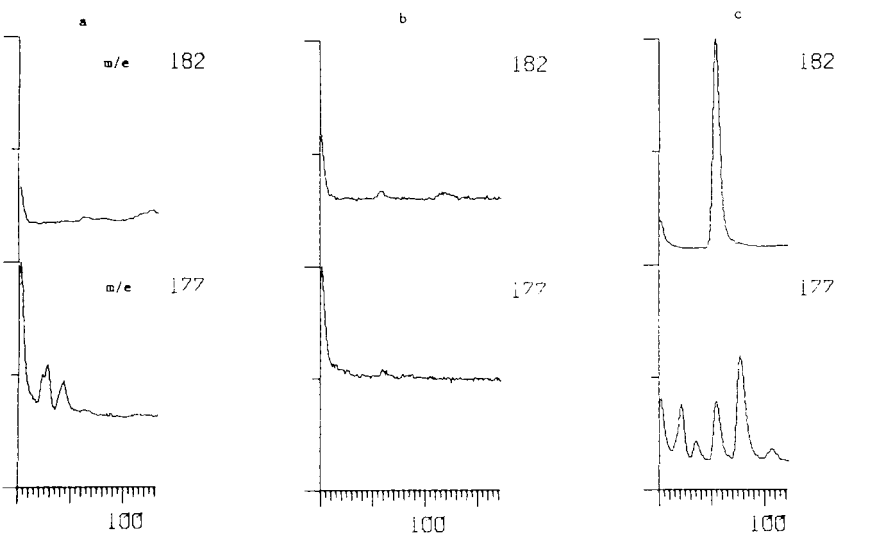

Fig. 3. SIM recording of *m/e* 177 and 182 of a control plasma sample (a) after one-step extraction; (b) after two-step extraction and (c) amphetamine plasma samples with two internal standards after one-step extraction. Details are given in the text. x-Axis denotes scan number and y-axis denotes ion current intensity.

a clean-up procedure, and no interference is noticed at *m/e* 177 or *m/e* 91 when used for increased sensitivity. The NCS derivative is superior to the dansyl derivative which is less volatile and pentafluoropropyl or N-trifluoroacetyl derivatives which do not give the molecular ion in the electron impact mode.

TABLE II

LEVELS OF AMPHETAMINE (ng/ml) IN PLASMA SAMPLES OF MONKEYS TREATED WITH d-AMPHETAMINE

Monkeys 1 and 2 (adult) were administered time release capsules nasogastrically 1.6 mg/kg every 12 h for 12 days. Samples were drawn at 12 noon 3 h after administration. Monkeys 3 and 4 (juvenile) were administered 0.5 mg/kg/day nasogastrically for 25 days.

No. of days after administration	Monkey 1	Monkey 2	Monkey 3	Monkey 4
0 (baseline)	0	0	0	0
1	50	77.5	40	72
2	120	166	90	72
3	108	100	n.d.*	n.d.
4	115	134	n.d.	n.d.
5	130	70	n.d.	n.d.
7	n.d.	n.d.	122	90
11	110	127	n.d.	n.d.
15			45	31
21			132	60
25			100	50

*n.d. = not done.

TABLE III

LEVELS OF AMPHETAMINE (ng/ml) IN CEREBROSPINAL FLUID SAMPLES OF MONKEYS TREATED WITH d-AMPHETAMINE

Values expressed as mean ± S.D. of two determinations. Monkeys were administered time release capsules at a dose level of 1.6 mg/kg/12 h.

Day after	Monkey 1	Monkey 2
3	83.1 ± 0.5	106.0 ± 1.0
5	93.0 ± 0.6	69.1 ± 1.8
11	100.6 ± 0.6	80.8 ± 0.7

TABLE IV

LEVELS OF AMPHETAMINE IN THE CAUDATE AND WHOLE BRAIN LESS CAUDATE OF RATS AFTER ADMINISTRATION OF d-AMPHETAMINE (5 mg/kg i.p.)

Values expressed as mean ± S.D. of two determinations. Number of animals = 3.

Min after administration	Caudate (μg/g)	Whole brain less caudate (μg/g)
30	2.25 ± 0.70	4.1 ± 1.5
120	0.42 ± 0.15	0.7 ± 0.3

Unlike the acyl derivatives which are not stable, the NCS derivatives could be stored indefinitely without loss due to decomposition.

We have stored a mixture of standards containing 20 ng/μl each of amphetamine-NCS and β-methylphenethylamine-NCS for over a year and compared it

with freshly prepared standards once a month and did not find any measurable decomposition. Further, the quantitative response in the GC-MS system was relatively constant for both isomers. Thus in 20 determinations of the same mixture the ratio of peak heights or peak areas of the two components in the mixture varied less than 5%, well within limits of experimental error in such biological determinations. The results from both internal standards agreed within ± 5%.

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Note

Improved gas—liquid chromatography—electron-capture detection technique for the determination of paracetamol in human plasma and urine

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Paracetamol is an easily available analgesic. Thus, poisoning by paracetamol is still a relatively common occurrence [1]. A quantitative, rapid, selective and sensitive method for the determination of paracetamol in biological fluids is essential for investigations such as poisoning due to acute overdose, research into factors affecting drug absorption in the gastro-intestinal tract when the drug is used as a marker, and studies on pharmacokinetics and bioavailability of this drug in various innovative dosage forms. A number of quantitative methods for the estimation of this drug in plasma or urine samples have been described but none have been ideal. The generally accepted gas—liquid chromatographic methods [2,3] suffer from interference peaks which often mask analytical peaks or prolong analysis time [4,5]. Hackett and Dusci [6] reported an improved procedure which involved column extraction with diethyl ether, acetylation and estimation by GLC with flame ionization detection. The extraction procedure can be time-consuming and the lowest limit for quantitation is about 5 μg/ml in a 2-ml sample of plasma which, however, is adequate for detecting paracetamol poisoning.

This paper reports a specific and sensitive GLC—electron-capture detection assay for paracetamol in human plasma and urine samples based on the method of assay for indomethacin in biological fluids previously described [7]. A preliminary report of the procedure was presented at the Pharmaceutical Conference [8].

METHODS

Apparatus

A Pye Series 104 gas chromatograph was used, fitted with a nickel 63 electron-capture detector and linked to a chart recorder (Hitachi Model 156). The detector was operated at a temperature of 330°, with a nitrogen purge flow-rate of 11.0 ml/min. A coiled glass column (1.5 m × 4 mm I.D.) packed with 3%

SP2100 coated on Supelcoport 100—120 mesh (Phase Separation, Queensferry, Great Britain) was used. The column was conditioned at 300° for 48 h and silanised in situ with 2 × 10 μl of hexamethyl disilane (HMDS) before use. The injector temperature was 300° and the oven temperature was 200°. White spot nitrogen was used as carrier gas at a flow-rate of 50 ml/min.

Centrifuge tubes (10 ml and 20 ml) with well-fitting screw caps were obtained from Sovirel (A.V. Howes, Great Britain); 10-μl SGE microsyringes from Chromatography Services (Wirral, Great Britain); Pipetman microsyringes from Anachem (Bedford, Great Britain), and for thin-layer chromatography, TLC plates coated with 0.3 mm silica gel, Merck GF254 from Merck (Darmstadt, G.F.R.).

Materials

The following chemicals were used: acetone, dichloromethane, diethyl ether, methanol and *n*-hexane (all of Analar grade and freshly redistilled); Sörensen phosphate buffer (1 *M*) pH 7.4; Analar ammonium sulphate and potassium carbonate (diethyl ether washed and dried); pentafluorobenzyl bromide (Pierce, Rockford, Ill., U.S.A.) stock solution for use was prepared by diluting the supplied material 1:1000 (v/v) in acetone; paracetamol (BDH, Poole, Great Britain), *n*-butyryl-*p*-aminophenol, the internal standard (R & R Lab., Hollywood, Calif., U.S.A.).

Treatment of glassware

It was found that all glassware should be cleaned and silanised before use according to the following procedure in order to eliminate impurities which might interfere with analytical peaks and minimise the loss of drugs which might absorb on to the wall of glassware. Evaporation tubes and screw-capped centrifuge tubes were cleaned in a 2% solution of RBS 25 (Chemical Concentrates (RBS), London, Great Britain) in water by soaking overnight, then rinsed with methanol (commercial grade) and hot tap water and then distilled water several times. The tubes were then dried in an oven at 105°. After cooling at room temperature, all the tubes were then silanised by rinsing with a 3% solution of HMDS in redistilled chloroform. They were then dried at 250° overnight. Silanisation of tubes should be repeated when necessary at monthly intervals.

Synthesis and characterisation of fluoro derivatives

The pentafluorobenzyl derivatives of paracetamol and its internal marker, *n*-butyryl-*p*-aminophenol were synthesised as follows: paracetamol or its internal marker ($1.6 \cdot 10^{-4}$ mole, 25 mg) was dissolved in re-distilled acetone (2.0 ml) in a micro round bottom flask (5.0 ml). Pentafluorobenzyl bromide ($3 \cdot 10^{-4}$ mole, 50 μl) and anhydrous potassium carbonate ($3.5 \cdot 10^{-4}$ mole, 50 mg) were added. The mixture was heated under reflux for 5 h at 50°. After cooling, the acetone in the reaction mixture was removed by a gentle stream of nitrogen. Distilled water (1.0 ml) was added to dissolve the potassium carbonate and the derivative was extracted into diethyl ether and subsequently recrystallised in acetone.

Paracetamol, *n*-butyryl-*p*-aminophenol and their pentafluorobenzyl deriva-

tives were characterised by thin-layer chromatography, GLC, infra-red spectroscopy and melting point determination.

Procedure for determination of paracetamol in biofluids

Duplicate samples of 0.5 ml of plasma or urine were pipetted into 20-ml glass centrifuge tubes and diluted with 0.5 ml of distilled water; 50 μl of internal marker (500 ng) solution, 1.0 ml of phosphate buffer (pH 7.4) and 1.0 g of ammonium sulphate were added. The tubes were each vortex mixed for 10 sec and 10 ml of an extracting solvent mixture [diethyl ether—dichloromethane (4:1)] were added. The tubes were then shaken by an automatic shaker (40 rpm) for 10 min followed by centrifugation at 1500 *g* for 10 min.

The organic layer was carefully transferred to a clean 10-ml screw-capped tube and evaporated to dryness at 45° under a stream of nitrogen. The walls of the tubes were then carefully rinsed using acetone (about 1.0 ml) which was subsequently evaporated to dryness. To each tube was added 25 mg of potassium carbonate and 0.5 ml of the stock solution of pentafluorobenzyl bromide. Pentafluorobenzylation was carried out in the well screw-capped tube at 60° for 30 min using a thermostatic water-bath. The reaction tubes were carefully agitated every 10 min during this period. The excess derivatising agent was then evaporated off under a stream of nitrogen at room temperature. To each tube was added 0.5 ml phosphate buffer and 0.5 ml *n*-hexane. The tubes were then mixed on an automatic shaker at a speed of 40 rpm for 10 min. An aliquot of the *n*-hexane layer (0.5—1.0 μl) was injected onto the gas chromatography column.

Standard solutions of paracetamol and its internal marker were prepared in methanol. These were diluted to give a series of solutions in plasma or urine (1 ml) covering the concentration range 50—1000 ng in a 0.5-ml sample of plasma or urine. The solutions were then analysed as described in the procedure, and the peak height ratios were plotted against the corresponding concentrations. The recovery of paracetamol from plasma or urine using the present extraction condition was well documented [9].

Reproducibility was checked by performing an anlysis on plasma samples containing paracetamol at concentrations of 50 and 500 ng per 0.5-ml sample.

The procedure was used to determine paracetamol concentrations in plasma and urine after a single oral dose of 1.0 g paracetamol (2 Panadol tablets of 0.5 g) was given to a healthy male subject.

RESULTS AND DISCUSSION

Table I summarises the characteristics of paracetamol, its internal marker *n*-butyryl-*p*-aminophenol and their pentafluorobenzyl derivatives. The melting points, thin-layer chromatographic R_F values, retention times of GLC in both electron-capture and flame ionization detection indicate that the pentafluorobenzylation reaction is successful. The absence of OH-stretching in the IR spectra of the derivatised paracetamol and internal marker also supports the view that the derivatisation procedure is correct although it was not possible to carry out a GC—mass spectrometry analysis on the corresponding peak.

Optimal formation of pentafluorobenzyl derivatives was found to occur at a

TABLE I

CHARACTERISTICS OF PARACETAMOL, *n*-BUTYRYL-*p*-AMINOPHENOL AND THEIR PENTAFLUOROBENZYL DERIVATIVES

Compound	Melting point (°C)	TLC* R_F	GLC retention time (min) using 10% Apiezon at 240°**		GLC retention time (min) using 3% SP2100 at 200°	
			FID	ECD	FID	ECD
Paracetamol	172	0.79	3.4 (TP)	–	1.8 (TP)	–
n-Butyryl-*p*-aminophenol	143	0.90	5.6 (TP)	–	3.4 (TP)	–
PFB-paracetamol	193	0.89	4.0	4.0	2.4	2.4
PFB-*n*-butyryl-*p*-aminophenol	162	0.98	6.2	6.2	4.6	4.6

*TLC system consists of silica gel plate (Merck GF 254) and solvent composition: ethyl-acetate—methanol—water—acetic acid (60:30:9:1) and detection using UV light and Dragendorff reagent.

**GLC system reported by Chan and Highley [8]. TP = Tailing peak.

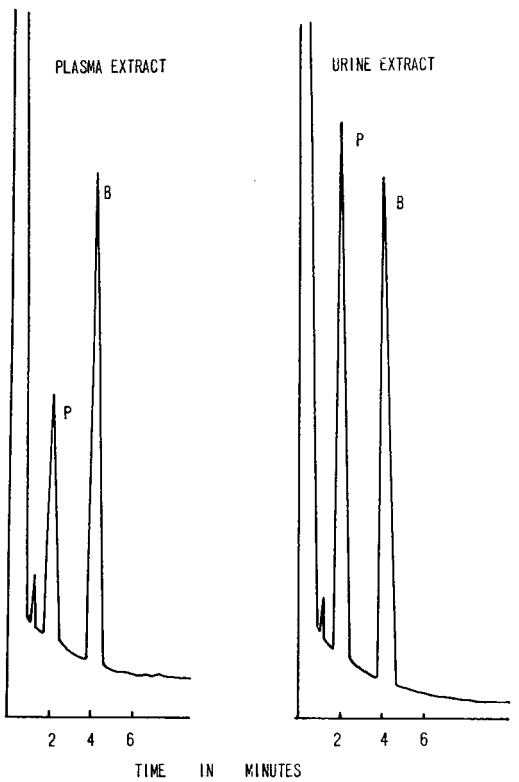

Fig. 1. Chromatograms of pentafluorobenzyl derivatives of paracetamol (P) and *n*-butyryl-*p*-aminophenol (B). Plasma extract: 200 ng per 0.5 ml; urine extract: 500 ng per 0.5 ml.

reaction temperature of 60° for a period of 30 min. This is in agreement with Walle's [10] suggestion of a reaction time of 5—120 min for the pentafluorobenzylation of barbituric acids and diphenylhydantoin.

In a previous report [8], 10% Apiezon L was used as a stationary phase for analysis. As the oven temperature was 240°, which is very close to the maximum recommended temperature for this stationary phase, column bleeding was observed. Further investigation suggested that 3% SP2100 on Supelcoport was considered satisfactory. This stationary phase has a high recommended temperature up to 375°.

Fig. 1 illustrates a typical GLC trace of paracetamol and *n*-butyryl-*p*-aminophenol, as their *o*-pentafluorobenzyl derivatives, extracted from both plasma and urine. Under the GLC conditions described the retention times of pentafluorobenzyl-paracetamol and pentafluorobenzyl-internal marker were 2.4 min and 4.6 min, respectively. The overall accuracy and reproducibility of the analytical procedure is satisfactory. The recoveries of paracetamol from plasma at 50 ng and 500 ng levels were 50.55 ± 2.41 ng and 494.66 ± 12.5 ng, respectively. The graph is linear over the range of 50 ng to 1000 ng per 0.5 ml of plasma and was found to be reproducible when repeated six times during the studies.

The present procedure provides a relatively selective and sensitive assay for the determination of paracetamol concentrations in biological fluids. Using this technique a pharmacokinetic study of an oral dose of Panadol (1 g) in a fasting subject was performed. The results obtained, i.e. an elimination $t_{1/2}$ of 2.69 h and a 24-h urinary recovery of 5.57% as the unchanged drug, compare favourably with previous reported data and indicate that the technique is reliable. Thus, this method has several advantages over those previously reported. Only a small sample of biological fluids (0.5—1.0 ml) is required for analysis. The high sensitivity of the assay allows a more thorough study of the fate of paracetamol in the body. The selectivity of the electron-capture detector gives a cleaner chromatogram and enables analysis of up to ten samples in an hour. A disadvantage is that all organic solvents used should be distilled twice.

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Note

Quantitation of pyridostigmine in plasma using high-performance liquid chromatography

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The quaternary ammonium compound, pyridostigmine, is the cholinesterase inhibitor most widely used in the treatment of myasthenia gravis. The magnitude of interindividual differences in absorption and disposition of pyridostigmine is not defined because of a lack of analytical methodology for routine quantitation in biological fluids. Calvey and Chan [1] reported a 100-fold difference in oral daily doses required to stabilize six myasthenics. Such wide variations in dosing requirements make it difficult to develop a "normal" dosing regimen. If, rather than dose, the plasma levels of pyridostigmine can be correlated to therapeutic response, individualized dosing regimens for optimal control of the disease and avoidance of toxicity can be developed. To develop such information, a simple, reliable and sensitive method for the assay of pyridostigmine in plasma is required.

Assay methods reported for the determination of cholinesterase inhibitors in biological fluids include radioisotopic studies [2—6], paper chromatography [7] and gas chromatography [1, 7—9]. Radioactive compounds are difficult to use in human studies and paper chromatographic techniques are slow and suffer from low sensitivity. The ionic character and low volatility of pyridostigmine do not permit direct quantitation by gas chromatography. The currently available methods involve thermal degradation and may lead to interference by other compounds.

In the present study, a rapid, sensitive and specific high-performance liquid chromatographic (HPLC) technique was developed for the quantitation of pyridostigmine in plasma.

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EXPERIMENTAL

Reagents and materials

Pyridostigmine bromide and neostigmine bromide were kindly provided by Hoffman-LaRoche (Nutley, N.J., U.S.A.). Glass-distilled acetonitrile and methylene chloride were purchased from Burdick & Jackson (Muskegon, Mich., U.S.A.). Glacial acetic acid, ammonium hydroxide, hydrochloric acid and perchloric acid were ACS reagent grade and obtained from Fisher Scientific (Dallas, Texas, U.S.A.). Sodium lauryl sulfate, USP was purchased from City Chemical (New York, N.Y., U.S.A.).

All glassware was treated with 2% trimethylchlorosilane in toluene (Pierce, Rockford, Ill., U.S.A.) for 10 min and rinsed twice with acetone and then water.

Drug extraction from plasma samples

Aliquots of plasma (1 ml) were pipetted into 13 × 100 mm culture tubes with PTFE-lined screw caps (Kimax K-45066A, Scientific Products, Grand Prairie, Texas, U.S.A.). After the addition of 2 μg of internal standard (25 μl of a solution of 80 μg/ml neostigmine bromide in water), 200 μl of 5 *N* hydrochloric acid was added. The sample was vortexed for 5 sec and then shaken with 10 ml of methylene chloride for 5 min on a shaker (Kahn Shaker, Precision Scientific, Chicago, Ill., U.S.A.). After centrifugation at 833 *g* for 5 min, the aqueous phase was transferred to another culture tube and the organic phase was discarded. The plasma protein precipitate was mixed with 2 ml of 0.2 *N* hydrochloric acid and the methylene chloride wash step repeated. The two aqueous portions were combined and 500 μl of 8 *M* perchloric acid added. The mixture was vortexed for 5 sec and extracted twice with 10 ml of methylene chloride. The two organic extracts were combined and evaporated to dryness in a sample concentrator (Model SC/27R, Brinkmann Instruments, Westbury, N.Y., U.S.A.) under a gentle stream of nitrogen. The sides of each tube were repetitively rinsed with 2, 1 and 0.5 ml of methylene chloride. The evaporated extract was redissolved in 100 μl of mobile phase and the entire sample injected into the liquid chromatograph.

Chromatography

The HPLC system consisted of a Model 995 pump and Model 970 variable-wavelength UV detector obtained from Tracor (Austin, Texas, U.S.A.); a Model 7120 injector (Rheodyne, Berkeley, Calif., U.S.A.); and a μBondapak C_{18} (particle size 10 μm) column obtained from Waters Assoc. (Milford, Mass., U.S.A.). The output from the detector was connected to a 10-mV recorder (Omniscribe, Model B-5118-4, Houston Instruments, Austin, Texas, U.S.A.).

Analyses were performed using a mobile phase of 37.5% acetronitrile in water containing 0.001 *M* sodium lauryl sulfate and 1% acetic acid with a final pH adjusted to approximately 4 with concentrated ammonium hydroxide. The system was operated at ambient temperature at a flow-rate of 1 ml/min. The UV detector was operated at a wavelength of 269 nm.

Calibration and reproducibility

Known quantities of pyridostigmine bromide (corresponding to 20.8—104.1 ng/ml of pyridostigmine) were added to blank plasma samples. Samples of 1 ml were then assayed for pyridostigmine. Calibration curves were constructed by plotting the peak height ratios between pyridostigmine and the internal standard, neostigmine, versus the amount of pyridostigmine added. The reproducibility of the analytical procedures was checked by determining the calibration curve on five different days.

Stability studies

The degradation of aqueous pyridostigmine bromide solutions (0.05 mg/ml) was followed at two pH (1 and 11) and two temperature values (25° and 70°) during a 3-h period using UV spectroscopy. The stability of pyridostigmine in plasma was determined over a 14-day period. Plasma samples containing 50 and 100 ng/ml of pyridostigmine bromide were stored at -20° and assayed periodically by HPLC using a plasma standard curve prepared on each assay day.

RESULTS AND DISCUSSION

The system reported here provides a reliable method for the extraction and quantification of pyridostigmine in plasma. The problems involved in extraction of quaternary ammonium compounds from an aqueous medium were overcome by forming a perchlorate ion-pair that partitioned into methylene chloride. The other ion-pairing agents studied, potassium iodide—glycine buffer [9], trichloroacetic acid [10] and sodium lauryl sulfate, were not as effective in extracting both pyridostigmine and neostigmine. The separation and detection problems result from attempting to resolve pyridostigmine (λ_{max} = 269 nm) from endogenous plasma constituents that absorb significantly in that region by including an ion-pairing agent, sodium lauryl sulfate, in the acetronitrile

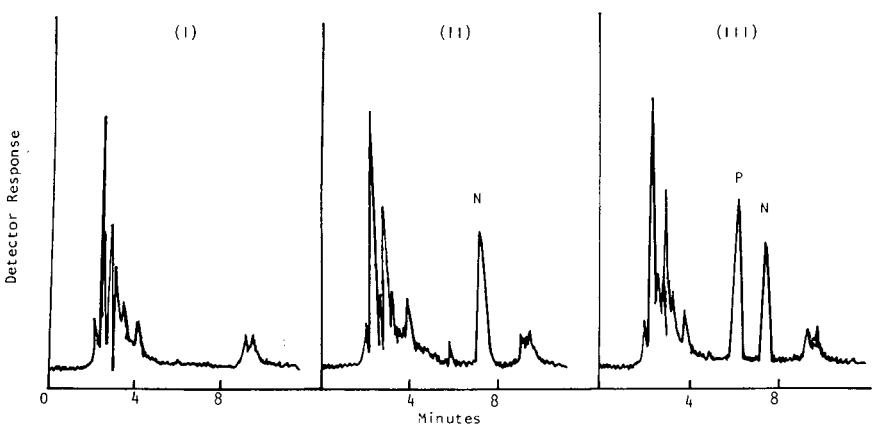

Fig. 1. Chromatograms of human plasma extracts using a 37.5% acetonitrile solution containing 0.001 *M* sodium lauryl sulfate and 1% glacial acetic acid with the pH adjusted to approximately 4 as the mobile phase. (I) Blank plasma; (II) plasma containing neostigmine (N); (III) plasma containing neostigmine (N) and pyridostigmine (P). Flow-rate at 1.0 ml/min; absorbance unit full scale (a.u.f.s.) at 0.02.

water mobile phase. Fig. 1 shows chromatograms typical of those obtained for the separation of pyridostigmine and the internal standard, neostigmine, following injection of plasma extracts. This figure demonstrates a slight interference in the pyridostigmine peak attributable to neostigmine. This interference was observed in extractions from both aqueous solutions and plasma samples. To compensate, the calculation of the peak height ratios used was:

$$\text{peak height ratio } (P/N) = \frac{H_P - (H_n \times r)}{H_n}$$

where r = peak height ratio of the interference to the internal standard, neostigmine, in blank plasma; H_P = peak height of pyridostigmine and H_n = peak height of neostigmine.

Fig. 2 shows the linear relationship obtained between the peak height ratio (P/N) and the concentration of pyridostigmine in plasma up to 694 ng/ml (equivalent to 1000 ng/ml of the bromide salt).

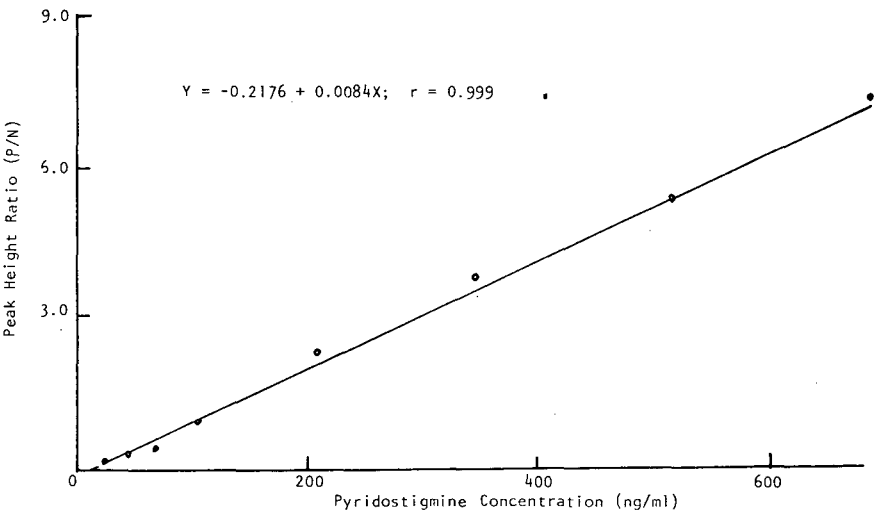

Fig. 2. Peak height ratios (P/N) as a function of the concentration of pyridostigmine in plasma.

When standard curves were constructed on five different days over a pyridostigmine concentration range of 20.8—104.1 ng/ml, an excellent linear relationship was obtained each time. The slopes of the calibration curves were quite reproducible with a relative standard deviation of 11.2%. The intercepts were considerably more variable and indicated that a standard curve should be prepared each day assays are to be run.

Plasma samples containing 34.7 and 69.4 of pyrisdostigmine per ml were frozen and then assayed on five different days over a two-week period. On each day a new standard curve was prepared. The mean values obtained were 37.1 and 69.2 ng/ml with a relative standard deviation of 2.8 and 3.2%, respectively, an indication of excellent reproducibility and stability of frozen plasma samples over a 14-day period.

The proposed assay procedure can be used to estimate levels below 20 ng/ml

by utilizing a plasma sample larger than 1 ml. However, for concentrations of 7 and 10 ng/ml, the relative standard deviations obtained were 19.7 and 20.5%, respectively.

Recovery of pyridostigmine following extraction from either aqueous solution or plasma was approximately the same. Mean recovery from aqueous solution was 69.4% and 63.9% from plasma.

The stability studies conducted in aqueous solution showed that pyridostigmine was stable in acid medium (pH 1) at both 25° and 70° for the length of the study (3 h). However, in alkaline solution (pH 11), pyridostigmine is extremely unstable even at 25°. During hydrolysis the pyridostigmine absorption spectra indicate a 1:1 transformation by the appearance of isobestic points at 260 and 287 nm. The absorbance of pyridostigmine at its λ_{max} (269 nm) decreases with a concomitant appearance of absorption maxima at 252 and 322 nm. Under alkaline conditions, hydrolysis of pyridostigmine at the ester linkage can be anticipated. This would lead to the formation of 3-hydroxy-N-methylpyridinium, a reported major metabolite of pyridostigmine [2, 4—6]. It is therefore advisable to avoid alkaline conditions in the assay of pyridostigmine. The gas chromatographic method of Calvey and Chan [1] was carried out at pH values of 10—12 and loss of pyridostigmine to hydrolysis is possible.

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Biomedical Applications

CHROMBIO. 402

Note

Determination of mitomycin C in biomedical specimens by high-performance liquid chromatography

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Mitomycin C (MMC), an antitumor antibiotic, is currently used in the treatment of various tumors [1]. The therapeutic effectiveness of MMC should be related to its concentration in blood, body fluids and tissues, which in turn depends on the dose, method, and schedule of administration. Although a number of clinical investigations have proved its effectiveness against cancer in the intestinal region, monitoring its concentration in the time course of the drug therapy has hardly been explored. A rapid and sensitive method of its assay is required for such monitoring.

High-performance liquid chromatography (HPLC) is widely used in the separation, identification and determination of a large number of compounds. Recently HPLC was used to separate MMC from other mitomycins and its polar conversion products [2]. We have been working on the analysis of anticancer agents in biomedical specimen by means of HPLC [3] and have found this technique can be applied with much success in the monitoring of MMC.

The present paper describes the HPLC analysis of MMC in biomedical specimens, which would give the necessary information for the optimal administration schedule.

MATERIALS AND METHODS

Mitomycin C used in this investigation was kindly supplied by Kyowahakkokogyo (Tokyo, Japan). All solvents for HPLC and chemicals were certified grade and products of Wako (Osaka, Japan).

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A Waters Assoc. high-pressure liquid chromatograph Model 204 was used. The chromatograph was equipped with a Model 6000 solvent delivery system, a Model U6K universal injector, and a Model 440 UV detector. A Waters Assoc. μBondapak C_{18}/Porasil (8—10 μm particle size, 300 × 3.8 mm I.D.) reversed-phase column was used. The mobile phase was methanol—water (35:65). The pump was generally run at a flow-rate of 1.0 ml/min at a pressure of 1800 p.s.i.

Standard curves obtained by plotting the peak heights against the amounts of tain 1.0—25.0 ng MMC per μl. Samples were injected into the chromatograph in aliquots of exactly 1.0 μl with a 10-μl Hamilton syringe.

Human serum, ascites and urine for analysis were collected from patients administered MMC. MMC-free samples were from a healthy adult man. To 1.0 ml of material, 0.1 ml of 0.5 *M* NaH_2PO_4 and 8 ml of ethyl acetate were added and the sample was extracted with vigorous shaking. The organic layer was separated by centrifugation and evaporated to dryness using a water-bath at 30° and a water-pump vacuum. The residue was dissolved in 40—100 μl of methanol for analysis by HPLC.

RESULTS AND DISCUSSION

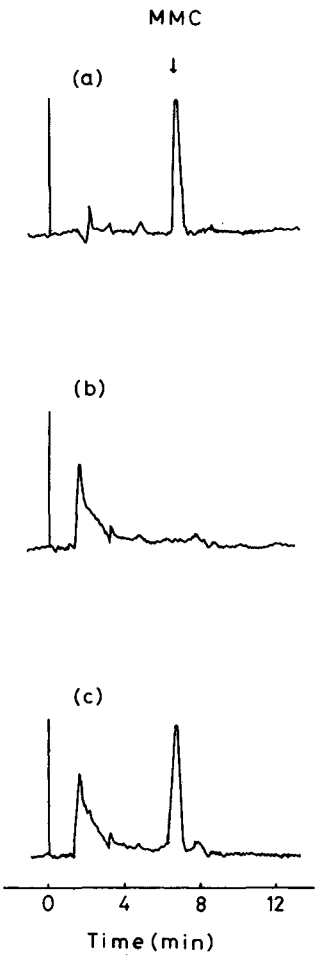
Fig. 1 shows chromatograms of (a) a standard MMC solution (10 ng/μl), (b) an extract of MMC-free serum, and (c) an extract of serum from a patient administered 10 mg of MMC. A sharp peak with a retention time of 6.6 min is readily identified as that of MMC. Column effluents were monitored at 365 nm. Comparable elution profiles were obtained by monitoring at 254 nm, though there was a slight interfering peak arising from endogenous serum components. Since detection at 365 nm gave higher absorbance and better resolution, the UV detector was operated at this wavelength for the determination of MMC.

Standard curves obtained by plotting the peak heights against the amounts of MMC injected were linear in the range 1.0—25.0 ng. It is estimated that 1.0 ng of MMC can easily be detected using the available detector. In the determination of MMC in solutions containing 10 ng, the standard deviation was 0.41 ng (n=7).

Appropriate amounts of MMC were added to MMC-free serum and these spiked standards were carried through the procedure. Plots of the peak heights against the amounts of spiked MMC gave straight lines, and recoveries of 93.8 ± 2.3% were obtained from comparison of the slopes with that obtained with the standard methanol solutions.

The present method permits the accurate determination of MMC in biological fluids at concentrations as low as 40 ng/ml and is suited for monitoring the drug in the therapeutic dose range (2—10 mg). The simple procedures allow a large number of analytical samples to be handled.

Fig. 2 shows examples of the time—concentration curves in the sera of a patient administered 10 mg MMC. These pharmacokinetic measurements of MMC in tumor-bearing patients are continuing. Correlation of the therapeutic effects to its concentrations in body fluids and tissues is under investigation. The details will be reported elsewhere.



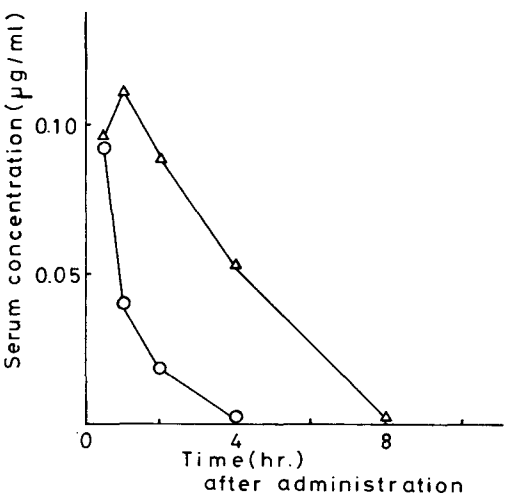


Fig. 1. Chromatograms of (a) methanolic solution of MMC (10 ng/μl), (b) an extract of MMC-free serum, and (c) an extract of serum collected 3 h after the intraperitoneal infusion of 10 mg of MMC to an adult man with intestinal cancer.

Fig. 2. Serum concentration of mitomycin C at various times after intravenous (○) or intraperitoneal (△) administration (10 mg) to a patient.

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