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CONTENTS

Contents (continued)

Contents (continued)

Book Review

JOURNAL

of

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QUANTITATION OF HALOGENATED AROMATIC COMPOUNDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Quantitation by gas chromatography—mass spectrometry and by gas chromatography—hydrogen flame ionization detection (GC—HFID) were compared for five classes of halogenated aromatic compounds. For the five classes of compounds studied (polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, polychlorinated diphenyl ethers, polychlorinated naphthalenes and polychlorinated biphenyls), relative peak areas based on both selected ion monitoring of ions in the molecular ion cluster, and total ion current peak areas gave good approximations to relative concentrations based on GC—HFID peak areas. Within each class of compounds, the molecular ion cluster peak areas are a fairly constant fraction of the total ion current peak areas, regardless of the number of chlorines in the molecule or the substitution pattern.

INTRODUCTION

Halogenated aromatic compounds occurring as environmental pollutants are often found as complex mixtures of classes, homologues, and isomers [1]. The quantitative analysis of such samples is complicated by coelution of related classes in typical fractionation schemes used during sample preparation. Further, some of these classes of compounds, for example the polychlorinated biphenyls (PCBs), are presently impossible to separate into individual, non-overlapping components even by capillary gas chromatography (GC) [2—6]. When several classes are present in the same sample, for example PCBs, poly-

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chlorinated diphenyl ethers (PCDPEs) and polychlorinated naphthalenes (PCNs), chromatographic separation into individual components becomes altogether out of the question. Under such circumstances quantitation by GC using electron-capture detection (ECD), hydrogen flame ionization detection (HFID) or mass spectrometry—total ion current (MS—TIC) gives invalid results due to superposition of components of differing detector responses.

There are 209 possible PCBs, 209 possible PCDPEs, and 75 possible PCNs; for the PCBs at least, over 130 of the 209 possible isomers have been observed in commercial preparations [5—7]. Standards are not available for all of the isomers that may occur in environmental samples, and it would be quite impractical to generate 493 individual calibration curves (209 for PCBs, 209 for PCDPEs and 75 for PCNs) even if all possible standards were available. Moreover, classes of compounds in addition to the three mentioned may also be present (for example the 135 possible polychlorinated dibenzofurans, PCDBFs, and the 75 possible polychlorinated dibenzo-p-dioxins, PCDBDs).

An approach to this problem based on a preliminary class fractionation (see Fig. 1) followed by GC—MS with selected ion monitoring (SIM) has been described previously [1]. Although this approach can deal with the co-chromatography problem, quantitation using this approach is only feasible if one can avoid the need for individual calibration curves for every compound present in the mixture. The present paper is concerned with a comparison of relative detector responses (HFID, TIC, SIM) to compounds of the classes found in Fractions 2 and 3 of the fractionation scheme shown in Fig. 1. The errors associated with the assumption that the relative detector response may be averaged for a given isomeric group are also considered.
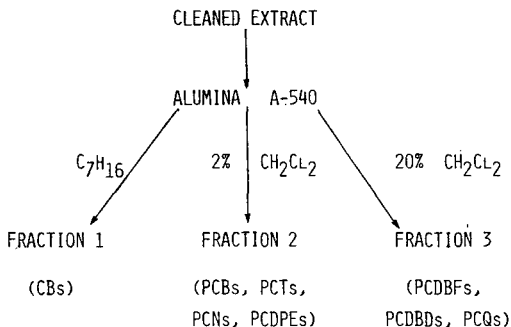
Fig. 1. Fractionation scheme.

EXPERIMENTAL

GC peak areas for fraction 2 mixtures were determined using a Varian 1200 gas chromatograph equipped with a hydrogen flame ionization detector, and fitted with a 2-m 3% OV-225 packed column. Fraction 3. GC—HFID analyses were done using capillary columns containing OV-1. Peak areas were measured with an Autolab System IV computing integrator. The chromatographic conditions have been detailed previously [1].

The analyses of fraction 2 and fraction 3 mixtures were performed on a VG Micromass 70/70F mass spectrometer, interfaced to a VG 2250 data system. The mass spectrometer was scanned from 670 to 20 a.m.u., at 1 sec per decade. The electron energy was 70 eV; source temperature was held at approximately 180°C. The mass spectrometer was equipped with a Hewlett-Packard HP 5710 gas chromatograph and a jet separator for packed column operation. A 2-m packed column containing 3% OV-225, programmed from 180 to 250°C, at 6°C/min, was used for the molecular ion cluster (MIC) and TIC analyses of fraction 2 mixtures. A capillary column containing Dexsil 410 (25 m) was used for the mixture analyses and the MIC and TIC determinations for the PCDBFs. An SE 30 (7 m) capillary column at 220°C, or programmed from 100 to 240°C at 10°C/min, or an OV-101 (11 m) capillary column programmed from 150 to 240°C at 10°C/min was used for the PCDBD analyses.

Molecular ion peak areas were determined by measuring the areas of the $[M+2]^+$ ion chromatograms, i.e., the ion containing one ^{37}Cl isotope, using the VG data system peak area program. The $[M+2]^+$ peak areas were then multiplied by weighting factors to give the areas in the entire MIC. The weighting factors were obtained from a table of relative probabilities of occurrence of the various isotope peaks [8]. TIC peak areas were determined from the peak areas of the total ion chromatogram and include all ion intensities in the entire mass range scanned.

Individual PCBs, PCDPEs, PCDBDs and PCDBFs were obtained from Analabs (North Haven, DE, U.S.A.) or RFR (Hope, RI, U.S.A.). The mixture of PCNs used was Halowax 1013 from Analabs. Solvents used were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

RESULTS AND DISCUSSION

Analysis of fraction 3 mixtures

PCDBDs. The results of the analyses of four dioxin mixtures are shown in Table I. If the HFID results are taken as the best approximation of the true relative concentrations, there is good correlation between the actual relative concentrations and those based on TIC peak areas and MIC peak areas. These relationships are shown in the following equations:

TIC% = 1.02 (HFID%) − 0.49, r = 0.9801, p < 0.001

M^+% = 0.89 (HFID%) + 2.49, r = 0.9494, p < 0.001

$[M+2]^+$% = 1.00 (HFID%) + 0.06, r = 0.9783, p < 0.001

where p is the probability that the correlation coefficients are not significantly different from zero. The regression equation is given below:

% TIC carried by the MIC = 0.87 (No. of chlorines) + 44.02, r = 0.3612, $0.1 < p < 0.2$

The low correlation coefficient indicates a poor correlation, so the % TIC carried by the MIC is not simply determined by the number of chlorines in the molecule.

TABLE I

RELATIVE PEAK AREAS BY HFID, M^+, $[M+2]^+$, AND TIC FOR FOUR DIOXIN MIXTURES

Component	HFID areas (±S.D.)	TIC areas (±S.D.)	MIC areas based on M^+ areas (±S.D.)	MIC areas based on $[M+2]^+$ areas (±S.D.)
Dioxin mixture A				
1-Cl$_1$	21.2±1.9	14.7±0.61	13.8±0.48	13.7±1.11
2,3-Cl$_2$	21.0±1.2	19.1±1.7	19.5±0.74	21.6±3.12
2,6,8-Cl$_3$	17.1±0.56	18.5±2.0	17.3±0.94	17.8±2.74
1,2,3,4-Cl$_4$	23.1±0.28	25.6±1.7	26.5±0.54	25.7±1.0
1,2,3,7,8-Cl$_5$	17.4±0.18	22.3±1.5	22.9±0.74	21.3±0.94
Dioxin mixture B				
2-Cl$_1$	9.27±0.30	6.7±1.2	7.1±0.25	7.0±0.97
2,7-Cl$_2$	16.40±0.37	17.4±3.4	14.4±1.0	15.5±1.36
2,3,7-Cl$_3$	14.70±0.10	13.8±2.6	15.5±0.65	13.7±1.8
2,3,7,8-Cl$_4$	8.09±0.11	7.1±1.1	8.0±0.47	8.1±0.53
1,2,3,7,8-Cl$_5$	51.6±0.87	55.1±3.6	54.9±1.6	55.8±3.8
Dioxin mixture C				
2,8-Cl$_2$	63.0±0.78	59.0±3.3	50.3±2.34	57.7±1.07
1,2,4-Cl$_3$	15.6±0.10	15.6±0.52	19.1±1.6	17.0±1.15
1,2,3,7,8-Cl$_5$	21.4±0.73	25.4±2.8	30.6±0.96	25.3±0.71
Dioxin mixture D				
1,2,4,7,8-Cl$_5$	27.5±1.6	24.5±1.4	26.4±0.46	24.1±0.67
1,2,3,6,7,8-Cl$_6$	19.0±0.11 }	52.0±2.1	48.3±1.9	50.7±0.78
1,2,3,7,8,9-Cl$_6$	28.1±0.30 }			
1,2,3,4,6,7,8-Cl$_7$	11.9±0.33	12.0±0.75	13.2±0.70	12.2±1.3
Octachloro-Cl$_8$	13.4±1.2	11.5±0.32	12.1±2.4	13.0±0.76

For there to be good correlations for MS—TIC peak areas with true relative concentrations, even for isomers containing different numbers of chlorines, the ionization cross sections for isomers containing different numbers of chlorines must be fairly constant. For molecular ion peak areas to also give good approximations to relative concentrations, independent of the number of chlorines in the molecule, then the portion of the TIC carried by the MIC must also be fairly constant. Alternatively, discrepancies may fortuitously cancel out.

The hydrogen flame ionization detector response to substituted aromatic compounds (peak area per μmole) generally tends to decrease as C—H bonds are replaced by C—Cl bonds. Although ways of correcting for this have been empirically developed for some compound classes [9], this has not been accomplished for PCDBDs due to lack of availability of a large number of highly pure individual standards in weighable quantities. Table I suggests that the relatively high response to monochloro PCDBDs and the relatively low response to highly chlorinated PCDBDs of the hydrogen flame ionization detector fortuitously cancel out when a least-squares regression line comparing HFID to TIC areas is calculated.

TABLE II

PERCENT OF THE TOTAL ION CURRENT CARRIED BY THE MOLECULAR ION CLUSTER FOR DIOXINS

Compound	Percent TIC carried by MIC ($\pm$ S.D.)
1-Cl$_1$	47.6 $\pm$ 2.46
2-Cl$_1$	46.9 $\pm$ 6.53
2,3-Cl$_2$	55.0 $\pm$ 7.02
2,7-Cl$_2$	38.8 $\pm$ 8.8
2,8-Cl$_2$	36.0 $\pm$ 1.04
1,2,4-Cl$_3$	45.3 $\pm$ 0.12
2,3,7-Cl$_3$	47.5 $\pm$ 9.3
2,6,8-Cl$_3$	48.4 $\pm$ 5.86
1,2,3,4-Cl$_4$	51.8 $\pm$ 3.46
2,3,7,8-Cl$_4$	49.9 $\pm$ 6.9
1,2,3,7,8-Cl$_5$	48.4 $\pm$ 4.26
1,2,4,7,8-Cl$_5$	49.9 $\pm$ 1.9
1,2,3,6,7,8-Cl$_6$ 1,2,3,7,8,9-Cl$_6$ }	46.1 $\pm$ 3.6
1,2,3,4,6,7,8-Cl$_7$	51.4 $\pm$ 2.4
1,2,3,4,6,7,8,9-Cl$_8$	53.1 $\pm$ 6.5
Mean	47.4 $\pm$ 4.9

TABLE III

RELATIVE PEAK AREAS BY HFID, M$^+$, [M+2]$^+$ AND TIC FOR TWO MIXTURES OF FURANS

Component	HFID areas ($\pm$ S.D.)	TIC areas ($\pm$ S.D.)	MIC areas based on M$^+$ areas ($\pm$ S.D.)	MIC areas based on [M+2]$^+$ areas ($\pm$ S.D.)
Furan mixture A				
2,3-Cl$_2$	9.99 $\pm$ 0.18	9.5 $\pm$ 3.2	8.3 $\pm$ 2.0	8.3 $\pm$ 1.6
2,4,6-Cl$_2$	17.70 $\pm$ 0.25	16.1 $\pm$ 2.9	16.4 $\pm$ 1.4	15.7 $\pm$ 1.4
2,3,8-Cl$_3$	8.58 $\pm$ 0.16	8.5 $\pm$ 2.3	9.4 $\pm$ 2.4	9.1 $\pm$ 1.9
2,3,6,8-Cl$_4$	10.40 $\pm$ 0.31	9.5 $\pm$ 2.0	10.2 $\pm$ 1.6	9.8 $\pm$ 2.1
2,4,6,8-Cl$_4$	15.10 $\pm$ 0.84	14.6 $\pm$ 1.8	14.9 $\pm$ 0.50	13.8 $\pm$ 1.8
1,3,4,7,8-Cl$_5$	23.7 $\pm$ 0.40	25.7 $\pm$ 3.2	24.5 $\pm$ 1.8	26.3 $\pm$ 0.56
2,3,4,6,7,8-Cl$_6$	14.5 $\pm$ 1.18	16.1 $\pm$ 3.2	16.2 $\pm$ 2.4	17.0 $\pm$ 1.5
Furan mixture B				
2,3,4,6,7,8-Cl$_6$	54.2 $\pm$ 0.62	58.7 $\pm$ 2.7	56.2 $\pm$ 3.6	58.7 $\pm$ 2.7
Octachloro-Cl$_8$	45.8 $\pm$ 0.62	41.3 $\pm$ 2.7	43.8 $\pm$ 3.6	41.3 $\pm$ 2.7

The portion of the TIC carried by the MIC for various isomers was studied, and the results are given in Table II. For these PCDBD's, the % TIC carried by the MIC was 47.4 $\pm$ 4.9%.

PCDBFs. A similar study was done on two mixtures of PCBDFs. The results of the mixture analyses are shown in Table III. Here again there was good cor-

relation between the HFID peak areas (taken as the best values), and the TIC and MIC peak areas, as shown in the following regression equations:

TIC% $= 1.03$ (HFID%) $- 0.58$, $r = 0.9894$

M^+% $= 1.01$ (HFID%) $- 0.25$, $r = 0.9963$

$[M+2]^+ = 1.03$ (HFID%) $- 0.62$, $r = 0.9870$

The % TIC carried by the MIC was calculated, and was fairly constant over all of the furan isomers studied (Table IV). The very poor correlation between the % TIC carried by the MIC and the number of chlorines in the molecule ($r = 0.0369$) indicates that these two parameters are independent.

TABLE IV

PERCENT OF THE TOTAL ION CURRENT CARRIED BY THE MOLECULAR ION CLUSTER FOR FURANS

Compound	Percent TIC carried by MIC ($\pm$ S.D.)
2,8-Cl_2	46.1$\pm$ 4.82
2,4,6-Cl_3	52.5$\pm$ 4.75
2,3,8-Cl_3	56.8$\pm$ 3.03
2,4,6,8-Cl_4	51.5$\pm$ 4.76
2,3,6,8-Cl_4	54.8$\pm$ 3.09
1,3,4,7,8-Cl_5	51.7$\pm$ 3.96
2,3,4,6,7,8-Cl_6	49.4$\pm$ 7.15
1,2,3,4,6,7,8,9-Cl_8	52.0$\pm$10.4
Mean	52.0$\pm$ 5.33

Analysis of fraction 2 mixtures

PCDPEs. Similar studies were done on a synthetic mixture of seven PCDPEs. Since no pure, individual standards were available, identification of the substitution pattern and relative molar response (RMR) calculations could not be done. The results of the mixture analysis are shown in Table V. Regression equations and correlation coefficients are given below:

TIC vs. FID   : TIC $= 1.05$ (FID) $- 0.49$; $n = 7$, $r = 0.9995$, $p < 0.001$

TIC vs. [M+2] : TIC $= 0.84$ [M+2] $- 2.15$; $n = 7$, $r = 0.9899$, $p < 0.001$

FID vs. [M+2] : [M+2] $= 0.58$ (FID) $+ 0.45$; $n = 7$, $r = 0.9888$, $p < 0.001$

PCNs. The chloronaphthalenes studied were also only available as a mixture (Halowax 1013) that was not completely resolved by GC (see Fig. 2). In addition, the same degree of resolution was not obtained by all of the methods of detection compared. To obtain comparable quantitation results, some poorly resolved peaks were combined and others, where the degree of chlorination was uncertain, were omitted (see Table IV). Regression equations and correlation coefficients are given below:

TIC $= 1.03$ (HFID) $+ 0.29$; $n = 9$, $r = 0.991$, $p < 0.001$

[M+2] $= 0.97$ (HFID) $+ 0.48$; $n = 7$, $r = 0.953$, $p < 0.001$

TABLE V

RELATIVE PEAK AREAS BY HFID, [M+2] AND TIC, AND PERCENT TIC CARRIED BY THE MOLECULAR ION FOR PCDPEs

Peak No.	PCDPE mix	HFID areas (± S.D.)	MIC areas based on [M+2] areas (± S.D.)	TIC areas (± S.D.)	Percent TIC carried by MIC
3	hexa	3.0±0.2	2.1±0.5	2.2±0.5	32.5
5	hepta	2.1±0.1	1.6±0.4	1.8±0.3	38.3
6	hepta	31.3±0.5	35.0±0.8	32.4±2.4	27.5
8	octa	34.2±0.5	41.7±1.6	35.6±1.4	37.6
9	octa	5.0±0.6	3.9±0.6	5.7±1.1	34.6
10	octa	6.2±0.2	4.2±0.6	5.7±1.1	23.6
12	nona	15.7±0.5	11.4±0.6	15.7±1.1	33.7
Mean					30.8±7.1

Calculations of % TIC carried by the MIC were also done on Halowax 1013. Since no pure standards were available, the substitution patterns of components in this mixture could not be determined. Moreover, since this mixture was much more complex than that of the PCDPEs, and some of the peaks were not completely resolved (see Fig. 2), even the number of chlorines in a particular peak could not always be determined. The results of calculating the % TIC carried by the MIC, assuming that the peak was due to a single component of the highest number of chlorines detected in the peak, are shown in Table VI.
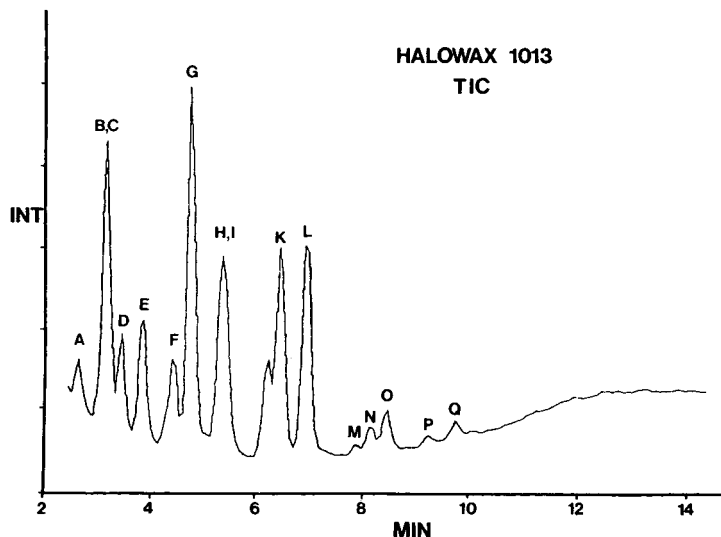
Fig. 2. GC—MS TIC chromatogram of Halowax 1013, a mixture of PCNs (0V-225, programmed from 180 to 250°C, at 6°C/min).

TABLE VI

RELATIVE PEAK AREAS BY HFID, [M+2] AND TIC, AND PERCENT OF THE TOTAL
ION CURRENT CARRIED BY THE MOLECULAR ION CLUSTER FOR PCNs

No. of Cl	Peak	HFID areas	MIC areas based on [M+2] area	MIC areas based on TIC area	Percent TIC carried by MIC
4	A	4.7	3.7	2.5	49.1
3	B			15.4	8.3
4	C				41.7
4	D				33.6
4	E	12.5	12.6	7.4	56.5
4	F				50.5
5	G				8.8
5	H, I	23.0	15.9	16.5	30.0
5	J, K	31.0	32.7	38.6	47.7
5	L	21.6	25.3	27.7	49.2
6	M, N, O	5.9	7.2	6.2	51.8
6	P	1.2	2.5	1.1	41.2
7	Q				52.2
Mean					48.0±6.8 (without peaks B and G)

TABLE VII

RELATIVE PEAK AREAS BY HFID, [M+2] AND TIC FOR PCBs

	HFID areas (±S.D.)	HFID areas (RMR-corrected) (±S.D.)	MIC areas based on [M+2] areas	TIC areas
PCB mix 1				
2,5,2',5'	18.05±0.23	16.64±0.21	12.2	14.7
2,4,6,2',4',6'	18.75±0.21	22.22±0.25	20.5	21.8
2,3,4,5	16.95±0.15	16.10±0.14	16.4	16.6
2,4,5,2',5'	19.12±0.43	18.40±0.41	17.6	19.1
2,3,6,2',3',6'	12.88±0.14	12.84±0.14	16.2	15.6
3,4,3',4'	14.28±0.074	13.80±0.072	15.8	13.6
PCB mix 2				
2,3,4,5	27.5±0.5	27.1±0.5	23.0	23.3
2,3,4,5,6	34.4±0.5	37.4±0.5	29.9	31.3
2,3,5,6,2',3',5',6'	17.2±0.1	18.7±0.2	25.0	22.7
2,3,4,5,2',3',4',5'	19.9±0.2	16.7±0.2	22.4	22.7
PCB mix 3				
3,4,3',4'	30.4±0.5	29.9±0.5	34.3	30.3
2,3,4,2',5'	26.8±0.5	23.9±0.4	21.7	23.6
2,3,4,2',3',4'	22.9±0.4	23.2±0.4	22.6	22.8
2,3,5,2',3',5'	20.4±0.3	22.9±0.4	21.3	23.2

As can be seen from Table VI, most of the values for the % TIC carried by the MIC fall in the range 40—57%. If one assumes that this value should be a constant for PCNs, as was found to be the case for PCDBDs, PCDBFs, and PCDPEs, and that values in this range represent true values for this constant, then peaks which give lower values may be interpreted as resulting from coeluting components with fewer chlorines. The effect of lower molecular weight coeluting components would be to increase the TIC peak area, but would leave the MIC area unchanged, thus leading to a lower ratio. One example of where this may have occurred is peak G, where the observed % TIC carried by the MIC is 8.8%.

In this way, if the % TIC carried by the MIC has been shown to be a constant for a certain class of compounds, the ratio can be used as a check on peak purity in a mixture, at least as to whether or not the peak is likely to contain only components with the same number of chlorines.

PCBs. Three mixtures of PCBs were analyzed, and the results of quantitation based on HFID peak areas, HFID ppeak areas corrected for RMR according to the empirical rules described previously [9], MIC peak areas based on $[M+2]^+$ peak areas, and TIC peak areas were compared (Table VII). The RMD-corrected HFID values were taken as the best approximation to the actual relative concentrations [9]. The regression equations and correlation coefficients obtained are given below:

TIC vs. RMR-corrected HFID values: TIC = 0.71 (RMR-corrected HFID) +
$$+ 6.38, n = 14, r = 0.9052, p < 0.001$$

[M+2] vs. RMR-corrected HFID values: [M+2] = 0.75 (RMR-corrected HFID)
$$+ 5.54, n = 14, r = 0.8047, p < 0.001$$

HFID vs. RMR-corrected HFID values: HFID = 0.69 (RMR-corrected HFID) +
$$+ 6.51, n = 14, r = 0.7874, p < 0.001$$

where p is the probability that the correlation coefficient is not significantly different from zero.

The RMR-corrected HFID values, RMR-corrected $[M+2]^+$ values, and the % TIC carried by the MIC's are given in Table VIII for the sixteen PCB isomers studied. There was no correlation observed between the number of chlorines in the molecule and the % TIC in the MIC, and the average value for the % TIC in the MIC was 37.9 ± 5.5.

CONCLUSIONS

For the five classes of compounds studied (PCDBDs, PCDBFs, PCDPEs, PCNs, and PCBs), relative peak areas based on both SIM of ions in the MIC, and TIC peak areas gave good approximations to relative concentrations based on GC—HFID peak areas. Within each class of compounds, the MIC peak areas are a fairly constant fraction of the TIC peak areas, regardless of the number of chlorines in the molecule or the substitution pattern.

What this must mean is that not only are the ionization cross sections fairly constant for a given compound class, but that the energy distributions must also be such that the fraction of molecular ions which are formed but which

TABLE VIII

RELATIVE PCB MOLAR RESPONSES FOR HFID AND [M+2] PEAK AREAS, AND THE PERCENT TIC CARRIED BY THE MOLECULAR ION CLUSTER

RMR = 0.132 (No. of chlorines) + 0.202, r = 0.9956

PCBs		RMR corrected HFID values	RMR-corrected [M+2] values	Percent TIC carried by MIC
Tri	2,3,5		1.17	42.7
	2,4,4' + 2,5,4'			49.7
	Mean			46.2 ± 5.0
Tetra	2,3,4,5	0.958	0.694	38.1
	2,4,2',4' + 2,5,2',5'			36.4
	2,5,2',5'	1.036	0.596	30.9
	3,4,3',4'	1.006	0.902	42.5
	Mean	1.021 ± 0.02	0.749 ± 0.22	36.9 ± 4.8
Penta	2,3,4,5,6	0.867	0.802	38.2·
	2,3,4,2',5'	0.994	0.757	30.6
	2,4,5,2',5'	1.000 (Reference)	1.000 (Reference)	34.4
	Mean	0.953 ± 0.08	0.853 ± 0.13	34.4 ± 3.8
Hexa	2,3,4,2',3',4'	1.036	0.731	33.0
	2,3,5,2',3',5'	0.928	0.731	30.6
	2,4,6,2',4',6'	0.819	1.125	39.6
	2,3,6,2',3',6'	0.976	1.418	38.5
	Mean	0.940 ± 0.09	1.001 ± 0.33	35.4 ± 4.3
Hepta from Aroclor 1254				
Octa	2,3,4,5,2',3',4',5'	1.120	1.398	39.7
	2,3,5,6,2',3',5',6'	0.867	1.122	44.0
	Mean	0.994 ± 0.18	1.260 ± 0.20	41.8 ± 3.0
Average of all PCBs studied		0.967 ± 0.08	0.957 ± 0.27	37.9 ± 5.5

have insufficient energy for fragmentation must also be fairly constant for a given class [10,11]. This fraction was fairly constant within each class, but varied from class to class (the % TIC carried by the MIC was 37.9 ± 5.5% for PCBs, 30.8 ± 7.1% for PCDPEs, 48.0 ± 6.8% for PCNs, 47.4 ± 4.9% for PCDBDs, and 52.0 ± 5.3% for PCDBFs). These results indicate that it should be possible to quantitate one member of a given compound class based on the calibration curve from SIM of a different isomer in the same class, even an isomer containing a different number of chlorines, and that the relative error introduced by doing this would be approximately 10—23%.

It should be noted that these % TIC carried by the MIC determinations were done on a magnetic sector instrument. The mass discrimination of a quadrupole mass spectrometer might lead to decreasing MIC values with increasing mass, or in this case, with increasing number of chlorines.

If the % TIC carried by the MIC were subject to a mass discrimination effect, this would in turn affect the use of MIC areas to approximate TIC peak areas.

Correction factors would probably be needed in order to use either TIC or MIC areas to determine relative concentrations, since these areas would be dependent on the mass distribution of the fragments. On a quadrupole mass spectrometer, even isomers with the same number of chlorines might not give constant MIC/TIC values if the mass distribution of the fragments differed significantly.

In the cases of the PCNs and PCDPEs, for which individual standards were unavailable, the possibility exists that the individual relative detector responses vary significantly for the hydrogen flame ionization detector but, fortuitously, in a similar manner to the TIC and SIM detectors. If this were the case there could be high correlation coefficients in spite of wide variations in responsivity. This possibility cannot be tested until a number of individual, isomeric PCDPEs and PCNs of appropriate chlorine numbers are available.

REFERENCES

1 P.W. Albro and C.E. Parker, J. Chromatogr., 197 (1980) 155.
2 J. Krupčík, P.A. Leclercq, A. Šímová, P. Suchánek, M. Čollák and J. Hrivňák, J. Chromatogr., 119 (1976) 271.
3 M. Zell, H.J. Neu and K. Ballschmiter, Chemosphere, No. 2/3 (1977) 69.
4 E. Schulte and L. Acker, Z. Anal. Chem., 268 (1974) 260.
5 D. Sissons and D. Welti, J. Chromatogr., 60 (1971) 15.
6 P.W. Albro, J.T. Corbett and J.L. Schroeder, J. Chromatogr., 205 (1981) 103.
7 P.W. Albro and C.E. Parker, J. Chromatogr., 169 (1979) 161.
8 J.H. Beynon, Mass Spectrometry and Its Applications to Organic Chemistry, Elsevier, New York, 1960, p. 298.
9 P.W. Albro and L. Fishbein, J. Chromatogr., 69 (1972) 273.
10 D.H. Williams and I. Howe, Principles of Organic Mass Spectrometry, McGraw-Hill, New York, 1972.
11 A.G. Harrison and C.W. Tsang, in G.R. Waller (Editor), Biomedical Applications of Mass Spectrometry, Wiley-Interscience, New York, 1972, pp. 135—156.

CHROMBIO. 1844

DIFFERENTIATION OF *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* FROM *HAEMOPHILUS APHROPHILUS* BY GAS CHROMATOGRAPHY OF HEXANE EXTRACTS FROM WHOLE CELLS

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SUMMARY

In the present study, differentiation of the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* has been made possible through gas chromatography of hexane extracts from their whole cells. While the investigated strains of *H. aphrophilus* were homogeneous, *A. actinomycetemcomitans* could be divided into three clusters of strains according to their gas chromatographic fingerprints. Myristic, palmitic and palmitoleic acid were major fatty acids in both *A. actinomycetemcomitans* and *H. aphrophilus*. In strain cluster III of *A. actinomycetemcomitans*, 13-methyltetradecanoic acid occurred as a fourth major fatty acid. Essentially no minor components were detected in cluster I of *A. actinomycetemcomitans* and in *H. aphrophilus*. In cluster II, the presence of minor components was moderate, in cluster III, marked.

INTRODUCTION

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are morphologically very similar Gram-negative facultative rods, indigenous to dental plaque [1]. Such plaque has been the source of infection in cases where these organisms have caused endocarditis [2]. *A. actinomycetemcomitans* has recently attracted much attention due to its suspected role as a major pathogen in periodontosis [3]. *H. aphrophilus* has not been implicated to the same extent, haemophili being considered by most authors

to have a low pathogenic potential in the periodontal pocket [4]. Taxonomic differentiation between these species is difficult and is based only on a few physiological characters [5]. In Bergey's Manual of Determinative Bacteriology [6], *A. actinomycetemcomitans* is designated as a species incertae sedis, uncertainty especially existing in the relationship to *H. aphrophilus*. A close phenotypic similarity has been found between *A. actinomycetemcomitans* and *H. aphrophilus* [7, 8], and inclusion of *A. actinomycetemcomitans* in the genus *Haemophilus* has been suggested [7].

Gas chromatography of cellular fatty acids is generally considered a useful tool in the differentiation between facultative bacteria (reviews: refs. 9 and 10). Usually, bound fatty acids, which are released by acid or alkaline hydrolysis and then derivatized before gas chromatographic analysis, are used for this purpose. Unfortunately, both hydrolysis and derivatization may cause artifacts. We have developed a direct method of gas chromatographic analysis of free, non-derivatized, whole-cell fatty acids from bacteria which circumvents these problems [11]. The present paper deals with the application of this method in the taxonomic differentiation of *A. actinomycetemcomitans* and *H. aphrophilus*.

MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, and the sources from which they were obtained, are shown in Table I. Strain ATCC 29522 was first obtained from Forsyth Dental Center, and later, for comparison, through the American Type Culture Collection, strains ATCC 33389, 33384 and 19415 through the American Type Culture Collection, and the remaining strains through Forsyth Dental Center. The organisms, which were maintained on blood agar plates kept in an atmosphere of 80% nitrogen, 10% carbon dioxide and 10% hydrogen, were transferred weekly. Mass cultivation occurred in Brain Heart Infusion® (Difco, Detroit, MN, U.S.A.), broth in air plus 10% carbon dioxide for five days at 37°C. All mass cultivations were made in duplicate, on different days.

Fatty-acid extraction

Lyophilized material from each of the two series of bacterial cultures was extracted twice with fresh *n*-hexane (E. Merck, Darmstadt, F.R.G.) in an all-glass Soxhlet apparatus furnished with a refluxing Liebig water condenser, each time for 3—4 h [11]. The two extracts from one culture were pooled, dried and stored at −20°C in an oxygen-free atmosphere.

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 (polydimethylsiloxane) capillary column used was 20 m × 0.22 I.D. with film thickness 0.14 μm and height equivalent to a theoretical plate (HETP) 0.25 mm. Helium served as carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was

TABLE I

LIST OF BACTERIA INVESTIGATED

Organism	Strain	Source	Site of origin
Actinobacillus	33384 (9710)	ATCC* (NCTC**)	Lung abscess
actinomycetemcomitans	29524	ATCC	Chest aspirate
	29523	ATCC	Blood
	29522§	ATCC	Mandibular abscess
	29522§§	ATCC	Mandibular abscess
	2112	FDC***	Periodontitis
	2097	FDC	Periodontitis
	2043	FDC	Periodontitis
	511	FDC	Periodontitis
	N27	FDC	Periodontosis
	Y4	FDC	Periodontosis
Haemophilus	33389 (5906)	ATCC (NCTC)	Endocarditis
aphrophilus	19415 (5886)	ATCC (NCTC)	Endocarditis
	655	FDC	Periodontitis
	654	FDC	Periodontitis

*American Type Culture Collection, Rockville, MD, U.S.A.
**National Collection of Type Cultures, London, U.K.
***Forsyth Dental Center, Boston, MA, U.S.A.
§Obtained through Forsyth Dental Center.
§§Obtained through American Type Culture Collection.

151.5 kPa. From the duplicate bacterial cultures, twenty runs were made on the gas chromatograph, ten from each pooled extract. The gas chromatographic profiles of the duplicate cultures differed by 1—2%. The bacterial fatty acids were identified tentatively by comparing their retention times with those of authentic standards, i.e. $C_{14:0}$ acid (myristic acid, Sigma, St. Louis, MO, U.S.A.), $C_{15:0}$ acid (pentadecanoic acid, Sigma), iso-$C_{15:0}$ acid (13-methyltetradecanoic acid, Larodan Fine Chemicals, Malmö, Sweden), anteiso-$C_{15:0}$ acid (12-methyltetradecanoic acid, Larodan), $C_{16:0}$ acid (palmitic acid, Sigma), $C_{16:1}$ acid (palmitoleic acid, Sigma). The identity of the bacterial fatty acids was confirmed by gas chromatography—mass spectrometry (GC—MS), cochromatography of authentic standards, proton nuclear magnetic resonance (NMR) spectrometry, and infrared spectroscopy.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a Carlo Erba 4200 gas chromatograph, a Micromass 7072F (Vg Micromass, Cheshire, U.K.) mass spectrometer, and a Vg data system 2200. The gas chromatograph was equipped with a glass capillary OV-1 methylsilicone column (20 m × 0.3 cm I.D.). Helium served as carrier gas. The column temperature was programmed from 100°C to 250°C at 10°C/min. Electron impact (EI) ionization spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 200 μA, ion-source temperature 240°C, and accelerating voltage 4 kV. Chemical ionization (CI) mass spectra were recorded with isobutane

as a reactant gas and with an ionizing energy of 50 eV. The other experimental conditions were as for EI ionization. High-resolution mass spectra were obtained at 70 eV from an MS902 double-focus spectrometer connected to an AEI computer (Scientific Apparatus, Manchester, U.K.).

Proton nuclear magnetic resonance spectrometry

For proton NMR analysis, a Varian EM360A spectrometer (Varian, Palo Alto, CA, U.S.A.) was used at 60 MHz with $Si(CH_3)_4$ (Fluka, Buchs, Switzerland) as internal standard.

Infrared spectroscopy

Infrared spectra were obtained with a Jasco IRA-1 diffraction grating infrared spectrophotometer. Samples, prepared by mixing dried extract onto infrared grade KBr (Merck), were pressed into 10-mm diameter pellets.

RESULTS

Yield of hexane-extractable material

The amount of hexane-extractable material from all the bacterial strains investigated is shown in Table II. The yield was highest in strains of *H. aphrophilus*. Oral strains of *A. actinomycetemcomitans* and of *H. aphrophilus* usually yielded more hexane-extractable material than did non-oral strains.

TABLE II

HEXANE-EXTRACTABLE MATERIAL

Values are expressed as percentage (w/w) of dried cells.

Actinobacillus actinomycetemcomitans		*Haemophilus aphrophilus*	
ATCC 33384	1.3	ATCC 33389	2.0
ATCC 29524	1.0	ATCC 19415	2.0
ATCC 29523	1.7	FDC 655	10.0
ATCC 29522*	2.5	FDC 654	6.0
ATCC 29522**	2.1		
FDC 2112	4.0		
FDC 2097	3.6		
FDC 2043	2.4		
FDC 511	4.0		
FDC N27	4.0		
FDC Y4	1.0		

*Obtained through Forsyth Dental Center.
**Obtained through American Type Culture Collection.

Gas chromatography

The gas chromatograms of the hexane extracts from the examined bacteria revealed major components only, or major components together with minor ones. Based on the distribution of all the components, the *A. actinomycetemcomitans* strains could be divided into three clusters. In the main cluster, i.e. cluster I, which consisted of strains ATCC 33384, 29524, and FDC 2112,

2043, 511, and N27, the major components were $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ acids. Minor components were present in negligible amounts, i.e. they constituted less than 0.1% (Fig. 1), which was confirmed when a ten-fold higher concentration of test substance was injected into the gas chromatograph (attenuator 2).

Cluster II of *A. actinomycetemcomitans* comprised strains ATCC 29523 and FDC 2097. The major components of these strains were the same as those detected in cluster I. In addition, a series of minor substances were present in moderate amounts (Fig. 2).

Cluster III of *A. actinomycetemcomitans* included strains ATCC 29522, obtained first through Forsyth Dental Center, and later directly through the American Type Culture Collection, and strain FDC Y4. The fatty-acid profiles of the two ATCC 29522 strains did not differ qualitatively. Characteristic of strain cluster III was the presence of a fourth major fatty acid: iso-$C_{15:0}$ acid. A series of minor components were also detected (Fig. 3). The iso-$C_{15:0}$ acid could be separated from anteiso-$C_{15:0}$ acid. At 130°C, the difference in retention time between these two isomeric acids was 0.16 min, at 133°C it was 0.13 min, and at 134°C, 0.12 min. The $C_{14:0}$, $C_{15:0}$, iso-$C_{15:0}$, and anteiso-$C_{15:0}$ acids were well separated at 133°C (Fig. 4). The difference in retention time between $C_{14:0}$ acid and $C_{15:0}$ acid was 1.92 min, between $C_{14:0}$ acid and anteiso-$C_{15:0}$ acid 1.25 min, and between $C_{14:0}$ and
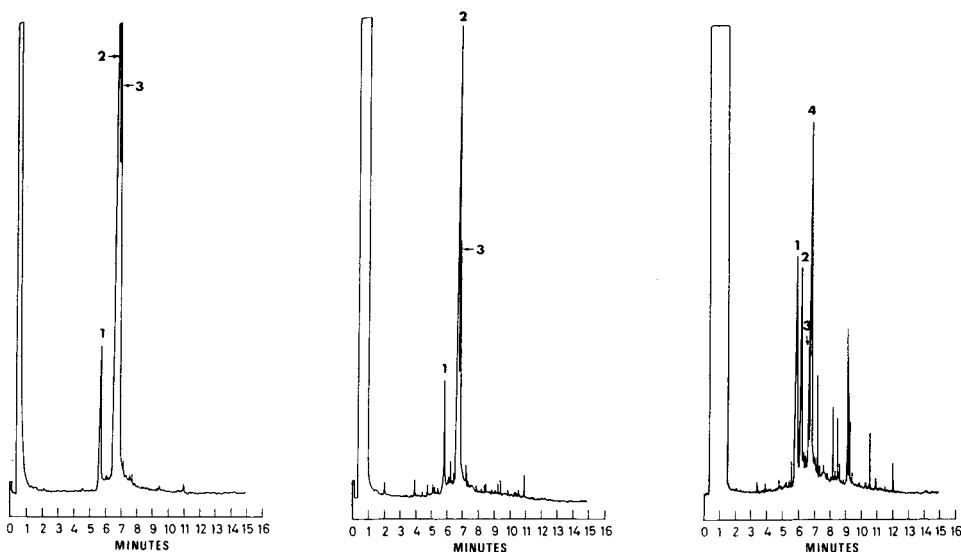
Fig. 1. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC 511, characteristic of cluster I. Program: hold 1 min at 110°C, then 110 to 340°C at 15°C/min. Injector temperature 210°C. Flame ionization detector temperature 210°C. Splitless injection. Paper speed 5 mm/min. Sample injected in hexane 0.1 μl. Attenuator 8. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$.

Fig. 2. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC 2097, characteristic of cluster II. Settings as in Fig. 1. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$.

Fig. 3. Gas chromatogram of *A. actinomycetemcomitans*, strain FDC Y4, characteristic of cluster III. Settings as in Fig. 1. 1 = $C_{14:0}$, 2 = iso-$C_{15:0}$, 3 = $C_{16:1}$, 4 = $C_{16:0}$.

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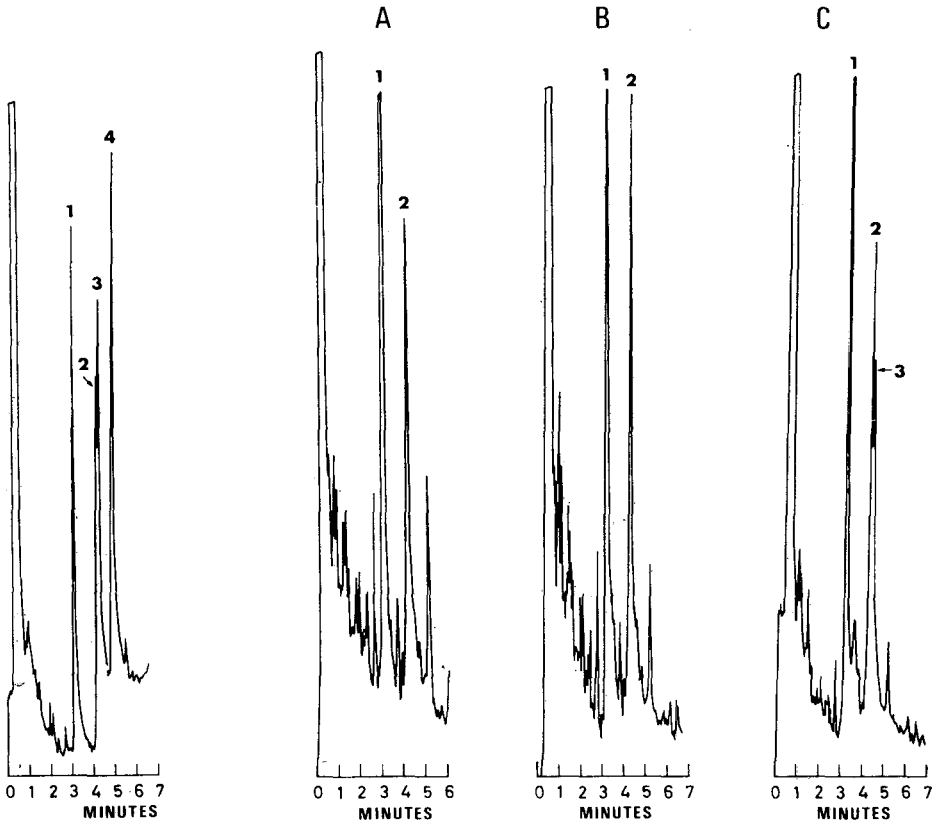
Fig. 4. Gas chromatogram of a mixture of saturated and unsaturated, branched and unbranched synthetic fatty acids. $1 = C_{14:0}$, $2 = $ iso-$C_{15:0}$, $3 = $ anteiso-$C_{15:0}$, $4 = C_{15:0}$. Isothermal, $133°C$. Otherwise settings as in Fig. 1.

Fig. 5. *A. actinomycetemcomitans*, strain FDC Y4, chromatographed alone (A) and cochromatographed with iso-$C_{15:0}$ acid (B) and anteiso-$C_{15:0}$ acid (C). $1 = C_{14:0}$, $2 = $ iso-$C_{15:0}$, $3 = $ anteiso-$C_{15:0}$. Isothermal, $133°C$. Otherwise settings as in Fig. 1.

iso-$C_{15:0}$ acid 1.14 min. In Fig. 5, the hexane extract from strain FDC Y4 has been chromatographed alone or cochromatographed with iso-$C_{15:0}$ or anteiso-$C_{15:0}$ acid.

The fatty acid profiles of the strains of *H. aphrophilus* investigated were similar to those of the strains in cluster I of *A. actinomycetemcomitans* (Fig. 6).

The quantitative distribution of the major fatty acids in each bacterial strain is shown in Table III. It was similar in the strains of cluster I and cluster II of *A. actinomycetemcomitans*. The quantitative distribution of these strains differed from that in the strains of cluster III of *A. actinomycetemcomitans* and in *H. aphrophilus*. There was further a marked difference in the fatty-acid content of cluster III of *A. actinomycetemcomitans* and *H. aphrophilus*. In cluster I and cluster II of *A. actinomycetemcomitans*, the $C_{16:1}$ acid was more abundant than the $C_{16:0}$ acid. The $C_{14:0}$ acid was lowest. In cluster III of *A. actinomycetemcomitans*, the $C_{16:0}$ acid was higher than the $C_{16:1}$ and
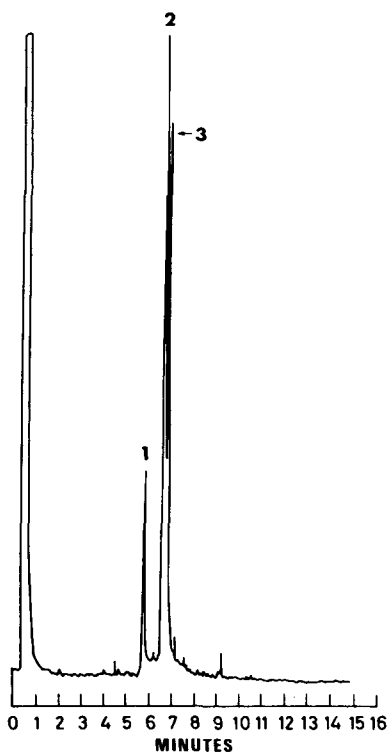
Fig. 6. Gas chromatogram of *H. aphrophilus*, strain FDC 654. 1 = $C_{14:0}$, 2 = $C_{16:1}$, 3 = $C_{16:0}$. Setting as in Fig. 1.

$C_{14:0}$ acids. Whereas iso-$C_{15:0}$ acid was a major fatty acid in all strains of cluster III, it constituted less than 0.1% in the other strains. In *H. aphrophilus*, the $C_{16:1}$ acid was higher than the $C_{16:0}$ acid, and $C_{14:0}$ was lowest.

Gas chromatography—mass spectrometry

The four major components of the hexane extracts were examined further by GC—MS (EI and CI mass spectra). In the spectra from $C_{14:0}$, $C_{16:1}$, $C_{16:0}$, and iso-$C_{15:0}$ acids, the fragment with m/e 60 is characteristic of fatty acids and a result of the McLafferty rearrangement [12]. The abundance of this fragment in the spectrum of $C_{14:0}$ acid was 62.8%, of $C_{16:1}$ acid 20.3%, of $C_{16:0}$ acid 62.1%, and of iso-$C_{15:0}$ acid 50.2%. With EI, the molecular ions were approximately 12% of the base peak in all the acids.

Proton nuclear magnetic resonance spectrometry

Non-saturated and saturated carboxylic acids were present in a mixture. The major peaks represented protons in a CH_3 group (δ 0.9) and CH_2 group (δ 1.25). Olefin protons occurred at δ 5.3. The signal from the acid proton (δ 8.5) was weak with a frequency of 550 Hz.

Infrared spectroscopy

A broad band between 3300 and 2500 cm^{-1} suggested O—H deformation

TABLE III

FREE FATTY ACIDS

Data are expressed as a percentage (w/w) of the total in the strain and are means of a total of twenty runs on two extracts obtained from cultures grown at different days.

Cluster	Actinobacillus actinomycetemcomitans		$C_{14:0}$	Iso-$C_{15:0}$	$C_{16:1}$	$C_{16:0}$
I	ATCC	33384	13.8		55.3	30.9
	ATCC	29524	17.8		57.8	24.3
	FDC	2112	10.3		45.1	44.6
	FDC	2043	16.4		58.6	24.9
	FDC	511	8.4		51.5	40.1
	FDC	N27	9.8		46.4	43.8
II	ATCC	29523	9.3		45.4	45.2
	FDC	2097	14.0		55.5	30.5
III	ATCC	29522*	20.8	9.0	26.8	43.4
	ATCC	29522**	23.7	7.9	30.4	38.0
	FDC	Y4	21.9	16.8	17.8	43.6
	Haemophilus aphrophilus					
	ATCC	33389	20.8		41.2	38.1
	ATCC	19415	21.6		46.1	29.9
	FDC	655	19.8		52.4	27.8
	FDC	654	24.2		44.4	30.8

*Obtained through Forsyth Dental Center.
**Obtained through American Type Culture Collection.

in the carboxylic acid. In the areas 2950 to 2920 cm^{-1}, and at 2850 cm^{-1}, three bands were seen suggesting C—H deformation. Intense bands in the 1715 cm^{-1} area represented stretching frequencies in dimerized C=O carboxylic acid groups. Moderate bands at 1280 cm^{-1} indicated deformation in the carboxylic acid C—O dimer. The absorption at 725 to 730 cm^{-1} was characteristic of CH_2 groups in long-chain (C $\geqslant$ 16) fatty acids.

DISCUSSION

Fatty acids of whole bacterial cells can be extracted either as bound fatty acids or as free acids [13]. In the present study, free fatty acids were chosen since previous investigations had revealed no difference in the content of bound fatty acids in A. actinomycetemcomitans and H. aphrophilus [14—16]. With our methods, A. actinomycetemcomitans proved to be rather heterogeneous and could usually be separated from the more homogeneous H. aphrophilus. These findings supported the idea of maintaining A. actinomycetemcomitans and H. aphrophilus in separate species, but suggested at the same time that A. actinomycetemcomitans might benefit from being divided into subspecies. This particularly applied to the cluster III strains. The heter-

ogeneity of *A. actinomycetemcomitans* demonstrated in the present study on cellular fatty acids parallelled its serological variety. Rabbit immune sera against *A. actinomycetemcomitans* contained two serotypes: (a) which was represented by ATCC 29523, and (b) represented by ATCC 29524, 29522, and FDC Y4 [17].

By means of GC, GC—MS, proton NMR, infrared spectroscopy, and by comparison of our results with existing literature data on fatty acids [18—20], we found the major fatty acids of *H. aphrophilus* to be myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and palmitoleic ($C_{16:1}$) acid. This was in complete agreement with the observations made by Braunthal et al. [14]. In *A. actinomycetemcomitans* the same acids were found, but we also detected 13-methyltetradecanoic acid (iso-$C_{15:0}$) as a fourth major acid in strain cluster III. Calhoon et al. [15] and Jantzen et al. [16], studying bound whole-cell fatty acids of *A. actinomycetemcomitans* and *H. aphrophilus*, detected 3-hydroxymyristic acid as a fourth major acid, but this acid was not a major distinguishing character in these bacteria. It is well known that in bacteria free fatty acids may differ markedly from bound fatty acids [9]. It is also recognized that a number of factors, e.g. growth medium and atmosphere, which differed in our study from that used by the above cited authors may have a profound influence not only on the quantitative, but also on the qualitative distribution of fatty acids in bacteria. Care must therefore be taken when comparing the gas chromatographic profiles of cellular fatty acids from bacteria cultured under different conditions. The extraction technique may also have a strong impact on the yield of fatty acids recovered from bacteria. Contrary to free fatty acids extracted with hexane, hydroxy-acids are covalently bound in the bacterial cell and may not be released but rather require hydrolysis with base or acid. Hydroxy-fatty acids could not be detected in the lipids extractable from bacterial cells with solvents such as ethanol and methanol—chloroform [21].

In cluster III of *A. actinomycetemcomitans*, iso-$C_{15:0}$ was a major fatty acid, while it was present only in trace amounts in the other bacterial strains investigated. It was high in strain ATCC 29522 whether it was obtained from Forsyth Dental Center or directly through the American Type Culture Collection. Recently, iso-$C_{15:0}$ acid was found as a major fatty acid in another CO_2-loving group of bacteria in dental plaque, viz. *Capnocytophaga*, where it constituted between 61 and 78% of the total [22]. Other gliding bacteria (the *Cytophaga—Flexibacter* group, the myxobacteria) also contain a significant amount of 13-methyltetradecanoic acid [23]. This acid is also a major fatty acid in oral and non-oral species of *Bacteroides* [24]. It is not possible at the moment to decide why the cluster III strains of *A. actinomycetemcomitans* were so high in iso-$C_{15:0}$ acid. Synthesis of branched-chain fatty acids in bacteria is believed to occur via α-keto acids, derived from endogenous precursors or from exogenous amino acid substrates, through acyl-CoA esters [25]. Mutant strains may produce less iso-branched acid than their origin. In *Bacillus subtilis* a mutant strain requiring L-leucine, which serves as the precursor for iso-acids, produced iso-$C_{15:0}$ and iso-$C_{17:0}$ fatty acids in much smaller proportions than did the parent strain [25].

At the moment, there is no universally accepted method for extracting lipids from whole bacterial cells, and the number of techniques currently used are legion [13]. The present study demonstrated that hexane extraction from lyophilized bacteria in a Soxhlet apparatus is a useful method for removing long-chain saturated and non-saturated, straight-chain as well as branched-chain whole-cell fatty acids from bacteria for taxonomic purposes. By standard addition of acid, this method recovered free fatty acids with an efficiency of 90% [11]. It was not necessary to increase the volatility of the recovered fatty acids through derivatization before gas chromatographic analysis. In fact, our direct method of gas chromatography made it possible not only to differentiate between the examined bacteria according to the major components present in their hexane extracts, but also according to the minor substances recovered. This meant that each bacteria could be grouped according to the total chemical fingerprint it left on the gas chromatogram. The simplicity, reproducibility and sensitivity of the present procedures suggested that they could be useful in future chemotaxonomic work on microorganisms, either as separate techniques or in combination with more well-established methods.

ACKNOWLEDGEMENT

REFERENCES

1   M. Kilian and R.C. Schiött, Arch. Oral Biol., 20 (1975) 791.
2   M.I. Page and E.O. King, N. Engl. J. Med., 275 (1966) 181.
3   S.S. Socransky, J. Clin. Peridontol., 6 (1979) 16.
4   W. Sims, Arch. Oral Biol., 17 (1972) 1029.
5   J. Slots, Arch. Microbiol., 131 (1982) 60.
6   J.E. Phillips, in R.E. Buchanan and N.E. Gibbons (Editors), Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, MD, 8th edn., 1974, pp. 373—377.
7   M. Kilian, J. Gen. Microbiol., 93 (1976) 9.
8   A.K. Broom and P.H.A. Sneath, J. Gen. Microbiol., 126 (1981) 123.
9   M.P. Lechevalier, CRC Crit. Rev. Microbiol., 5 (1977) 109.
10 N. Shaw, Advanc. Appl. Microbiol., 17 (1974) 63.
11 I. Brondz, I. Olsen and T. Greibrokk, J. Chromatogr., 274 (1983) 299.
12 W. Kemp (Editor), Organic Spectroscopy, Macmillan Press, London, Basingstoke, 2nd edn., 1978, pp. 185—218.
13 D.B. Drucker (Editor), Microbiological Applications of Gas Chromatography, Cambridge University Press, Cambridge, London, 1981, pp. 183—256.
14 S.D. Braunthal, S.C. Holt, A.C.R. Tanner and S.S. Socransky, J. Clin. Microbiol., 11 (1980) 625.
15 D. Calhoon, W.R. Mayberry and J. Slots, J. Clin. Microbiol., 14 (1981) 376.
16 E. Jantzen, B.P. Berdal and T. Omland, Acta Pathol. Microbiol. Scand. Sect. B, 88 (1980) 89.
17 R.J. Genco, J. Slots, C. Mouton and P. Murray, in D.W. Lambe, Jr., R.J. Genco and K.J. Mayberry-Carson (Editors), Anaerobic Bacteria. Selected Topics, Plenum Press, New York, London, 1980, pp. 277—293.

18 C.J. Pouchert, in the Aldrich Library of Infrared Spectra, Aldrich Chemical Company, Inc., Library of Congress, catalogue card No. 73-143382, 1970, pp. 221—251.

19 S. Stenhagen, S. Abrahamsen and F.W. McLafferty (Editors), Atlas of Mass Spectral Data, Wiley, New York, 1969, p. 1564.

20 S. Stenhagen, S. Abrahamsen and F.W. McLafferty (Editors), Register of Mass Spectral Data, Wiley, New York, 1974, pp. 1781—1782.

21 T. Kaneshiro and A.G. Marr, J. Lipid Res., 3 (1962) 184.

22 S.B. Dees, D.E. Karr, D. Hollis and C.W. Moss, J. Clin. Microbiol., 16 (1982) 779.

23 E. Fautz, G. Rosenfelder and L. Grotjahn, J. Bacteriol., 140 (1979) 852.

24 E. Miyagawa, R. Azum and T. Suto, J. Gen. Appl. Microbiol., 25 (1979) 41.

25 T. Kaneda, Can. J. Microbiol., 12 (1966) 501.

CHROMBIO. 1823

A HIGHLY SENSITIVE DETERMINATION OF INDIVIDUAL SERUM BILE ACIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH IMMOBILIZED ENZYME

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SUMMARY

We have developed a highly sensitive method for the simultaneous determination of individual bile acids in serum using high-performance liquid chromatography (HPLC) combined with immobilized 3α-hydroxysteroid dehydrogenase. Both the HPLC column and the immobilized-enzyme column are suitable for use with alkaline solutions necessary in working with this enzyme system. With this method we were able to determine simultaneously fifteen different serum bile acids.

INTRODUCTION

Bile acids are synthesized from cholesterol and conjugated with glycine or taurine in the liver cells. Conjugated bile acids are excreted into the small intestine where about 30% of them are deconjugated and converted into secondary bile acids by intestinal bacteria. Most bile acids are reabsorbed from the terminal ileum by a process of active transport and are returned to the liver via the portal vein. This constitutes the enterohepatic circulation. In vivo, most bile acids are present as conjugates of glycine or taurine with only traces of bile acid present in the unconjugated form. In determining serum bile acids, it

would be most advantageous not only to be able to determine total concentration but also the concentrations of each individual bile acid since these patterns may yield clinical information in liver disease. Mashige et al. [1] in 1976 reported a simple fluorometric method for determining total serum bile acid concentration and the method became popular among clinicians [2]. In 1978, we reported a method for measuring individual 3α-hydroxy bile acids in serum using high-performance liquid chromatography (HPLC) combined with enzymatic and fluorometric analyses [3]. Because our method was a flow system using enzyme solution, it was expensive. In 1979 Okuyama et al. [4] combined the HPLC method with an immobilized-enzyme system, but it was upon our principle. Although this solves an economic problem, it was difficult to establish optimum conditions for the enzymatic reactions.

In the present paper, we report our experience with a method utilizing a new HPLC column and a new immobilized-enzyme column both of which are suitable for use at the alkaline pH which is optimum for 3α-hydroxysteroid dehydrogenase enzyme activity. In our system the coenzyme NAD fed as the reagent is reduced to NADH by the immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) in the presence of bile acids as substrate eluting from the HPLC column, and the fluorescence intensity of the NADH thus produced is measured.

EXPERIMENTAL

Materials used for the HPLC column

Tetraethyleneglycol diacrylate (Sin Nakamura Chem. Co., Wakayama, Japan) and tetramethylolmethane triacrylate (Sin Nakamura Chem. Co.) were prepared as monomers. Toluene and benzoyl peroxide were used for the polymerization.

Materials used for immobilized-enzyme column

Beaded cellulose (Cellulofine GC-200 m, Seikagaku Kogyo, Tokyo, Japan) was adopted as solid support because of its chemical stability at alkaline pH and superior mechanical properties. The enzyme was 3α-HSD extracted from *Pseudomonas putida* and purified (Kyowa Hakko, Tokyo, Japan) [5,6].

Eluent

Triammonium phosphate was dissolved in distilled water to make 0.5% and 1.5% (w/v) triammonium phosphate solution. The 0.5% solution was adjusted to pH 9.1 and the 1.5% solution to pH 9.7 with ammonium hydroxide solution. Each solution was mixed with acetonitrile. Three kinds of eluent were made. The volume ratio (v/v) of eluent I was 13:87 (acetonitrile—0.5% triammonium phosphate solution), eluent II was 15:85 (acetonitrile—1.5% solution) and eluent III was 24:76 (acetonitrile—0.5% solution).

Reagents

β-NAD$^+$, 200 mg/l (Sigma, St. Louis, MO, U.S.A.), 25 mg/l EDTA, 2.3 g/l sodium pyrophosphate, 2.72 g/l sucrose and 500 μl/l mercaptoethanol were dissolved in 0.1 mol/l hydrazine hydrate (pH 9.5).

Standard materials

Sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) were purchased from P.-L. Biochemicals (Milwaukee, WI, U.S.A.). Sodium salts of ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) were a gift from Tokyo Tanabe Pharmaceutical (Tokyo, Japan). As an internal standard (I.S.), we used N'-($3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholanoyl) glutamate (GLU-CA, Lot No. 9271), a gift from Sekisui Kagaku (Osaka, Japan).

Instruments

A schematic diagram of the method is shown in Fig. 1. HPLC was carried out on an ALC 204 type Waters' system (Waters Assoc., Waltham, MA, U.S.A.). The detector used was a Fluorichrom fluorescence detector (Shimazu Seisakusho, Kyoto, Japan) with an excitation wavelength of 350 nm and an emission wavelength of 460 nm.
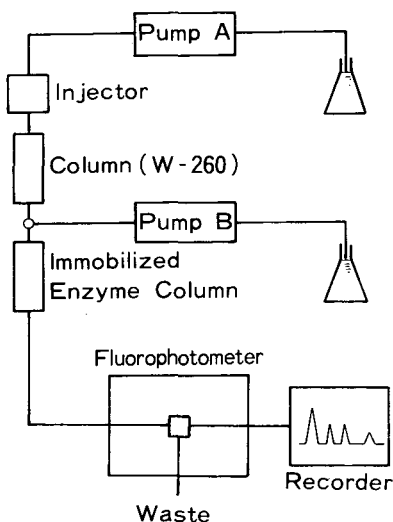
Fig. 1. Flow diagram of the system. Eluent flows from pump A at 1 ml/min, and reagent from pump B at 1 ml/min.

Preparation of W-260 column

Co-polymer beads were synthesized by a standard suspension polymerization method. The aqueous portion of the polymerization mixture was prepared in a reaction flask (5 l) by adding 60 g of polyvinylalcohol as dispersing agent to 2.5 l of distilled water. The monomer phase consisted of 250 g of tetraethyleneglycol diacrylate, 50 g of tetramethylolmethane triacrylate, 300 g of toluene

(diluent), and 3.5 g of benzoyl peroxide. When the aqueous phase reached 50°C, the monomer—diluent—initiator solution was added with stirring. Temperature was maintained at 80°C for 10 h. When the polymerization was complete, the resin beads were filtered and washed with boiling water to remove the dispersing agent. After sieving, about 25 g of the fraction of diameter 10—15 μm were extracted with methanol in order to remove residual diluent and unreacted monomers. The porous beads thus obtained were packed into a stainless-steel column (25 cm × 79 mm I.D.). A slurry containing 10 g of gel in about 100 ml of water was added to a column packing vessel. A total of 120 ml of water was pumped before completing the column packing.

Preparation of immobilized-enzyme column

The activation of beaded cellulose by the addition of CNBr was followed by the coupling reaction with 3α-HSD. The reactions were performed according to the procedure described by March et al. [7]. The enzyme-bound beads were packed into a stainless-steel column (10 × 0.4 cm I.D.) and it was referred to as the immobilized-enzyme column. In this immobilizing method, the yield of immobilized enzyme was over 60%, and the enzyme activity of the gel was stable for two weeks at 15°C in phosphate buffer, pH 11, containing 20% acetonitrile and 0.2% mercaptoethanol.

Preparation of serum material

Serum was collected from a peripheral vein after overnight fasting and frozen (−20°C) until analyzed for bile acids. A 0.5-ml volume of serum was diluted with 4.5 ml of 0.9% NaCl containing 0.1 M of NaOH. This solution was applied to a column of Amberlite XAD-7 resin (0.2 g, Lot No. 3933, Rohm and Haas, Philadelphia, PA, U.S.A.). After the serum solution was applied, the column was washed with 5 ml of methanol at the rate of five drops a minute. Then the eluent was evaporated to dryness and the residue was re-dissolved in 0.5 ml of methanol containing 2500 ng/ml N'-(3α,7α,12α-trihydroxy-5β-cholanoyl) glutamate for analyzing.

Measurement procedure

Aliquots (100 μl) of the samples were applied to the W-260 column from the injector with a microsyringe (Hamilton, Reno, NV, U.S.A.). Eluent was passed through at a rate of 1 ml/min. In order to shorten the retention time, the mobile phase was changed from eluent I to eluent II at 30 min after injection and from eluent II to eluent III at 68 min. Reagent was passed through at a constant rate of 1 ml/min. Eluent and reagent together flow into the immobilized-enzyme column, where 3α-hydroxysteroids are converted to 3-ketosteroids and NAD is converted into NADH. This reaction is carried out at 27°C in a water bath. NADH produced by this reaction is measured by the fluorophotometer connected to the immobilized-enzyme column. The chart speed is 2.5 mm/min. Fig. 2 shows a standard chromatogram.

Standard curves

Four concentrations of a mixture of each of the standard bile acids were made: 10, 5, 2.5 and 1.25 μmol/l. These samples were dissolved in distilled
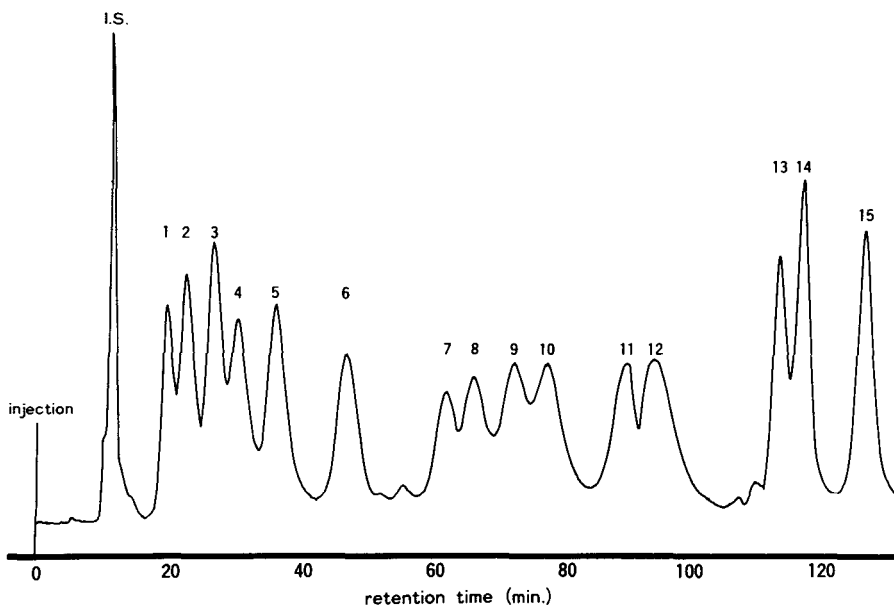
Fig. 2. Standard chromatogram of individual bile acids (125 ng each). I.S. = N'-(3α,7α,12α-trihydroxy-5β-cholanoyl) glutamate, 1 = cholic acid, 2 = glycocholic acid, 3 = taurocholic acid, 4 = ursodeoxycholic acid, 5 = glycoursodeoxycholic acid, 6 = tauroursodeoxycholic acid, 7 = chenodeoxycholic acid, 8 = deoxycholic acid, 9 = glycochenodeoxycholic acid, 10 = glycodeoxycholic acid, 11 = taurochenodeoxycholic acid, 12 = taurodeoxycholic acid, 13 = lithocholic acid, 14 = glycolithocholic acid, 15 = taurolithocholic acid.

water and treated with Amberlite XAD-7. The standard curves were established by plotting the peak height against the amount of each bile acid.

Recovery experiments

Recovery experiments were performed by adding a mixture of standard samples to the sera of healthy subjects. This mixture contained 5 μmol/l of each bile acid. The sera with added standards were then treated with Amberlite XAD-7.

Reproducibility

As an intra-assay reproducibility, the same samples were determined six times within a day, and for inter-assay reproducibility the same samples were determined during another six days. The coefficients of variation (C.V., %) were calculated from the mean values and S.D. values.

RESULTS

Fig. 2 shows a chromatogram of a mixture of free, glycine- and taurine-conjugates of each of five bile acids. The separation of these bile acids was satisfactorily obtained in the order CA, GCA, TCA, UDCA, GUDCA, TUDCA, CDCA, DCA, GCDCA, GDCA, TCDCA, TDCA, LCA, GLCA and TLCA. The

resolution value of TCDCA and TDCA, in which separation was most difficult, was 0.7. Fig. 3 shows the standard curve of each bile acid. Linear correlations were obtained between the peak height and the amount of each bile acid.

Table I indicates the recovery ratios and reproducibility. Satisfactory recovery ratios were obtained ranging between 88.7% and 103.8%. The intra-assay C.V. value of each bile acid ranged from 0.3 to 4.5% in six replicate determinations and from 0.6 to 7.8% in six replicate determinations for inter-assay C.V. The sensitivity limits of each bile acid by this method ranged from 0.01 to 0.04 μmol/l.
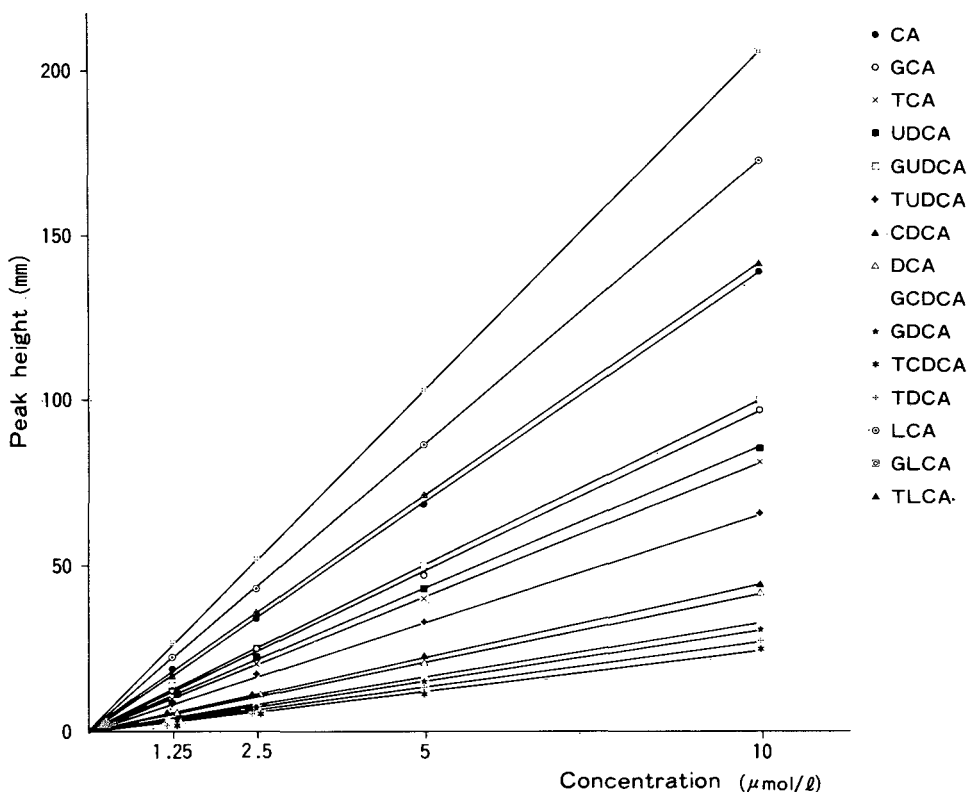
Fig. 3. Standard curves of individual bile acids.

A normal-serum analysis by this method is presented in Fig. 4. Also an example of serum analysis in a patient with gallstones is presented in Fig. 5. A typical bile acid pattern of extrahepatic cholestasis was obtained. Normal values analyzed in this system are compared in Table II with values obtained from other methods including our previous HPLC method using 3α-HSD reagent solution [3, 8—10].
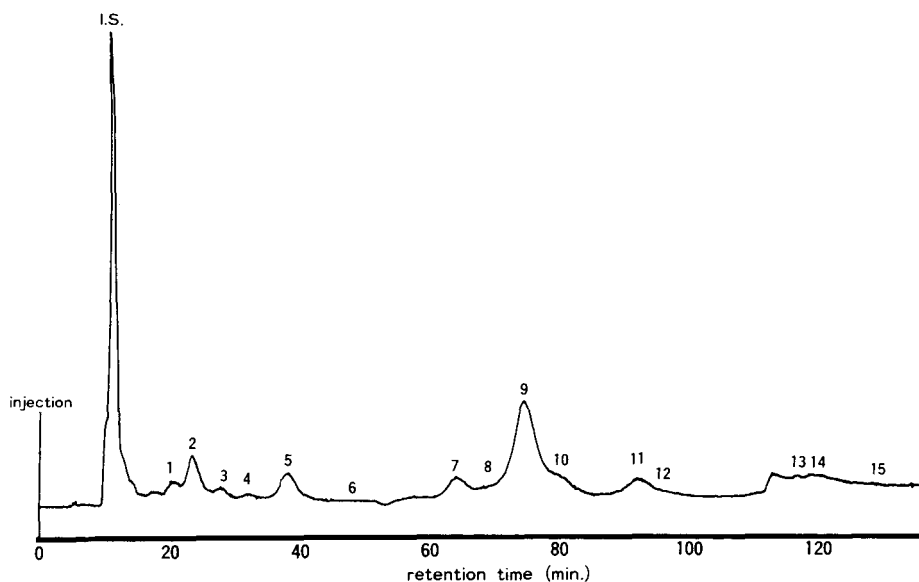
Fig. 4. A bile acid pattern of normal serum. Peak numbers as in Fig. 2.

TABLE I

REPRODUCIBILITY AND RECOVERY

Bile acids	Reproducibility of each bile acid assay		Recovery rate (%)
	Intra-assay C.V. (%) ($n = 6$)	Inter-assay C.V. (%) ($n = 6$)	
Cholic acid	0.6	2.9	89.2
Glycocholic acid	2.1	2.5	96.9
Taurocholic acid	1.7	5.0	92.4
Ursodeoxycholic acid	2.7	0.6	95.9
Glycoursodeoxycholic acid	2.9	3.8	90.9
Tauroursodeoxycholic acid	0.2	5.5	90.4
Chenodeoxycholic acid	2.3	4.6	88.7
Glycochenodeoxycholic acid	2.7	3.4	96.2
Taurochenodeoxycholic acid	4.5	3.8	94.7
Deoxycholic acid	2.3	1.3	92.5
Glycodeoxycholic acid	4.2	4.5	89.0
Taurodeoxycholic acid	0.3	7.0	103.8
Lithocholic acid	2.7	6.8	98.9
Glycolithocholic acid	2.3	2.5	102.9
Taurolithocholic acid	2.1	7.8	94.7

DISCUSSION

The data indicate that our HPLC method and immobilized-enzyme column operate well under the severely alkaline conditions which are optimum for 3α-hydroxysteroid dehydrogenase activity. Columns could be used more than

TABLE II

NORMAL VALUES OF SERUM BILE ACIDS*

Values are given in μmol/l (mean ± S.E.M.).

Author and method	No.	Total	UDCA group			CA group			CDCA group			DCA group			LCA group		
			UDCA	GUDCA	TUDCA	CA	GCA	TCA	CDCA	GCDCA	TCDCA	DCA	GDCA	TDCA	LCA	GLCA	TLCA
Present method HPLC (using immobilized enzyme)	12	3.90 ±0.85	0.08 ±0.03	0.14 ±0.05	0.07 ±0.02	0.26 ±0.05	0.30 ±0.08	0.21 ±0.03	0.75 ±0.20	0.97 ±0.38	0.45 ±0.13	0.20 ±0.06	0.23 ±0.04	0.23 ±0.05	0.02 ±0.01	0.01 ±0.00	0.01 ±0.00
Baba et al. [3] HPLC (using enzyme solution)	8	2.88 ±0.74	0.07 ±0.04			0.14 ±0.05	0.16 ±0.07	0.16 ±0.05	0.42 ±0.16	0.47 ±0.05	0.36 ±0.08	0.30 ±0.05	0.46 ±0.26	0.36 ±0.08	N.D.	N.D.	N.D.
Demers and Hepner [8] RIA**	25						0.27 ±0.03			0.20 ±0.03			0.06 ±0.01				
Sandberg et al. [9] GLC** (ranges)	18	0.9– 6.9		N.D.***			0.09–1.95			0.15–3.9			0.18–1.35			N.D.	
Sino et al. [10] GC–MS** (mean)	4			1.02			0.55			1.81			1.12			0.11	

* Abbreviations, see text.
** RIA = radioimmunoassay, GLC = gas—liquid chromatography, GC—MS = gas chromatography—mass spectrometry.
*** N.D. = not determined.
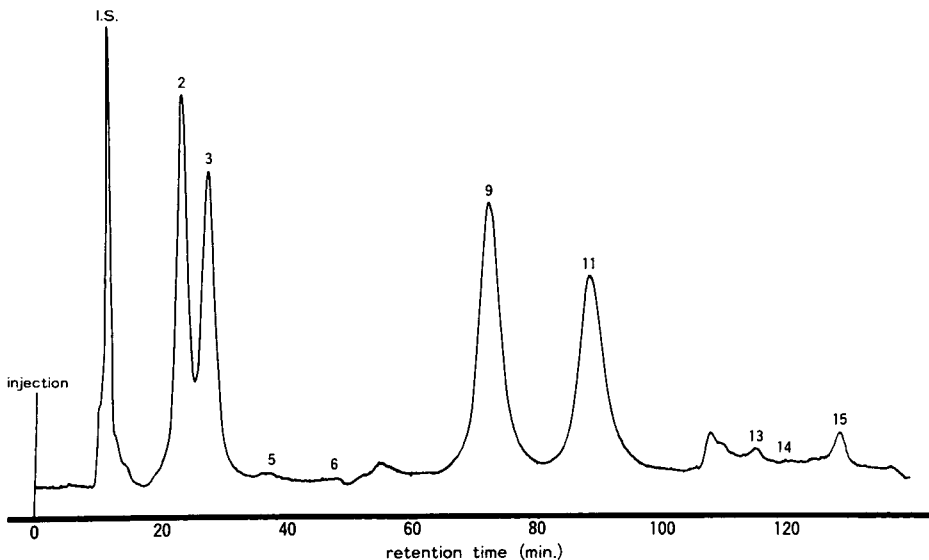
Fig. 5. A bile acid pattern of serum in the patient with gallstones. Peak numbers as in Fig. 2.

300 times without a significant change in their operating characteristics. The enzyme which we used was purified from *Pseudomonas putida* instead of *Pseudomonas testeroni*. The former enzyme is specific for the 3α-hydroxy group whereas the latter enzyme has some activity towards the 3β-hydroxy group as well as the 3α-hydroxy group [11]. The acetonitrile used in our eluent probably decreases the life span of the immobilized enzyme to some extent. Okuda and Hashimoto [12] reported a method using methanol instead of acetonitrile for the eluent. Although their method separated many bile acids, it did not separate TCDCA and TDCA completely. It may be possible in the future to devise methods which combine excellent separation with long enzyme life span. Until then, the present method appears to provide the best separation specificity and sensitivity available for the analysis of serum bile acids. We hope that the ability to analyze quickly and specifically individual circulating bile acid species will become clinically useful in differentiating various kinds of liver disease. We have shown the reproducibility of this method and have defined its practicality in clinical use. We have also established normal values against which we can compare values in patients with various kinds of liver disease.

REFERENCES

1 F. Mashige, K. Imai and T. Osuga, Clin. Chim. Acta, 70 (1976) 79.
2 T. Osuga, F. Mitamura, F. Mashige and K. Imai, Clin. Chim. Acta, 75 (1977) 81.
3 S. Baba, K. Suminoe, R. Uenoyama and Y. Kameno, Med. Biol., 97 (1978) 219.
4 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata. Chem. Lett.,
   (1979) 1433.
5 T. Uwajima, K. Takayama and S. Terada, Agr. Biol. Chem., 42 (1978) 1577.
6 T. Uwajima and S. Terada, Agr. Biol. Chem., 43 (1979) 1521.
7 S.C. March, I. Parikh and P. Cuatrecasas, Anal. Biochem., 60 (1974) 149.
8 L.M. Demers and G.W. Hepner, Amer. J. Clin. Pathol., 66 (1976) 831.
9 D.H. Sandberg, J. Sjövall, K. Sjövall and D.A. Turner, J. Lipid Res., 6 (1965) 182.
10 M. Shino, M. Nezu and Y. Abe, J. Pharm., 99 (1979) 421.
11 J. Boyar, N. Baron and P. Talalay, J. Biochem., 4 (1965) 9.
12 H. Okuda and E. Hashimoto, Acta Hepatol. Jap., 23 (1982) 89.

CHROMBIO. 1830

FLUOROMETRIC DETERMINATION OF HYPOXANTHINE AND XANTHINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ENZYME REACTORS

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SUMMARY

A selective and sensitive assay of hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography coupled with immobilized-enzyme reactors was developed. The separations were achieved by reversed-phase liquid chromatography. Hydrogen peroxide produced from hypoxanthine and xanthine by immobilized xanthine oxidase was determined fluorometrically using immobilized peroxidase and *p*-hydroxy-phenylacetic acid. Immobilized enzymes were prepared by intermolecular cross-linking to controlled-pore glass. Assay of allopurinol was also possible by the present method. The method was applied to serum and urine. The detection limits of hypoxanthine and xanthine were approximately 50 and 120 pg per injection, respectively.

INTRODUCTION

The determination of hypoxanthine (Hyp), xanthine (Xan), and/or allopurinol, which is a potent xanthine oxidase inhibitor used in the treatment of gout, in biological fluids has been required for various pharmacological and physiological reasons [1—3]. As a part of these studies, we needed a rapid, accurate and sensitive assay for the substrates of xanthine oxidase because we wished to analyze a large number of samples containing various amounts of these compounds.

Recently, high-performance liquid chromatography (HPLC) has become popular for the assay of nucleosides and bases involving Hyp and Xan [4—10]. Unfortunately, such methods were unsuitable for the rapid assay of the compounds of interest. On the other hand, enzymatic methods for the deter-

mination of Hyp and Xan have been published [11, 12]. However, these methods do not permit the simultaneous assay of Hyp and Xan.

We have already reported a simultaneous and specific determination of Hyp and Xan using HPLC coupled with enzyme reactors [13—15]; uric acid produced from Hyp and Xan by immobilized xanthine oxidase (IXO) was determined at 290 nm. However, we could not determine Xan in normal serum accurately due to the lack of adequate sensitivity and selectivity of this method (the detection limit of Xan was 0.5 μmol/l). Hartwick et al. [4] found Xan in normal serum in the concentration range 0.542—4.7 μmol/l.

In this report, we describe a sensitive fluorometric determination of Hyp and Xan using HPLC coupled with enzyme reactors. The assay employed the following principles as the post-column reactions [16]:

$$Hyp + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} Xan + H_2O_2$$

$$Xan + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + H_2O_2$$

$$H_2O_2 + p\text{-hydroxyphenylacetic acid} \xrightarrow{\text{peroxidase}} \text{fluorescent compound}$$
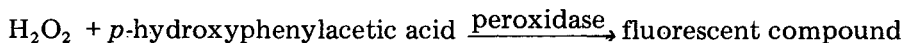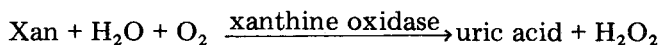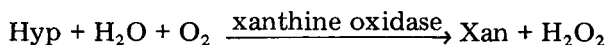
Various substrates of peroxidase have been reported [17]; we chose p-hydroxyphenylacetic acid because of the lower cost and the lower fluorescence blank than the others.

The present method may easily be instituted as a routine laboratory procedure in the investigation of purine metabolism.

EXPERIMENTAL

Chemicals

Standard solution of Hyp, Xan, allopurinol (Nakarai Chemicals, Kyoto, Japan) and uric acid (Merck, Darmstadt, F.R.G.) were made by dissolving them in diluted sodium hydroxide and diluting to the desired volume with 0.01 M phosphate buffer (pH 5.5) before use. p-Hydroxyphenylacetic acid (Nakarai Chemicals) purified by sublimation was dissolved in 0.1 M phosphate buffer (pH 10). Enzymes used were xanthine oxidase (cow's milk, 0.4 units/mg protein, Boehringer-Mannheim-Yamanouchi, Tokyo, Japan), peroxidase (horseradish, 264 units/mg protein, Toyobo, Tokyo, Japan) and catalase (bovine liver, 37,000 units/mg protein, Sigma, St. Louis, MO, U.S.A.). An aminopropyl controlled-pore glass (80—530 Å mean pore diameter, Electro-Nucleonics, Fairfield, U.S.A.) was used as a carrier for the immobilization of enzymes. All other reagents were of analytical reagent grade.

Apparatus

The liquid chromatograph used was a Model LC-3A (Shimadzu, Kyoto, Japan). Solutes were monitored with a variable-wavelength ultraviolet (UV) detector Model SPD-2A (Shimadzu) and a fluorescence spectrometer Model RF-500LC (Shimadzu) equipped with an integrator Model C-R1A (Shimadzu). Reagent for the enzymatic reaction was added with a mini-micro pump Model KHD-16 (Kyowaseimitu, Tokyo, Japan).

Preparation of immobilized enzymes

The immobilization procedure for xanthine oxidase and catalase was the same as that reported previously [14]; enzyme was attached to an aminopropyl controlled-pore glass by the intermolecular cross-linking method.

The immobilization of peroxidase was achieved by cleaving its carbohydrate residues with periodic acid followed by coupling the enzyme to aminopropyl controlled-pore glass. A 5-mg portion of peroxidase was dissolved in 2 ml of 0.05 M phosphate buffer (pH 7). This solution was mixed well with 1 ml of 5 μM periodic acid in water for 4 h at room temperature, then the mixture was dialyzed overnight against 500 ml of 0.05 M phosphate buffer (pH 7). The dialyzed solution was added to 0.5 g of aminopropyl controlled-pore glass and allowed to react at 4°C overnight. The glass beads carrying enzyme were filtered and washed with 100 ml of cold water followed by washing with 100 ml of 1 M sodium chloride and 1000 ml of cold water.

IXO and immobilized peroxidase (IPO) were filled into stainless-steel tubes, 5 × 0.21 cm and 15 × 0.21 cm, respectively, for HPLC; PTFE tubes, 5 × 0.1 cm, were used for other experiments.

Chromatographic conditions

A pre-column (1.0 × 0.4 cm) and an analytical column (20 × 0.4 cm) were prepared with Nucleosil 5 C_{18} (5 μm, Macherey, Nagel & Co., Düren, F.R.G.) using a high-pressure slurry-packing technique.

The separations were performed by eluting with 0.01 M phosphate buffer (pH 5.5) containing 2% (v/v) acetonitrile at a flow-rate of 0.7 ml/min.

Sample purification

Serum purification was performed by the procedure described by Khym [18]. A 0.5-ml portion of serum was mixed with an equal volume of a 6% (w/v) solution of trichloroacetic acid, and the mixture was centrifuged for 15 min at 1500 g. An appropriate volume of the supernatant was added to an equal volume of 0.5 M tri-n-octylamine in 1,1,2-trichlorotrifluoroethane. After mixing and centrifuging, 5 or 10 μl of the aqueous solution were injected into the analytical column.

Recovery experiments were carried out by adding known amounts of Hyp and Xan to control serum. The recoveries of Hyp and Xan were 93 ± 9.9% ($n = 4$) and 96 ± 9.2% ($n = 4$), respectively.

Urine purification was performed by the following method. A 1-ml aliquot of urine was heated for 10 min at 70°C and mixed with 1 ml of 1 M phosphate buffer (pH 9) and filtered; 0.5 ml of this solution was adjusted to neutral pH by adding 4.5 ml of 0.01 M phosphate buffer (pH 5). A 10-μl volume of this solution was injected. Recoveries were approximately 100%.

RESULTS AND DISCUSSION

Reactor properties

The properties and the conversion efficiencies of IXO and IPO were calculated from UV-absorptiometric and fluorometric determinations, using a flow-injection method. The pH optima of IXO and IPO were confirmed
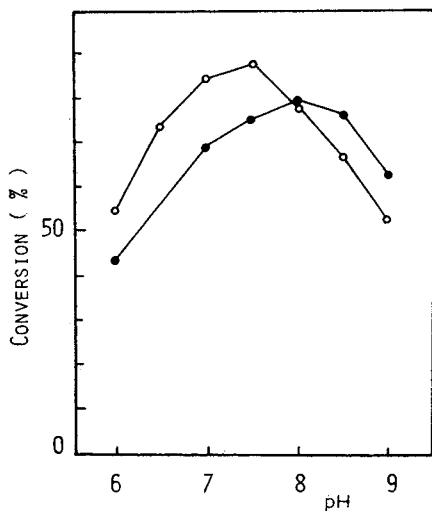
Fig. 1. Effects of pH on the conversion efficiency of IXO (○) and IPO (●).

as 7.5 and 8.0, respectively (Fig. 1). The conversion efficiencies as a function of the amount of acetonitrile in the solvent were studied. Acetonitrile contents lower than 3% (v/v) did not degrade the activities of either immobilized enzyme. The effect of temperature on conversion efficiencies was also studied and there was no significant difference between optimum (37°C) and room (25°C) temperature. Both immobilized enzymes retained their performance without decrease in activity for more than a month.

Inclusion of immobilized-enzyme reactors into the chromatographic system

A diagram of the HPLC system coupled with enzyme reactors used in this study is shown in Fig. 2. After separation on the analytical column, the effluent was adjusted to pH 7.5 by adding 0.1 M phosphate buffer (pH 10) with pump 2 at a flow-rate of 0.35 ml/min; Hyp and Xan were oxidized to hydrogen peroxide and uric acid in the IXO reactor. The effluent from the IXO reactor was adjusted to pH 8.0 by adding 0.1 M phosphate buffer (pH 10) containing 1.6 mM p-hydroxyphenylacetic acid with pump 3 at a flow-rate of 0.46 ml/min; p-hydroxyphenylacetic acid was oxidized by hydrogen
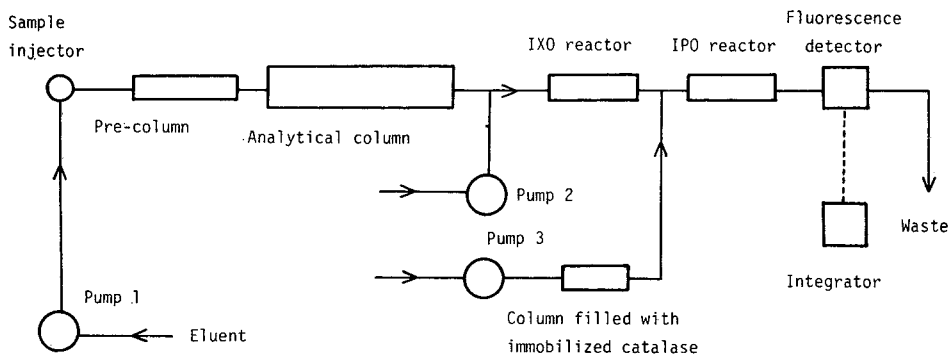
Fig. 2. Flow diagram of HPLC coupled with the immobilzed-enzyme reactors.
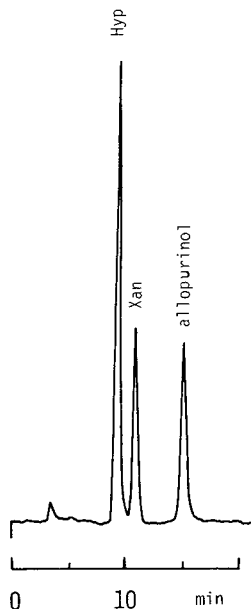
Fig. 3. Chromatogram of a mixture containing 400 ng/ml each of uric acid, Hyp, Xan and allopurinol obtained using the present method. Column: Nucleosil 5 C_{18}, 20 × 0.4 cm. Eluent: 0.01 M phosphate buffer (pH 5.5) containing 2% (v/v) acetonitrile. Flow-rates from pump 1, pump 2 and pump 3: 0.7, 0.34 and 0.45 ml/min, respectively. Injection volume: 10 μl. Fluorescence detection: excitation 320 nm, emission 407 nm.

peroxide, produced from Hyp and Xan, in the IPO reactor to form a highly fluorescent compound, which has an excitation maximum of 320 nm and an emission maximum of 407 nm. Then, the column (1.0 × 0.4 cm) filled with immobilized catalase was used to reduce the blank fluorescence in the solution containing p-hydroxyphenylacetic acid.

Fig. 3 shows the chromatogram of a standard solution containing uric acid, Hyp, Xan and allopurinol using the present system. There was a little peak broadening in the reactors.

Chromatograms of biological fluids

Fig. 4. shows chromatograms of a serum extract from a normal subject. Chromatogram A was obtained using the system illustrated in Fig. 2 and chromatogram B was obtained when the IXO reactor was removed. These show that the selective assay of Hyp and Xan is performed within 15 min and that the peaks of both Hyp and Xan are eluted free from other fluorescent compounds. Chromatogram C was obtained at an UV absorbance of 254 nm when all the reactors had been removed and shows that the simultaneous quantitative determination of Hyp and Xan is impossible.

Fig. 5 shows chromatograms of urine from a normal subject. The compound eluting at 33 min which reacts with xanthine oxidase to produce hydrogen peroxide, was not determined in this study.
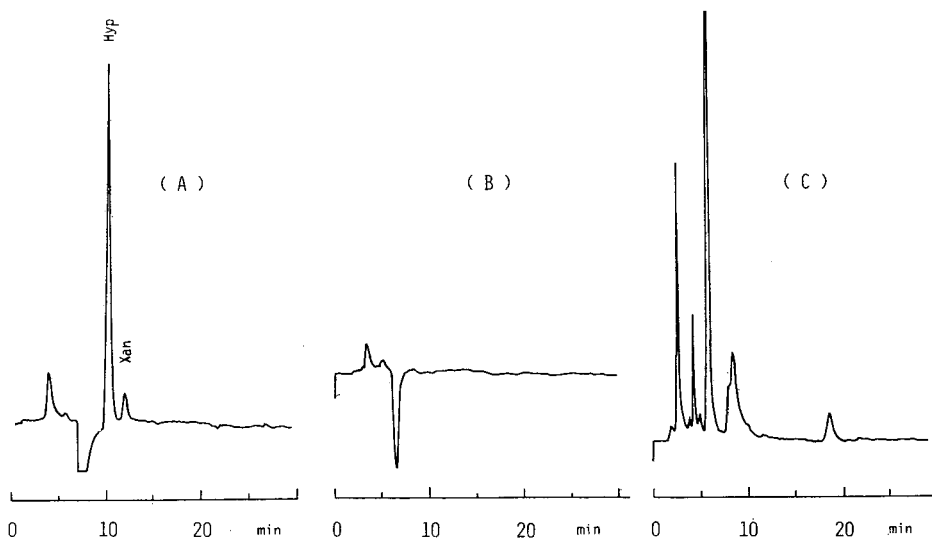
Fig. 4. Chromatograms of a serum extract from a normal subject obtained by the present method (A), when the IXO reactor is removed from the system (B), and with UV absorbance at 254 nm when all reactors are removed (C). Chromatographic conditions as in Fig. 3.
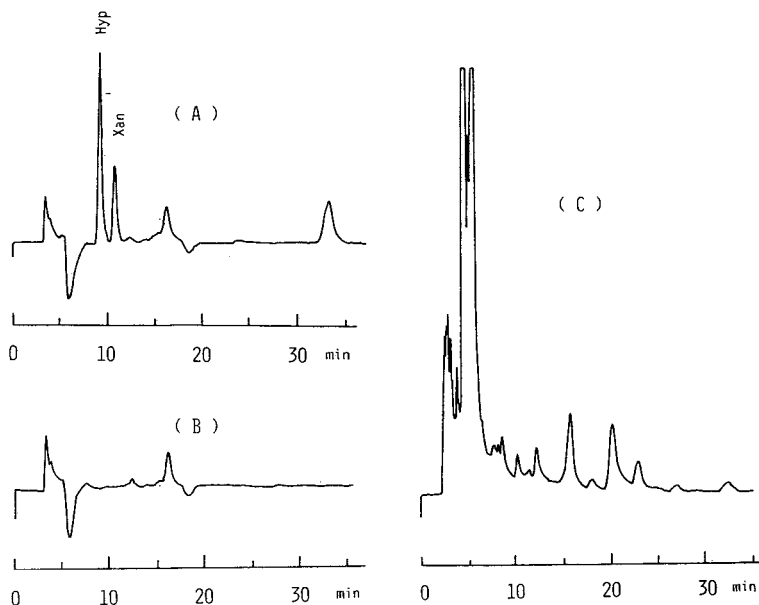
Fig. 5. Chromatograms of urine from a normal subject. Conditions as in Fig. 4.

Analysis of Hyp and Xan

The linearity of calibration curves of concentration versus peak area was excellent for both Hyp and Xan, ranging from 0.74 to 13.15 μmol/l. The average coefficients of variation for the normalized peak area over this range of Hyp and Xan concentrations were 2.87 and 3.48%, respectively.
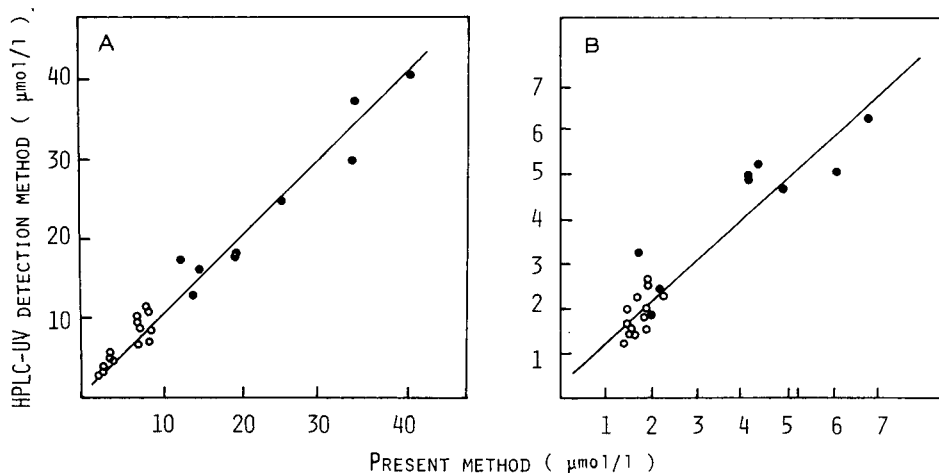
Fig. 6. Comparison of the results of Hyp (A) and Xan (B) in serum from normal (○) and gouty (●) subjects obtained by two methods.

TABLE I

DETERMINATION OF HYP AND XAN IN BIOLOGICAL FLUIDS FROM NORMAL SUBJECTS

Results are expressed as mean ± S.D. (n = 4).

Subject	Serum		Urine	
	Hyp (μmol/l)	Xan (μmol/l)	Hyp (μmol/l)	Xan (μmol/l)
1	4.04 ± 0.331	1.51 ± 0.151	58.70 ± 1.403	49.44 ± 2.051
2	8.60 ± 0.514	2.43 ± 0.092	144.52 ± 1.896	103.81 ± 22.056
3	3.60 ± 0.558	1.64 ± 0.237	190.51 ± 7.516	135.03 ± 3.550
4	7.71 ± 0.433	1.97 ± 0.145	85.15 ± 4.952	78.89 ± 4.464
5	8.16 ± 1.124	2.56 ± 0.316	101.76 ± 11.381	82.44 ± 1.775
Mean	6.42	2.02	116.13	89.92

We compared the results of Hyp and Xan in serum from normal subjects and gouty subjects obtained by the present method (x) with those using the HPLC—UV detection method (y) of Hartwick et al. [4]. Hyp and Xan in serum from gouty subjects have been known to exist at high levels compared with those in normal subjects; we found Hyp and Xan in serum from gouty subjects (n = 9) in the concentration ranges 12.93—41.14 μmol/l and 2.10—6.84 μmol/l, respectively. As shown in Fig. 6, the results obtained by these two methods were correlated closely: for Hyp y = 0.99x + 0.08 (r = 0.99), and for Xan y = 0.93x + 0.05 (r = 0.93).

The results for the determination of Hyp and Xan in biological fluids from normal subjects are presented in Table I. The values for Hyp (6.20 μmol/l) and Xan (2.04 μmol/l) in serum were slightly lower compared with those described by Hartwick et al. [4]: 7.16 μmol/l for Hyp and 2.62 μmol/l for Xan. The sensitivities of determination for Hyp and Xan were approximately

50 and 120 pg per injection, respectively. The assay of allopurinol, which reacts with xanthine oxidase to ·produce hydrogen peroxide, was also possible with a sensitivity of 130 pg per injection. Therefore, we expect that the present method is suitable for studying the influence of allopurinol on purine metabolism.

CONCLUSION

The present method was troublesome; however, it offered several advantages dependent on increasing the sensitivity and selectivity. The rapid assay was performed without using a gradient technique. The chromatographic columns were used for a large number of samples without showing any deterioration because it was possible to analyze small volumes (5 or 10 μl) of the diluted sample.

The mechanism and function of reducing the blank fluorescence by immobilized catalase will be discussed in the future.

REFERENCES

1 R. Parker, W. Snedden and R.W.E. Watts, Biochem. J., 115 (1969) 103.
2 T.T. Hayashi, D. Gill, H. Robbins and R.E. Sabbagha, Gynecol. Invest., 3 (1972) 221.
3 G.B. Elion, A. Kovensky, G.H. Hitchings, E. Metz and R.W. Rundles, Biochem. Pharmacol., 15 (1966) 863.
4 R.A. Hartwick, A.M. Krstulovic and P.R. Brown, J. Chromatogr., 186 (1979) 659.
5 G.J. Putterman, B. Shaikh, M.R. Hallmark, C.G. Sawyer, C.V. Hixson and F. Perini, Anal. Biochem., 98 (1979) 18.
6 W. Voelter, K. Zech, P. Arnold and G. Ludwig, J. Chromatogr., 199 (1980) 345.
7 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704.
8 A. McBurney and T. Gibson, Clin. Chim. Acta, 102 (1980) 19.
9 R.J. Simmonds and R.A. Harkness, J. Chromatogr., 226 (1981) 369.
10 R. Boulieu C. Bory, P. Baltassat and C. Gonnet, J. Chromatogr., 233 (1982) 131.
11 D.G. Gardiner, Anal. Biochem., 95 (1979) 377.
12 T. Sumi and Y. Umeda, Clin. Chim. Acta, 95 (1979) 291.
13 R. Tawa, M. Kito and S. Hirose, Chem. Lett., (1981) 745.
14 R. Tawa, M. Kito, S. Hirose and K. Adachi, Chem. Pharm. Bull., 30 (1982) 615.
15 M. Kito, R. Tawa, S. Takeshima and S. Hirose, J. Chromatogr., 231 (1982) 183.
16 G.G. Guilbault, P. Brignac, Jr. and M. Zimmer, Anal. Chem., 40 (1968) 190.
17 G.G. Guilbault, P.J. Brignac, Jr. and M. Juneau, Anal. Chem., 40 (1968) 1256.
18 J.X. Khym, Clin. Chem., 21 (1975) 1245.

CHROMBIO. 1824

DIFFERENTIAL ISOELECTRIC FOCUSING PROPERTIES OF CRUDE AND PURIFIED HUMAN α_2-MACROGLOBULIN AND α_2-MACROGLOBULIN—PROTEINASE COMPLEXES

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SUMMARY

The isoelectric focusing (IEF) properties of human α_2-macroglobulin (α_2M) and α_2M—proteinase complexes from crude and partially purified preparations were studied by column IEF. The average isoelectric point (pI) of the major form was 6.5 for α_2M from crude plasma but was 5.3 for purified samples. Following preincubation with either trypsin, chymotrypsin or pancreatic elastase the crude α_2M—proteinase complexes displayed pI values ranging from 5.3 to 6.1 and the purified α_2M—proteinase complexes ranged from pH 6.0 to 6.1. A comparison of recoveries for focused crude or purified α_2M and trypsin-preincubated α_2M indicated enhanced recovery for the trypsin-preincubated samples suggesting that the binding of the proteinase enhances the stability of α_2M. α_2M thus displays column IEF properties which appear to be dependent upon the state of purity of the molecule. These findings are of particular significance to investigators concerned with using expressions of altered α_2M electrophoretic patterns for clinical diagnostic purposes in such diseases as multiple sclerosis, diabetes and cystic fibrosis.

INTRODUCTION

The human serum glycoprotein α_2-macroglobulin (α_2M) is a relatively non-specific proteinase inhibitor which has been found to inhibit to varying degrees the activated forms of most of the known endopeptidases (for reviews see refs. 1 and 2). The broad specificity displayed by α_2M toward proteinases appears

central to its proposed regulatory role in a number of important physiological processes including blood coagulation, fibrinolysis, and inflammatory processes [3]. In addition, the inhibitory effect of $\alpha_2 M$ on several in vitro, proteinase-mediated immunological processes has suggested a possible regulatory role for $\alpha_2 M$ in various immune responses [4].

Several investigators have demonstrated by isoelectric focusing (IEF) that $\alpha_2 M$ displays multiple forms but the reported number of forms and their respective isoelectric points differs significantly. The isoelectric point (pI) of the principal form of $\alpha_2 M$ ranges from 4.2 [5] to 5.4 [6,7]. Frénoy and Bourillon [8] resolved four forms of $\alpha_2 M$ with pI values ranging from 4.97 to 5.50. Rosén et al. [5] reported seven forms with pI values ranging from 4.1 to 4.9. Significantly different separations of $\alpha_2 M$ from $\alpha_2 M$—proteinase complexes by IEF have also been obtained depending upon the system employed [9].

Since knowledge of the electrophoretic forms of $\alpha_2 M$ is particularly important for studying possible abnormalities of $\alpha_2 M$ in diseases such as cystic fibrosis [10], diabetes [11] and multiple sclerosis [12], the present study has utilized column IEF to compare the number, pI values and relative amounts of the forms of $\alpha_2 M$ from crude and purified samples. IEF was employed for our studies on $\alpha_2 M$ because this high-resolution technique has proven to be very useful for analytical and diagnostic purposes as well as for preparatively separating proteins with different pI values [13]. Liquid media were used to minimize the amount of molecular sieving which can occur for very large molecules like $\alpha_2 M$ [1,2] in solid media such as polyacrylamide [7,9] and, to a lesser extent, agarose [5]. Our findings suggest that the IEF profiles of $\alpha_2 M$ differ in crude and purified states and that the binding of proteinases enhances the stability of $\alpha_2 M$.

MATERIALS AND METHODS

General

Sources of chemicals and other materials were as follows: α_1-antitrypsin (human), N-benzoyl-L-tyrosine ethyl ester, α-chymotrypsin (type II), elastase (porcine, type I, 2× crystallized suspension), N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanalide, trypsin (bovine, type III), trypsin inhibitor (soybean, type I-S), trypsin inhibitor (turkey eggwhite, type II-T) from Sigma (St. Louis, MO, U.S.A.); α_2-macroglobulin (human, rabbit antiserum) and tosyl-L-arginine methyl ester hydrochloride from Calbiochem-Behring (La Jolla, CA, U.S.A.); p-toluene sulfonyl-L-arginine from ICN K and K Labs. (Plainview, NY, U.S.A.).

Ampholytes were from LKB Instruments (Rockville, MD, U.S.A.) and from Bio-Rad Labs. (Richmond, CA, U.S.A.). Blue Sepharose C1-6B was from Pharmacia (Piscataway, NJ, U.S.A.). Agar double-immunodiffusion plates were from Hyland Diagnostics (Deerfield, IL, U.S.A.).

Blood was obtained from healthy human donors and plasma prepared according to the method of Harpel [14]. Plasma was dialyzed 18 h at 0—4°C against 0.05 M Tris—HCl buffer, pH 8.0, containing 0.02% sodium azide and stored at 2°C or at −20°C.

Assays of proteinase-binding activity of α_2M

All assay procedures were based upon the hydrolysis of a low-molecular weight substrate by α_2M-bound proteinase in the presence of an excess of an inhibitor specific for the unbound proteinase and each assay varied by less than 10%.

The assay for α_2M-bound trypsin is based upon the method of Ganrot [15]. In each duplicate tube, 10—40 μl of plasma were brought to a final volume of 50 μl with 1 mM hydrochloric acid, preincubated at 22°C for 1 min with 10 μl of 10 mg/ml trypsin, and preincubated 1 min longer with 40 μl of 10 mg/ml soybean trypsin inhibitor. The reaction is initiated by mixing a 10-μl sample aliquot with 1 ml of freshly prepared 1 mM tosyl-L-arginine methyl ester in 0.1 M Tris—HCl, pH 8.0, containing 10 mM calcium chloride. The increase in absorbance at 247 nm is recorded at 30-sec intervals for 3—4 min at 22°C. A unit of activity is defined as a change in absorbance of 0.01 per min at 247 nm which corresponds to the hydrolysis of 5 nmoles of substrate. The assay was linear with respect to amount of plasma (10—50 μl) and time (0.5—10.0 min).

The assay for α_2M-bound chymotrypsin was developed from the method of Walsh and Wilcox [16] using benzoyl tyrosine ethyl ester as substrate. In each duplicate tube, 10—40 μl of plasma is brought to a final volume of 50 μl with 0.1 M Tris—HCl buffer, pH 7.8, containing 0.1 M calcium chloride, preincubated at 22°C for 1 min with 10 μl of 10 mg/ml chymotrypsin in 1 mM hydrochloric acid, and preincubated 1 min longer with 40 μl of 10 mg/ml turkey eggwhite trypsin inhibitor. The reaction is initiated by mixing a 20-μl aliquot of sample with 0.50 ml of 1 mM substrate in 50% (w/w) aqueous methanol brought to 1 ml final volume with the same Tris buffer. The increase in absorbance at 253 nm is recorded at 30-sec intervals for 3—4 min at 22°C. A unit of activity is defined as a change in absorbance of 0.01 per min at 253 nm. The assay was linear with respect to amount of plasma (10—80 μl) and time (0.5—10.0 min).

The colorimetric assay of α_2M-bound elastase activity was developed from the method of McGillivray et al. [17] using N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanalide as substrate. In each duplicate tube, a 5-μl aliquot of plasma in 1120 μl of 0.2 M Tris—HCl, pH 8.0, is preincubated for 5 min at 37°C in a shaking water bath with 15 μl of elastase suspension, diluted 1:10 in the same Tris buffer. This solution is mixed with 60 μl of 10 mg/ml α_1-antitrypsin and preincubated 5 min longer at 37°C. The reaction is initiated by mixing the entire sample aliquot with 150 μl of 10 mM substrate in the Tris buffer, terminated after 20 min with concentrated glacial acetic acid, and the absorbance at 410 nm determined. A unit of activity is defined as an absorbance change of 0.01 per min at 410 nm. The assay was linear with respect to amount of plasma (1—10 μl) and time (5—30 min).

All absorbances were read on a Beckman Model 24 spectrophotometer. Assay of α_2M activity after IEF was measured as for plasma samples, except that aliquot volumes were adjusted to be in the linear range of the assay.

Purification of α_2M

α_2M was partially purified by a procedure based upon that of Virca et al.

[18]. Human plasma (5 ml), stored at 2°C for 2—5 days after collection, was chromatographed at 22°C on a bed (1.0 × 100 cm) of Cibicron Blue Sepharose using 0.05 M Tris—HCl, pH 8.0, containing 0.02% sodium azide, at a flow-rate of 5—7 ml/h. Fractions were stored at 2°C as collected. $\alpha_2 M$ was detected by double immunodiffusion against $\alpha_2 M$ antisera to be in fractions from the void volume. All fractions containing $\alpha_2 M$ were combined and concentrated from about 18 ml to 0.5 ml in a collodion bag apparatus using a collodion bag (Schleicher and Schuell, Keene, NH, U.S.A.) with 25,000 MW cut off. Concentrated $\alpha_2 M$ samples were stored at 2°C. The purified $\alpha_2 M$ samples were determined by SDS-polyacrylamide gel electrophoresis, using the method of Laemmli [19], to contain only minor contamination (less than 5%).

Isoelectric focusing

IEF was performed at 2—4°C in a pH 3.5—10 gradient essentially as previously described [20] using a 40-ml column apparatus. Ampholytes (2%) (pH 3.5—10) were used in a gradient of 0—67% sucrose. The starting amperage was 2.5—3.0 mA at a constant voltage of 600 V. Focusing was conducted for 15 h or until the amperage became constant at 0.5 mA.

Samples of plasma or purified $\alpha_2 M$ of comparable trypsin-binding activity (about 2000 units) were focused in all experiments (except where indicated due to limited materials). Samples of proteinase-bound $\alpha_2 M$ were prepared for focusing by preincubating an excess of proteinase with plasma samples at 37°C or with purified $\alpha_2 M$ samples at 22°C for 30 min. The migration of $\alpha_2 M$ in the gradient was monitored by proteinase-binding activity and by use of agar double-immunodiffusion plates in which 5 μl of IEF aliquots were diffused against 5 μl of $\alpha_2 M$ antiserum.

Recovery of $\alpha_2 M$ following IEF was estimated by measuring the $\alpha_2 M$ trypsin-binding activity of pooled focusing fractions, pH 4.5—8.0, dialyzed against 0.05 M Tris—HCl, pH 8.0 at 2°C for 12 h. Since it was found that recoveries were lower for small amounts of focused $\alpha_2 M$ activity (< 1000 units), all reported recovery data were based upon IEF of 2000 units of $\alpha_2 M$ trypsin-binding activity.

RESULTS

Fig. 1a depicts the results of IEF of human plasma assayed for $\alpha_2 M$ trypsin-binding activity. The profile of activity is representative of profiles obtained for six different plasma samples. Examination of these profiles indicated that a small percentage of the total integrated, recovered activity (6—27%) focused between pH 4.5 and 6.0, and the main isoelectric form in this region had a pI value of 5.4—5.7. The greatest percentage of the total integrated, recovered activity for all six samples was from pH 6.0 to 8.0 (73—94%). The major isoelectric form had a pI centered around 6.5 and ranging from 6.2 to 6.8.

A comparison of the proteinase-binding activity in Fig. 1a (trypsin) with Fig. 1b (chymotrypsin) and Fig. 1c (elastase) demonstrates that similar focusing profiles were obtained (with regard to the presence of a single main peak above pH 6.0) when $\alpha_2 M$ from the same donor was assayed with each of these three proteinases. The total recovery of $\alpha_2 M$ trypsin-binding activity was 26—58% of the activity applied to the IEF column (average recovery = 44%).
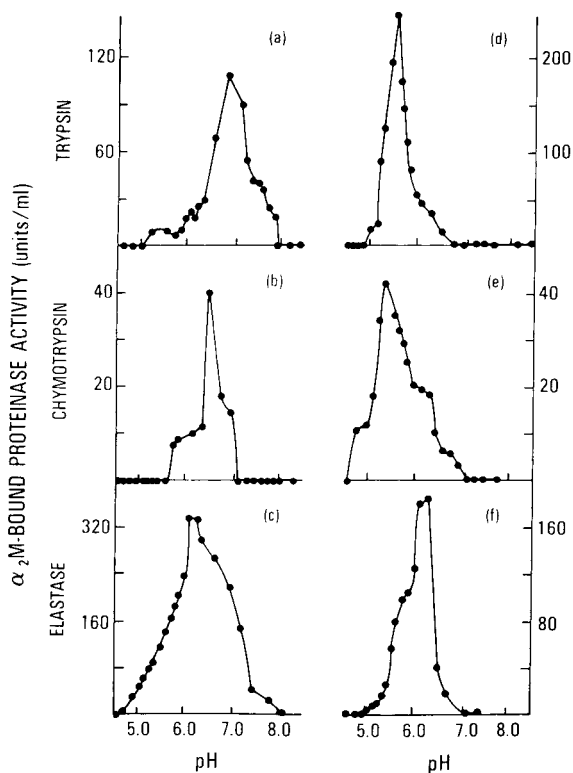
Fig. 1. IEF of α_2M in crude plasma before (a—c) and after (d—f) preincubation with various proteinases. (a, d) Trypsin-binding activity; (b, e) chymotrypsin-binding activity; (c, f) elastase-binding activity. Samples of plasma equivalent to 2000 units of trypsin-binding activity were focused in (a—e) and 1000 units were focused in (f). See Materials and methods section for details.

Fig. 1d—f (each profile representative of three different plasma samples) depicts the results of IEF of plasma samples preincubated prior to focusing with saturating amounts of trypsin (Fig. 1d), chymotrypsin (Fig. 1e) or elastase (Fig. 1f). For the trypsin- and chymotrypsin-preincubated samples, the α_2M-bound proteinase focused to a lower pI value relative to the nonpreincubated sample (Fig. 1a and b). For α_2M-bound trypsin the pI value of the principal isoelectric form ranged from 5.5 to 5.7 and for α_2M-bound chymotrypsin from 5.3 to 5.7. Under saturating conditions the α_2M-preincubated elastase displayed no appreciable shift in pI to a lower pH (Fig. 1f). However, under subsaturating conditions, the α_2M-bound elastase displayed a profile similar to that seen for the other α_2M-bound proteinases studied (data not shown).

In contrast to the recovery of α_2M obtained for IEF of plasma, the recovery of α_2M for IEF of proteinase-preincubated plasmas was substantially enhanced. The typical total recovery of α_2M-bound trypsin activity was 61—74% (average recovery = 70%).

In Fig. 2 are shown the results of IEF of α_2M and α_2M—proteinase complexes employing purified α_2M samples. A comparison of the α_2M profiles for focused
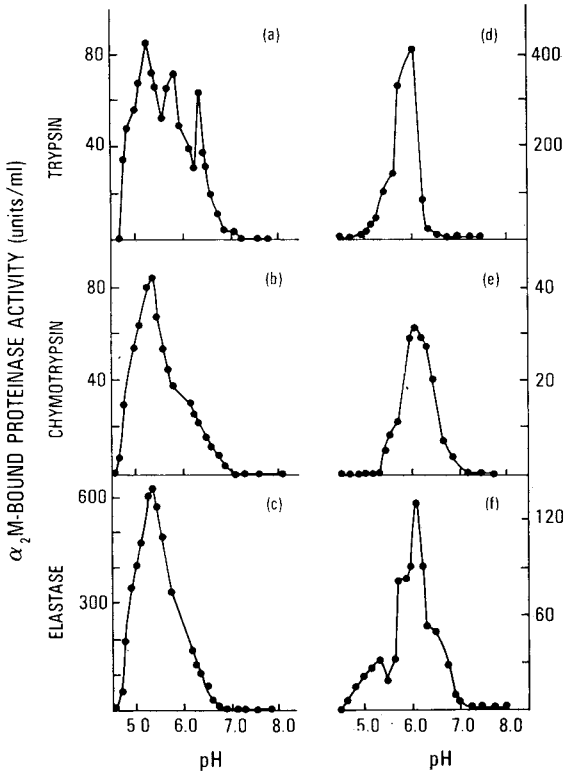
Fig. 2. IEF of purified α_2M before (a—c) and after (d—f) preincubation with various proteinases. (a, d) Trypsin-binding activity; (b, e) chymotrypsin-binding activity; (c, f) elastase-binding activity. Samples of α_2M equivalent to 2000 units of trypsin-binding activity were focused in (a—d) and 800 units were focused in (e) and (f). See Materials and methods section for details.

plasma in Fig. 1 with those for purified α_2M in Fig. 2 indicates that the pI values of the α_2M isoelectric forms are quite different. The principal form of α_2M from purified samples had a pI value of about 5.3 when assayed with either trypsin, chymotrypsin or elastase (Fig. 2a, b and c, respectively). Better resolution of minor forms was obtained using the trypsin assay and forms with pI values at 5.8 and 6.4 were also resolved.

For purified α_2M samples preincubated with either trypsin, chymotrypsin or elastase, the principal isoelectric form for each α_2M—proteinase complex displayed a neutral shift in pI value to pH 6.0—6.1 (Fig. 2d, e and f, respectively), when compared to the corresponding nonpreincubated sample.

The recovery of α_2M activity following IEF of purified α_2M samples was similar to that seen for IEF of plasma. The typical recovery of α_2M was 56% for the uncomplexed α_2M and essentially full recovery was obtained for the trypsin-preincubated α_2M.

Throughout these studies double immunodiffusion using α_2M antisera was employed to determine the distribution of α_2M antigen in samples of focused plasma or purified α_2M. In each experiment the presence of proteinase-binding

activity always coincided with the distribution of antigenically cross-reactive material.

Several other findings relative to the IEF studies were noteworthy. No endogenous trypsin, chymotrypsin or elastase activity was detected in samples of plasma or focused plasma. The focusing profiles were very reproducible regardless of the duration of focusing (15—36 h), the amount of α_2M focused (1550—3000 units of trypsin-binding activity), the supplier of ampholytes (LKB or Bio-Rad), or previous history of plasma samples (fresh or frozen for 1—12 weeks). For comparable studies, similar recoveries of α_2M were obtained for each proteinase employed. However, recoveries were not as good when low levels of α_2M activity (< 1000 units) were focused.

DISCUSSION

Column IEF of human α_2M in crude and purified states demonstrated that both α_2M and α_2M—proteinase complexes displayed markedly different focusing properties depending upon the degree of purity of the α_2M. For purified samples, the pI value of the major α_2M form was 5.3 which is in close agreement with previous findings for column IEF [6, 8, 21] and polyacrylamide gel IEF [7, 22]. The form we observed at pH 5.8 appears to correspond to the secondary form at pH 5.5 observed by Frénoy and Bourrillon [8].

In agreement with previous studies employing polyacrylamide gel IEF [9, 22], a neutral shift in pI was observed for purified α_2M samples preincubated with proteinases prior to IEF. However, we were unable to confirm the observation of Barret et al. [9] using column IEF that purified α_2M and α_2M—proteinase complexes do not differ appreciably in pI.

In contrast to the close agreement seen in many IEF studies employing purified α_2M samples, widely differing results have been obtained for IEF of crude α_2M. Rosén et al. [5] employed agarose-crossed immunoelectrofocusing of whole serum to resolve α_2M into seven peaks with pI values ranging from 4.1 to 4.9. The main form had an apparent pI of 4.2. In contrast, Ohlsson and Skude [23] identified only one α_2M peak with a pI value of 5.0 by employing two-dimensional polyacrylamide gel IEF. In the present study, the main α_2M form focused to a pI value ranging from 6.2 to 6.8 when monitored by trypsin-binding activity and double immunodiffusion against α_2M antisera. In addition, preincubation of plasma with trypsin or chymotrypsin prior to IEF elicited an acid shift to pI values ranging from 5.5 to 5.7 for trypsin and from 5.3 to 5.7 for chymotrypsin. We thus did not observe the consistent basic shift in pI value to pH 6.0 for α_2M—proteinase complexes as observed by Ohlsson and Skude [23]. Taken together, these diverse findings suggest that there may be system-related factors [24] as well as unidentified interactions of α_2M with other molecules in plasma which are causing the different observed electrophoretic migrations of crude α_2M.

A number of studies we performed supports the validity of the isoelectric focusing patterns we observed in the crude state. The focusing profiles for both α_2M and α_2M—proteinase complexes were very reproducible regardless of the duration of focusing, the amount of plasma focused, the brand of ampholytes employed, or the previous history of the plasma. In addition, the substantial

recoveries obtained for trypsin-preincubated plasma samples (61—74%) indicate that the focusing profiles for crude α_2M are representative of the majority of the examined proteinase-binding activity. The distinct differences in the focusing profiles for nonpreincubated α_2M (Fig. 1a, b) and α_2M preincubated with trypsin or chymotrypsin (Fig. 1d, e) suggest that the migration of non-preincubated α_2M to pI values more neutral than previously reported [5, 23] was not due to association of α_2M with proteinases. This is supported by the absence of endogenous trypsin, chymotrypsin or elastase activity in either plasma or focused plasma.

A systematic examination of IEF recoveries for α_2M trypsin-binding activity demonstrated similar recoveries for crude and purified α_2M samples indicating no loss of α_2M stability to IEF due to purification. Consistently enhanced recoveries were observed for both crude and purified trypsin-preincubated samples which suggests that formation of the α_2M-proteinase complex enhances the in vitro stability of α_2M.

The present column IEF study indicates that the pI values and focusing properties of α_2M and α_2M-proteinase complexes appear dependent upon the state of purity of the molecule. The present findings are of particular significance to investigators concerned with using expressions of altered α_2M electrophoretic patterns for clinical diagnostic purposes. In addition, our findings indicate the general need for caution in characterizing the isoelectric forms of other macromolecules which may also display variable IEF properties depending upon their state of purity.

REFERENCES

1   P.C. Harpel, in D.H. Bing (Editor), The Chemistry and Physiology of the Human Plasma Proteins, Pergamon Press, New York, 1979, pp. 385—399.
2   P.M. Starkey and A.J. Barret, in A.J. Barret (Editor), Proteinases in Mammalian Cells and Tissues, North-Holland, Amsterdam, 1977, pp. 663—696.
3   P.M. Starkey, in D. Collen, B. Wiman and M. Verstraete (Editors), The Physiological Inhibitors of Blood Coagulation and Fibrinolysis, North Holland, Amsterdam, 1979, pp. 221—230.
4   K. James, Trends Biochem. Sci., 5 (1980) 43—47.
5   A. Rosen, K. Ed and P. Åman, J. Immunol. Meth., 28 (1979) 1—11.
6   M. Schönenberger, R. Schmidtberger and H.E. Schultze, Z. Naturforsch., 13b (1958) 761—772.
7   J.M. Jones, J.M. Creeth and R.A. Kekwick, Biochem. J., 127 (1972) 187—197.
8   J.-P. Frénoy and R. Bourrillon, Biochim. Biophys. Acta, 371 (1974) 168—176.
9   A.J. Barrett, M.A. Brown and C.A. Sayers, Biochem. J., 181 (1979) 401—418.
10 G.B. Wilson and H.H. Fudenberg, Pediatr. Res., 10 (1976) 87—96.
11 K. James, J. Merriman, R.S. Gray, L.J.P. Duncan and R. Herd, J. Clin. Pathol., 33 (1980) 163—166.

12 S.C. Rastogi and J. Clausen, Clin. Chim. Acta, 107 (1980) 141—144.
13 P.G. Righetti, Laboratory Techniques in Biochemistry and Molecular Biology, Vol. II. Isoelectric Focusing. Theory, Methodology and Applications, Elsevier Biomedical Press, New York, 1983.
14 P.C. Harpel, J. Exp. Med., 138 (1973) 508—521.
15 P.O. Ganrot, Clin. Chim. Acta, 14 (1966) 493—501.
16 K.A. Walsh and P.E. Wilcox, in G.E. Permran and L. Lorand (Editors), Methods in Enzymology, Academic Press, New York, 1970, pp. 31—41.
17 D.H. McGillivray, D. Burnett, S.C. Afford and R.A. Stockley, Clin. Chim. Acta, 111 (1981) 289—294.
18 G.D. Virca, J. Travis, P.K. Hall and R.C. Roberts, Anal. Biochem., 89 (1978) 274—278.
19 U.K. Laemmli, Nature, 227 (1970) 680—685.
20 J.A. Alhadeff, A.L. Miller, D.A. Wenger and J.S. O'Brien, Clin. Chim. Acta, 57 (1974) 307—313.
21 U. Hamberg, P. Stelwagen and H.-S. Ervast, Eur. J. Biochem., 40 (1973) 439—451.
22 M. Parsons and G. Romeo, Clin. Chim. Acta, 100 (1980) 215—224.
23 K. Ohlsson and G. Skude, Clin. Chim. Acta, 66 (1976) 1—7.
24 M.A. Bridges, D.A. Applegarth and J. Johannson, Clin. Chim. Acta, 121 (1982) 167—172.

CHROMBIO. 1822

THE USE OF THIOL—DISULPHIDE EXCHANGE CHROMATOGRAPHY FOR THE AUTOMATED ISOLATION OF α_1-ANTITRYPSIN AND OTHER PLASMA PROTEINS WITH REACTIVE THIOL GROUPS

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SUMMARY

A method has been developed for the rapid isolation of α_1-antitrypsin and other thiol proteins from plasma by an automated chromatography system. The thiol-proteins are initially bound to matrix-linked activated thiol-compounds by an SH—SS interchange reaction. The mixed disulphides are then reduced in two steps and subfractionated by passage through Blue-Sepharose and AH-Sepharose columns. The rate of the interchange reactions varies with the microenvironment of the reacting thiols. α_1-Antitrypsin is recovered with 95% purity in 60% yield within two days from 1 l of plasma.

INTRODUCTION

Current methods for the isolation of α_1-antitrypsin from plasma are usually designed to recover below 100 mg in each cycle and most procedures include ion-exchange chromatography [1—4]. Fractions containing the bulk (80—90%) of α_1-antitrypsin are selected after each chromatographic step from the elution diagrams. Since this entails partial exclusion of the most basic and acid fractions at each chromatography step, the final product will contain α_1-antitrypsin with a microheterogeneity that differs from that of native plasma [5]. We describe here a method principally based on thiol—disulphide interchange reactions [6] for semiautomatic isolation of α_1-antitrypsin with retained microheterogeneity in high yield and in weekly gram quantities. The fractionation procedure is monitored by a programmable controller.

MATERIALS AND METHODS

L-Cysteine · HCl and 2-mercaptoethanol were purchased from BDH (Poole, U.K.) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol from Sigma (St. Louis, MO, U.S.A.). AH-Sepharose 4B, Blue-Sepharose CL-6B, cyanogen bromide (CNBr)-activated Sepharose 4B, heparin-Sepharose CL-6B, thiopropyl-Sepharose 6B, thiol-Sepharose 4B and dextran sulphate (sodium salt) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and polyethyleneglycol M 4000 from Kebo (Stockholm, Sweden).

Antisera to all major plasma proteins were available at the laboratory.

Agarose gel electrophoresis, immunoelectrophoresis and electroimmunoassay were used for screening purposes and for quantitation of specific proteins.

κ -Chain-Sepharose

κ-Chains isolated from urine of myeloma patients were activated to κ-thionitrobenzoate (κ-TNB) [5]. κ-Chains (5 g) were dissolved in 75 ml Tris—HCl (1 M, pH 8) with Na_2EDTA (0.1 M) and saturated with DTNB. The solution was kept at room temperature for 4 days to obtain mainly monomeric κ-TNB. The amount of the mixed disulphide κ-TNB required for Sepharose-coupling was passed through 8—10 volumes Sephadex G 75, equilibrated with 0.1 M $NaHCO_3$ containing 0.5 M sodium chloride, to separate κ-TNB from TNB and DTNB. κ-Chains (1 g) with TNB-blocked COOH-terminal cysteinyls were conjugated with 20 g CNBr-activated Sepharose 4B as recommended by the manufacturer. About 40 μmol κ-chains (23,000 dalton) were bound per 100 ml Sepharose.

Plasma preparations

Plasma was available from the hospital blood bank. Low (LD) and very low density (VLD) lipoproteins were removed from plasma, either by passage through a heparin-Sepharose column (2 ml plasma per ml heparin-Sepharose) [7] which allowed recovery of a series of proteins, or by precipitation at 4°C with 20 ml dextran sulphate (10%) and 100 ml (1 M) calcium chloride per l plasma [8]. Plasma was mildly reduced by adding 2-mercaptoethanol to a concentration of 0.02 M, to cleave mixed disulphides and interprotein SS-bridges. All low-molecular thiol compounds were removed by dialysis overnight with running tap-water. Polyethyleneglycol was added to a concentration of 4% (w/v) at 4°C, and the plasma was centrifuged 1 h later. Salts were added to the supernatant in a final concentration of 0.05 M Tris, 0.2 M sodium chloride, 5 mM Na_2EDTA and 3 mM NaN_3, and the pH was adjusted to 8.1—8.0 with 6 M hydrochloric acid under vigorous stirring. This diluted plasma was stored frozen until the fractionation was started. The plasma albumin content had decreased from 45 to about 30 g/l.

Solutions for chromatography

Buffer 1: 0.05 M Tris buffer, pH 8.1, containing 0.2 M sodium chloride, 5 mM Na_2EDTA and 3 mM NaN_3. Buffer 2: 0.05 M sodium phosphate buffer, pH 5.5, containing 0.5 M sodium chloride. Reducing solution 1: 1 mg DTNB and 0.25 mg dithiothreitol per ml buffer 1. Reducing solution 2: buffer 1 with

20 mM 2-mercaptoethanol. DTNB solution of κ-chain reactivation: 0.5 mg DTNB per ml buffer 1. Buffer 1 with additional 1.3 M sodium chloride was used as a regeneration solution for ridding Blue-Sepharose and AH-Sepharose of proteins.

EXPERIMENTAL

The flow system for the fractionation of thiol-proteins

The principle design is shown in Fig. 1 and Table I. Plasma rid of VLD- and LD-lipoproteins is reduced, dialyzed, and pumped through a κ-chain Sepharose column with DTNB-activated thiol groups. α_1-Antitrypsin and some other plasma proteins are retained in the column by SH—SS exchange reactions. The retained (SS-linked) proteins are later eluted by a two-step reduction. Albumin is removed by passing the primary eluate through a Blue-Sepharose column. The eluate is pumped on through an AH-Sepharose column which retains pre-albumin and other minor contaminants, while α_1-antitrypsin and a mixed Ig-fraction pass through to be recovered as eluate 1. These proteins are separated by ammonium sulphate fractionation. Other thiol-proteins are recovered in eluates II, III and IV.

All solution inlets are furnished with filters. Transmission tubes (0.075 mm), glass T connectors and nylon nipples of Technicon design are used throughout as links.

Arrows in Fig. 1 indicate direction of flow. The Sepharose columns are
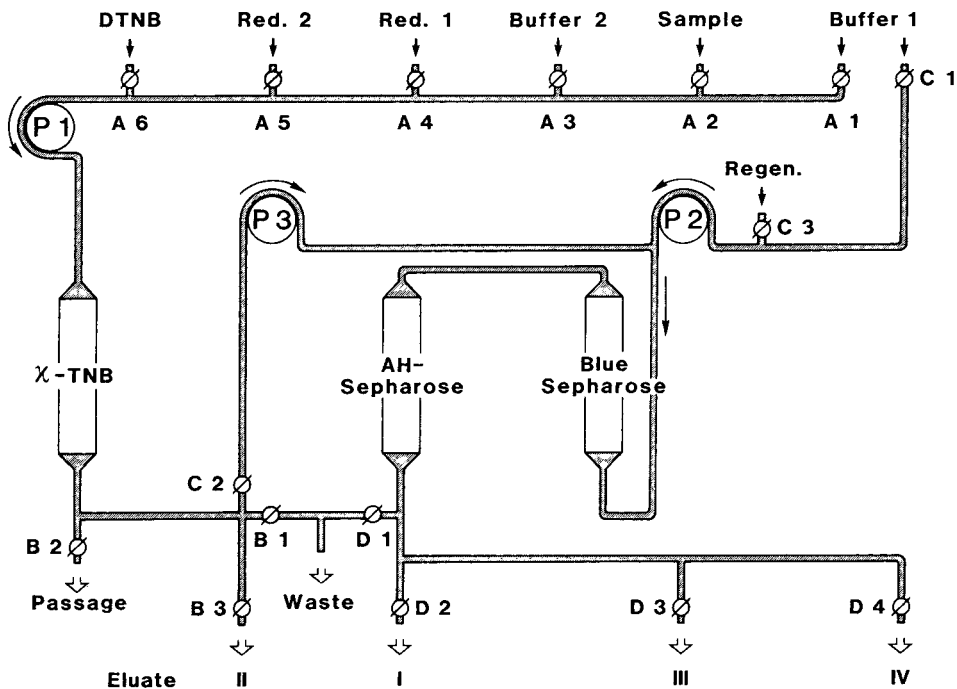
Fig. 1. Scheme of flow system for retention elution and subfractionation of thiol-proteins. A, B, C and D indicate valves and P pumps. The activation time and order of pumps and valves are given in Table I. For details see text.

TABLE I

PROGRAMME FOR FLOW SYSTEM GIVEN IN FIG. 1

Standard activation programme with a pump flow-rate for 1 and 3 of 180 ml/h, and 110 ml/h for P2; 450 ml κ-Sepharose, 150 ml Blue-Sepharose and 170 ml AH-Sepharose columns suitable for fractionation of 500 ml de-lipidated, mildly reduced plasma (final volume 800 ml). Time is given in minutes.

Step	Time	Valve	P1	Valve	Valve	P2	P3	Valve
1	230	A1	+	B1				
2	120	A2	+	B1				
3	155	A2	+	B2				
4	120	A1	+	B2				
5	115	A3	+	B1				
6	230	A1	+	B1				
7	55	A4	+	B1	C1	+		D1
8	65	A1	+	B1	C1	+		D1
9	70	A5	+		C2		+	D1
10	40	A1	+	B1	C1	+		D1
11	120	A1	+	B3	C3	+		D2
12	125	A6	+	B1	C1	+		D3
13	85	A6	+	B1	C1	+		D4

loaded from the bottom upwards, their inlets being provided with 10-ml bubble traps with a clamped tube as top outlet. The inlet and outlet of each column is connected to a mechanically adjustable double-channel four-port valve (SRV-4, Pharmacia), which permits the channel system to be flushed by cleansing solutions that bypass the columns. These details have been excluded from Fig. 1.

The three chromatography tubes (300 mm × 50 mm I.D.) are furnished with adaptors at top and bottom (Pharmacia A 50). The 16 valves were of type: Skinner valve B10 (6 W, 24/50 V) (New Britain, CT, U.S.A.). The heat generated in the activated valves may raise the temperature of the valve body to some 55°C, which may cause accumulation of a thin ring of protein precipitate inside the rim of the valve membrane. Since this precipitate may finally disturb the valve function after 25—50 h, valves of latch type are therefore preferred at positions A2 and B2, through which the bulk of heat labile plasma protein passes.

Three LKB multiplex peristaltic pumps are used. Pumps 1 and 3 are calibrated to the same flow-rate (180 ml/h) and pump 2 slower (110 ml/h). The pump tubes are exchanged prophylactically every third month, no flow-rate adjustment being needed in between. A Mitsubishi programmable controller, Melsec-K, activates pumps and valves in accordance with an easily exchangeable programme from a Melsec-K7PUE programming unit. The controller is equipped with two KYOI units (each a 16-point element I/O unit).

The columns are emptied and repacked every second month, assuming four complete cycles to be run each week. The κ-chain-Sepharose and the Blue-Sepharose have been used for two years without apparent loss in exchange capacity, while the AH-Sepharose had to be replaced within three months (= 12—15 regeneration cycles). Increasing amount of prealbumin in eluate 1

Practical comments

De-aeration of the solutions is unnecessary if bubble traps are mounted at the column inlets. The reducing solutions 1 and 2 are prepared daily and retain sufficient reducing capacity overnight without having to be flushed with nitrogen. No disturbing build-up of pressure has been observed in the tube system during repetitive cycles, when the flow-rate was kept at 3 ml/min and the LD lipoproteins are carefully removed from the plasma. The Sepharose packing stabilizes after a few cycles, and some millimetres of free fluid layer are observed above the filters of the inlet adaptors. These need not be adjusted. Smooth flow through the columns is indicated if an even yellow zone passes when DTNB is pumped through.

The outlet from the AH-Sepharose was connected to a fraction collector, and the samples were analysed by electroimmunoassay to find the optimal period for activation of valve D2. Identical daily volumes in the receptacles of the four eluate outlets indicate correct flow and valve functions.

Development of the method for fractionation of thiol proteins

The SH—SS interchange reaction, on the passage of mildly reduced plasma at pH 7—8, was used to compare the efficiency of DTNB-activated thiopropyl Sepharose ($-O-CH_2-CHOH-CH_2-SH$), glutathione Sepharose (-GluCysGly), and κ-chain Sepharose (Glu-Cys) [6] in retaining both albumin and α_1-antitrypsin. Both proteins have one reactive thiol group, but the ratio of the plasma concentrations of mercaptoalbumin and α_1-antitrypsin is above 15 to 1. The results showed that the interchange reaction with the activated thiopropyl group was roughly proportional to the albumin:α_1-antitrypsin ratio in the passing solution, while activated glutathione bound to Sepharose retained α_1-antitrypsin 30 times faster than it did albumin, and activated κ-chains enriched α_1-antitrypsin 150 times faster than they did albumin, at a flow-rate of the protein solution through the column of 3 ml/min. Since the amount of albumin retained is increased by recycling the sample, or by retarding the flow, the solution was passed through only once to keep the albumin retention at a minimum. We have shown previously [6] that SS-linked plasma proteins vary slightly in their sensitivity to reduction; thus, further enrichment of α_1-antitrypsin to albumin could be achieved by a two-step reduction (elution) programme. The coupling reaction used in the column

$$\alpha_1\text{-AT(s}^-) + \text{TNB(ss)}\kappa \rightleftharpoons \alpha_1\text{-AT(ss)}\kappa + \text{TNB}^-$$
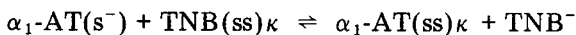
was forced to the left by excess TNB$^-$ in reducing solution 1. Any released α_1-AT(s$^-$) formed a soluble mixed disulphide with DTNB in reducing solution 1 according to

$$\alpha_1\text{-AT(s}^-) + \text{DTNB} \rightleftharpoons \alpha_1\text{AT(ss)TNB} + \text{TNB}^-$$
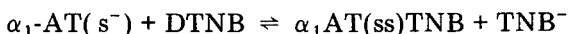
The mixed disulphide was eluted in front of and overlapping TNB$^-$. In the second elution step, 0.02 *M* 2-mercaptoethanol released all remaining κ-linked plasma proteins. This eluate (II) was harvested without further purification

through valve B3, while the solution with the proteins first released was transferred to a Blue-Sepharose column, the size of which was chosen to efficiently remove albumin. The third column was loaded with AH-Sepharose, which had been found to link prealbumin stronger, compared with α_1-antitrypsin, than other anionic exchangers. The proteins remaining in the solution after its passage through the AH-Sepharose column, were harvested through valve D2 (eluate I) and consisted mainly of α_1-antitrypsin and immunoglobulins. The latter are quantitatively precipitated by 1.97 M ammonium sulphate.

The AH-Sepharose and Blue-Sepharose were regenerated by the passage of a strong salt solution which released proteins that were collected in the consecutive eluates III and IV — eluate III containing proteins from the AH-Sepharose with some admixture of proteins from the Blue-Sepharose column. The bulk of the latter are recovered in eluate IV.

Proteins in all four eluates were precipitated with ammonium sulphate in two steps (1.97 M and 3.08 M) with intervening centrifugation.

RESULTS

After repeated activation and reduction of the κ-chain Sepharose with DTNB and 2-mercaptoethanol, 210 μmol TNB$^-$ per l Sepharose was recovered on reduction. During each loading with plasma the total amount of thiol proteins linked per l κ-Sepharose was about 25 μmol. The average recovery of plasma proteins in ten experiments is given in Table II. The plasma used in these experiments had been de-lipidated by the passage of heparin-Sepharose. The proteins reported in the tables are those which were obtained in mg quantities when loading a 450-ml κ-chain column with 300 ml plasma.

TABLE II

MAJOR PROTEINS RECOVERED FROM EACH SH—SS CYCLE WITH ACTIVATED κ-CHAINS

Values are mg protein recovered from 300 ml plasma in eluate.

Proteins*	Eluate I		Eluate II		Eluate III		Eluate IV	
	a	b	a	b	a	b	a	b
α_1-Antitrypsin	4	235	0.3	0.4	3	24	—	—
Albumin	0.2	1	13	87	8	79	1	44
Prealbumin	—	1	(1)	6	3	14	trace	—
α_2-Macroglobulin	18	—	37	—	72	—	2	—
IgA	12	—	50	—	32	—	1	—
IgM	20	—	41	—	16	—	0.5	—
IgG	10	—	12	—	3	—	—	—
C3	0.3	—	0.8	—	4	—	0.05	—
C4	0.08	—	0.4	—	3	—	0.1	—
Hc-globulin	0.08	0.02	0.3	—	0.3	0.05	0.05	0.01
HDL	(0.9)**		(3)		(11)		(1)	

*Protein content of each eluate (I—IV) is given after precipitation with 1.97 M and 3.08 M ammonium sulphate as a and b, respectively.
**Values between brackets indicate atypical immunoreactivity.

The following proteins were searched for but not found: orosomucoid, antichymotrypsin, inter-α-trypsin inhibitor, ceruloplasmin, antithrombin III, α_2-antiplasmin, Cl-esterase inhibitor, HS-globulin, prothrombin, plasminogen, pseudocholinesterase, fibronectin, haemopexin, thyroxin-binding globulin, sex hormone-binding globulin, P-component, CRP, α_2-microglobulin, β-microglobulin, C4-binding globulin. The following average percentages were obtained for the recovery of each protein, when calculated from the protein load: α_1-antitrypsin (66%), prealbumin (23%), IgM (32%), α_2-macroglobulin (17%), IgA (16%), transcortin (10%), HC- or α_1-microglobulin (9%), C4 (4%), albumin (2%), C3 (2%), HDL (2%), haptoglobins (1%), IgG (0.8%), transferrin (0.3%), Gc-globulin (trace), LDL (trace).

The recovery given for IgM in Table II may be too high, since we did not check the molecular size, and since the fractions were not strongly reduced before immunochemical estimation. The recovery values for C3, C4 and HDL are also approximate, because the electroimmunoassay analyses indicated atypical immunologic reactivity with partial blockage of antigenic determinants. Generally, the agarose gel electrophoretic patterns of the final four fractions agreed well with the concentrations of the various protein species identified in them. However, a major α_1-component of low solubility in eluate II, and the major α_2-fraction of low solubility in eluate IV have not yet been identified.

The proteins in eluate II are cleaved off from the κ-chains together with TNB^- by 2-mercaptoethanol, and eluted with excess 2-mercaptoethanol, mainly as free thiol proteins, while the proteins in eluates I, III and IV are recovered as mixtures of mixed disulphides with DTNB, or as SS-linked mixed proteins. This may explain why slightly atypical electrophoretic mobility and broad electrophoretic zones are obtained. The more anodal position of prealbumin and albumin in eluate III than in eluate II is due to the negative charge added by linked TNB^- (not shown).

The various proteins are converted within 24 h to mixed Cys-disulphides by the addition of 0.1 M freshly prepared cysteine $\cdot$ HCl under pH adjustment to 7. This reaction is accelerated by the addition of activated carbon which adsorbs TNB^-. The proteins are stored as stabile mixed disulphides with cysteine in 0.1 M glycine of pH 6—7, 0.05 M Na_2EDTA 0.15 M sodium chloride and 3 mM NaN_3. The purity of α_1-antitrypsin in eluate Ib and of mercaptoalbumin in IVb is 95% or higher. The microheterogeneity of the final α_1-antitrypsin fraction agreed on isoelectric focusing with that of α_1-antitrypsin in the original plasma (not shown).

DISCUSSION

Covalent chromatography of thiol proteins, based on 4,4-dithiodipyridine activated glutathione linked to Sepharose, was introduced by Brocklehurst et al. [9]. The rate of the SH—SS interchange reaction is primarily determined by the pK of the protein thiols, which must be borne in mind when designing experiments. These pK values are not easy to estimate, as they vary with the micro-environment of the thiol on the protein surface [10]. Our early finding of κ-chains, linked by SS-bonds to plasma proteins, suggested that κ-chains

reacted faster with α_1-antitrypsin than prealbumin, and prealbumin than albumin, when the plasma concentration of the various proteins was considered [11]. Electrostatic influence from the micro-environment of the reactive thiols seems to effect the SH—SS interchange rate strongly, judging by the sequences of the three proteins which we now know. The reaction rates at neutral pH with κ-chains, of the thiols of α_1-antitrypsin (232) (—His—Cys—Lts—) [12], of prealbumin (10) (—Lys—Cys—Pro—), and of albumin (34) (—Gln—Cys—Pro), decrease in corresponding order. The adjacent Glu to Cys in κ-chains seems to facilitate the interchange reaction of proteins with Cys and adjacent basic residues in the sequence. The same series of reaction rates has been observed for κ- and λ-chains (C-terminals: —Glu—Cys—Ser) and the three proteins, but the rate is much slower for λ-chains, suggesting that the ultimate seryl induces some steric hindrance.

It may be considered sophisticated to utilize terminal κ-chain thiols as SH—SS exchangers, but these small proteins are available in large amounts in the urine of many myeloma patients, and are easily recovered in any clinical laboratory. While their large size limits the usefulness of κ-chains as SH—SS exchangers, their fast reaction with α_1-antitrypsin makes them preferred for the isolation of α_1-antitrypsin with native microheterogeneity — at least until more efficient, but similarly selective, SH—SS exchangers have been synthesized. A peptide of the four to six C-terminal amino acids of κ-chains may be a more efficient exchanger than the complete κ-chain. Substitutions around the two reacting cysteinyls affect the SH—SS exchange rate, and an optimal electrostatic effect may exist for each specific protein; thus, it would be advantageous to have a range of thiol peptides to chose between for use in this type of chromatography.

The protein recoveries presented in Table II refer to proteins with reactive thiols in pooled and stored human plasma; thus, complementary studies have to be performed on freshly collected, individual plasma before any conclusions may be drawn about the physiological state of the proteins. The large amount of α_2-macroglobulin, C3 and C4 obtained, may have been caused by thiols released on cleavage of their labile thiol esters after blood sampling. Though plasma IgA is well known for formation of SS-linked complexes in plasma, IgM is not, so the large amount recovered was unexpected. Further studies of the effect of the mild reduction (0.02 M 2-mercaptoethanol) on the various native IgM species are necessary before any valid conclusions may be drawn about the physiological implications of our findings.

REFERENCES

1 R.E. Roll and R.H. Glew, J. Biol. Chem., 256 (1981) 8190.
2 T.F. Busby and J.S. Gau, Arch. Biochem. Biophys., 177 (1976) 556.

3   R. Pannell, D. Johnson and J. Travis, Biochemistry, 13 (1974) 5439.
4   M. Morii, S. Odani, T. Koide and T. Ikenaka, J. Biochem., 83 (1978) 269.
5   C.-B. Laurell, J. Pierce, U. Persson and E. Thulin, Eur. J. Biochem., 57 (1975) 107.
6   C.-B. Laurell, J. Chromatogr., 159 (1978) 25.
7   E.J. McKay and C.-B. Laurell, J. Lab. Clin. Med., 95 (1980) 69.
8   M. Burstein and H.R. Scholnik, Adv. Lipid Res., 11 (1973) 67.
9   K. Brocklehurst, J. Carlsson, M.P.Y. Kierstan and E.M. Cook, Biochem. J., 133 (1973) 573.
10 G.H. Snyder, M.J. Cennerazzo, A. Karalis and D. Field, Biochemistry, 20 (1981) 6509.
11 C.-B. Laurell and E. Thulin, Immunochemistry, 11 (1974) 703.
12 R.W. Carrell, J.-O. Jeppsson, C.-B. Laurell, S.O. Brennan, M.C. Owen, L. Vaughan and D.R. Boswell, Nature (London), 298 (1982) 329.

CHROMBIO. 1833

NACHWEIS EINER EINNAHME VON HASCHISCH IN BIOLOGISCHEM MATERIAL*

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SUMMARY

Detection of hashish intake in biological material

In order to detect hashish intake, urine, blood and serum were analysed for the main components of hashish, i.e., tetrahydrocannabinol (THC), cannabidiol, cannabinol and the decomposition product of THC, THC-carboxylic acid. After extraction and silylation, the samples were analysed by gas chromatography—mass spectrometry with multiple ion detection. The Emit-st-system® is used as a pretest for urine.

EINLEITUNG

Wegen ihrer psychotropen Wirkung fallen Zubereitungen von Haschisch (z.B. Marihuana, gepresster Haschisch oder Haschöl) unter das Betäubungsmittelgesetz (BtMG). Die Hauptinhaltsstoffe sind Cannabinol (CBN), Cannabidiol (CBD) und Tetrahydrocannabinol (THC), wobei dem letzteren die eigentliche Haschischwirkung zugeschrieben wird [1]. Im forensischen Bereich ist es nötig, die Einnahme von Haschisch im Blut oder Urin nachzuweisen, z.B. wegen eines Vergehens gegen das BtMG oder z.B. wegen eines Verkehrsdeliktes. Obwohl nach Einnahme von Haschisch eine Vielzahl von Abbauprodukten im Urin auftreten [2,3], beschränkt sich der Nachweis im Routinebetrieb auf die drei Hauptinhaltsstoffe CBN, CBD und THC, sowie auf

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das Hauptabbauprodukt von THC, 11-nor-Δ^9-Tetrahydrocannabinol-9-carbon-säure (THC-COOH). Es gibt hierfür radioimmunologische Verfahren (RIA) [4,5] und enzymimmunologische Verfahren (Emit®) [6,7], wobei die letzteren speziell auf THC-COOH eingestellt sind. Da diese immunologischen Methoden nur als Vortest verwendet werden können, weil damit keine eindeutige Aussage möglich sind [8], müssen die Befunde mit einer zweiten Methode abgesichert werden. In der Literatur sind hierfür Verfahren für THC und THC-COOH beschrieben und zwar mit Gaschromatographie (GC) [9] und Hoch-leistungsflüssigkeitschromatographie (HPLC) [10,11]. Um eine grössere Sicher-heit zu bekommen, werden zur Identifizierung Trennverfahren gekoppelt mit einem Massenspektrometer eingesetzt. Off-line wurde eingesetzt Flüssigkeits-chromatographie—Massenspektrometrie (LC—MS) [12, 13] und am häufigsten on-line GC—MS [14—16]. Auch HPLC kombiniert mit Radioimmunoassay wurde eingesetzt [17].

Unser Problem war, den Haschischnachweis so zu führen, dass er schnell durchgeführt und in die Laborroutine eingebaut werden konnte. Ausserdem sollten alle drei Hauptinhaltsstoffe sowie die THC-COOH gleichzeitig und sicher nachgewiesen werden. Wir haben uns entschieden, als Vortest das Emit-st-System® einzusetzen. Zur Absicherung und zur quantitativen THC-Bestimmung verwenden wir eine GC—MS-Kopplung und Multiple-Ion-Detektion (MID). Mit dem beschriebenen Verfahren können schnell eindeutig abgesicherte Befunde erhalten werden, ohne dass eine spezielle Aufarbeitung notwendig ist.

EXPERIMENTELLER TEIL

Geräte und Gerätebedingungen

(a) Emit-st-Photometer und Emit-st Reagenzien Cannabinoide (Syva-Merck, Darmstadt, B.R.D.).

(b) GC-MS-System 5985 (Hewlett-Packard). Quarzkapillarsäule 25 m OV-1 (Hewlett-Packard); 0.3 mm I.D. Ofen: Temperaturprogramm 150°C 1 min, dann 20°C pro min bis 280°C. Einspritzblock: 250°C. Splitlose Injection. Träger-gas: Helium, Vordruck 0.7 bar, Ionenquellentemperatur 200°C; GC—MS-Inter-face-Temperatur: 280°C; SEV-Spannung: altersabhängig 1600—3000 V.

Zeitprogramm für MID: CBD 4.4—4.8 min, Massen: 390.2, 351.2, 337.2 und 301.2; THC 4.8—5.0 min, Massen: 386.2, 371.3, 315.2 und 303.2; CBN 5.0—5.3 min, Massen: 382.3 und 367.3; THC-COOH 6.3—6.6 min, Massen: 488.4, 473.4, 398.3 und 371.3. Material: Blut- bzw. Serumproben und Urinproben von Personen, bei denen der Verdacht bestand, dass Haschisch eingenommen wurde.

Aufarbeitung

Emit-st. Für die immunologische Bestimmung wird der Urin nach pH-Kontrolle (pH 5—7) direkt eingesetzt.

GC—MS Urin. Für die qualitative Analyse von THC, CBN, CBD und THC-COOH im Urin werden je nach vorhandener Menge 5—20 ml Urin soweit nötig mit dest. Wasser auf 20 ml aufgefüllt und dann mit 1.5 ml konz. NH₃ alkalisiert. Diese Lösung wird auf eine Extraktionssäule Extrelut® (Merck, Darmstadt, B.R.D.) gegeben. Zur Glucuronidspaltung werden 5—18 ml Urin

soweit nötig mit dest. Wasser auf 18 ml aufgefüllt und mit 2 ml konz. HCl 20 min am Rückfluss gekocht. Anschliessend wird mit 3 ml konz. NH_3 alkalisiert und diese Lösung auf eine Extraktionssäule Extrelut gegeben.

Beide Säulen werden mit 40 ml Dichlormethan—Isopropanol (85:15) eluiert und die organischen Phasen eingedampft [18]. Die Rückstände werden dann getrennt analysiert.

GC—MS Blut bzw. Serum. Zu 1—3 ml Blut oder Serum werden 2 ml ges. Ammoniumsulfatlösung gegeben. Diese Mischung wird in einem Kunststoffzentrifugenröhrchen mit 2 ml Hexan geschüttelt. Nach Zentrifugation wird die Hexanphase abpipettiert, mit Na_2SO_4 getrocknet und eingedampft.

Silylierung

Zur GC—MS-Untersuchung werden die eingedampften Rückstände in Eppendorf-Reaktionsgefässen silyliert mit 50 μl Silylierungsmittel [15% N-Methyl-N-trimethylsilyl-trifluoracetamid (MSTFA, Macherey-Nagel) in Pyridin, dem noch einige Tropfen Chlorsilan zugesetzt sind]. Die Lösung wird im geschlossenen Gefäss 10 min bei 60°C gehalten. Aus dieser Lösung werden 3 μl eingespritzt.

ERGEBNISSE UND DISKUSSION

Emit-Verfahren

Bei Verdacht einer Einnahme von Haschisch werden die Urinproben zunächst mit dem Emit-st-System geprüft. Negative Proben werden ausgeschieden, positive Proben werden mit GC—MS abgesichert. Mit dem Emit-st-System können nur Einzelproben untersucht werden und eine Automatisierung ist nicht möglich. Der Zeitaufwand pro Probe beträgt 3—5 min. Der Vorteil des Systems liegt darin, dass die Reagenzien gefriergetrocknet und deshalb bis zu einem Jahr haltbar sind.

Als Erfassungsgrenze für den Emit-st-Test sind 200 ng THC-COOH pro ml Urin angegeben. Welche Menge THC eingenommen werden muss, um diesen Spiegel zu erreichen, ist uns nicht bekannt. Nach unseren Erfahrungen reicht die Erfassungsgrenze aus bei missbräuchlicher Einnahme und bei solchen Fällen, wo nach Einnahme von Haschisch Ausfallserscheinungen festgestellt werden, z.B. Verkehrsdelikten. Zur Erfassung geringerer Konzentrationen müsste der Extraktionsrückstand untersucht werden.

Das Verfahren ist eingestellt auf THC-COOH, es werden jedoch über Kreuzreaktionen auch andere Metaboliten und die Hauptinhaltsstoffe von Haschisch erfasst.

Nach Angaben von Syva-Merck beträgt die Empfindlichkeit für CBN und für die hydroxilierten Metaboliten von THC etwa die Hälfte, für THC und CBD etwa ein Viertel gegenüber THC-COOH.

GC—MS

In der angegebenen Literatur wird der Nachweis von CBN, CBD und THC direkt oder nach Trimethylsilylierung, der Nachweis von THC-COOH nach Silylierung, nach Methylierung und anschliessender Trimethylsilylierung oder nach Umsetzung mit Heptafluorbuttersäureanhydrid (HFBA) durchgeführt. Wir haben uns für die Trimethylsilylierung entschieden, weil damit alle vier
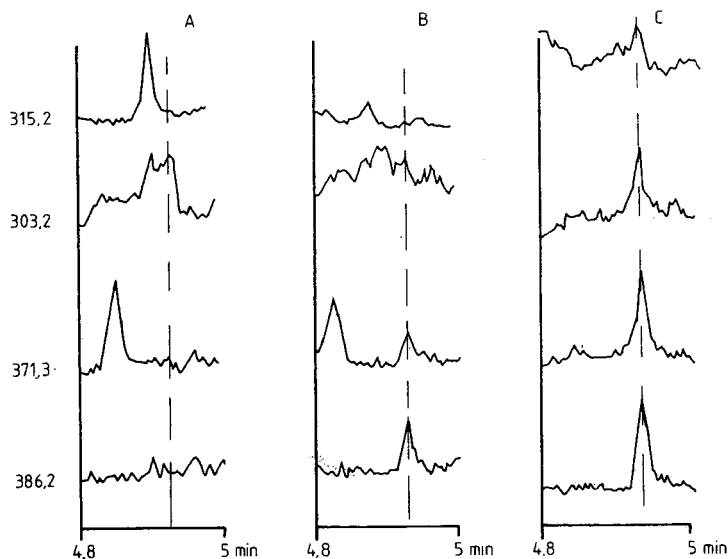
Fig. 1. Massenspuren von silyliertem THC. A, Negative Serumprobe; B, 8 ng/ml im Serum; C, Reinsubstanz THC 10 ng/ml.
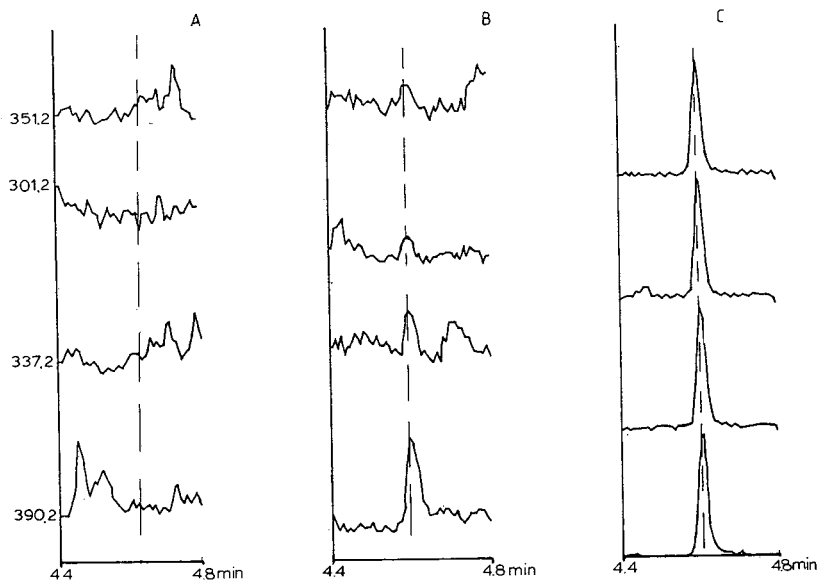
Fig. 2. Massenspuren von silyliertem CBD in Urin. A, Negative Probe; B, schwacher Befund; C, starker Befund. Die Bestimmungen wurden nur qualitativ durchgeführt.

Substanzen gleichzeitig und empfindlich erfasst werden. Die Umsetzung mit HFBA haben wir ebenfalls durchgeführt. Dieses Verfahren liefert zwar höhere Massen, das überschüssige HFBA verringert jedoch innerhalb kurzer Zeit die Trenneigenschaften der Säule. Zur Schonung der Säule musste deshalb ein zusätzlicher Aufarbeitungsschritt durchgeführt werden, was das Verfahren
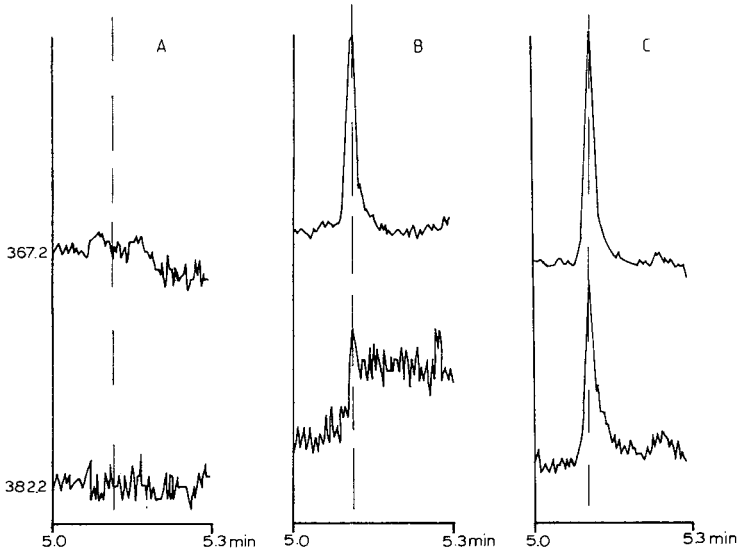
Fig. 3. Massenspuren von silyliertem CBN im Urin. A, Negative Probe; B, schwacher Befund; C, starker Befund. Die Bestimmungen wurden nur qualitativ durchgeführt.
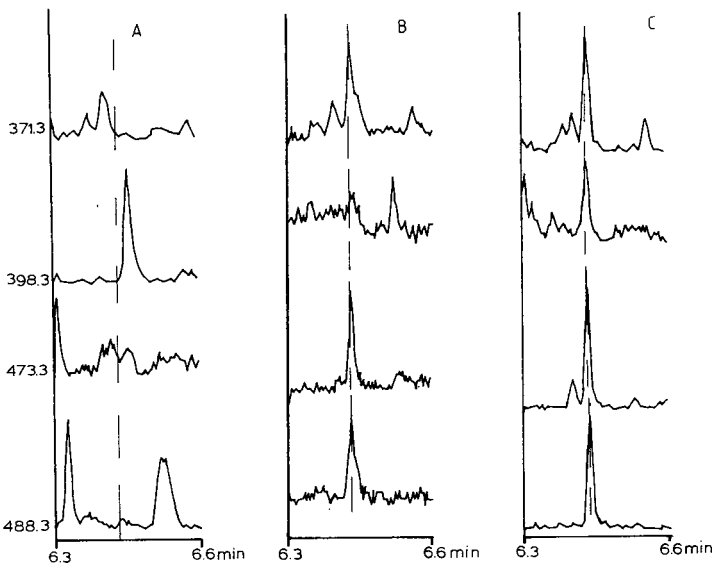
Fig. 4. Massenspuren von silyliertem THC-COOH im Urin. A, Negative Probe; B, schwacher Befund; C, starker Befund. Die Bestimmungen wurden nur qualitativ durchgeführt.

wieder aufwendiger machte. Es war deshalb für unsere Routineuntersuchungen nicht geeignet.

Die spezifischen Massen für die Trimethylsilylverbindung und die relativen Intensitäten sind in Lit. 19 angegeben; für CBN, das in dieser Liste fehlt, lauten sie 367 (100) und 382 (12). Fig. 1—4 zeigen die Massenspuren der Trimethylsilylverbindungen.

Um abgesicherte Befunde zu erhalten, die auch für forensische Zwecke Verwendung finden können, ist es notwendig, auf mehrere Massen einer Verbindung zu prüfen. Neben der Retentionszeit und der spezifischen Masse kann dann für die Identifizierung zusätzlich noch das Verhältnis der Massen zueinander verwendet werden.

In Tabelle I sind die Kovats-Indices der silylierten Substanzen angegeben, da die zur Bestimmung der Retentionszeit nötigen Reinsubstanzen nicht immer zur Verfügung stehen.

TABELLE I

KOVATS-INDICES DER SILYLIERTEN VERBINDUNGEN IM TEMPERATUR-PROGRAMM

Verbindung	Kovats-Index
CBD	2272
THC	2361
CBN	2445
THC-COOH	2762

Bei der Untersuchung der Urinproben auf THC-COOH konnten wir bisher ca. 90% der positiven Emit-st-Befunde bestätigen. Wir führen die Untersuchung durch sowohl von direkt extrahiertem Urin als auch von Urin nach Hydrolyse. Nach unseren Erfahrungen findet man manchmal die Hauptmenge von THC-COOH im direkten Extrakt, manchmal im Extrakt nach Hydrolyse, vermutlich abhängig von zeitlichen Abstand zwischen Einnahme und Probeentnahme. Da bei der direkten Extraktion wesentlich weniger Störpeaks auftreten als nach Hydrolyse, machen wir beide Untersuchungen parallel. Ausserdem kann in diesen Extrakten dann noch auf weitere Suchtmittel geprüft werden, z.B. auf Cocain oder Tilidin im direkten Extrakt und auf Morphin im hydrolysierten Extrakt [20]. Aus diesen Gründen extrahieren wir auch nicht im Sauren. Die Ausbeute von THC-COOH bei der alkalischen Extraktion ist ohne weiteres ausreichend und so auch in der Literatur beschrieben [2]. Stellvertretend für die verschiedenen Substanzen wurde die Wiederfindungsrate für THC-COOH im Urin bestimmt, da es sich dabei auch um das Hauptausscheidungsprodukt handelt. Sowohl im direkten Extrakt als auch nach Hydrolyse betrug die Wiederfindung 70—75%, so dass durch die Hydrolyse keine Verluste auftraten. Die Erfassungsgrenze betrug für alle Substanzen 5—10 ng/ml.

Bei den für Urin positiven Emit-Befunden, die wir mit GC—MS absichern konnten, fanden wir zusätzlich in ca. 50% aller Fälle CBN, zu einem geringeren Prozentsatz CBD und vereinzelt THC.

Im Blut oder Serum untersuchen wir quantitativ auf THC. Die Bestimmung erfolgt durch externe Standardisierung über Zusatzversuche. Die Erfassungsgrenze beträgt ca. 5 ng/ml, die Wiederfindungsrate ca. 40%. Es ist somit möglich, die Einnahme von Haschisch im Blut trotz der schnell abfallenden Spiegel mehrere Stunden lang nachzuweisen [4,5,12]. Die Ergebnisse einiger Fälle sind in Tabelle II aufgeführt. Bei Fall A und B handelt es sich um einen

TABELLE II

QUANTITATIVE THC-BESTIMMUNGEN IN BLUT/SERUM

	A Blut	B Blut	C Serum	D Serum
THC (ng/ml)	21	4	5	1. 103 2. 4

Mordfall, wobei A der Täter und B das Opfer waren. In der Wohnung waren Haschutensilien gefunden worden. Bei Fall C handelt es sich um einen Verkehrsdelikt, wo aufgrund des positiven Urinbefundes die Spiegel-bestimmung durchgeführt worden war. Weitere Einzelheiten zu diesen Fällen waren nicht bekannt. Besonders interessant ist Fall D. Hier hat ein junger Mann beim Eintreffen der Polizei angeblich ca. 15 g gepressten Haschisch verschluckt. Er musste wegen entsprechender Symptome im Krankenhaus behandelt werden. Die erste Blutprobe wurde ca. 4 h nach Einnahme, die zweite ca. 16 h nach Einnahme entnommen. Der erste Wert von 103 ng/ml ist stark erhöht gegenüber dem Maximalspiegel von ca. 40 ng/ml, der nach dem Rauchen einer Marihuana-Zigarette 5—10 min nach Rauchbeginn erreicht wird [4]. Dass grössere Mengen von Haschisch eingenommen worden sein müssen, zeigt auch Fig. 5, wo im Serum neben THC noch CBD, CBN und THC-COOH deutlich nachweisbar waren. Die Abbildung zeigt die Summe der Einzelionen für jede Verbindung, die mit einem systemeigenen Rechnerprogramm aufsummiert wurden. Ob es sich bei den zusätzlichen Peaks um weitere Haschischabbau-
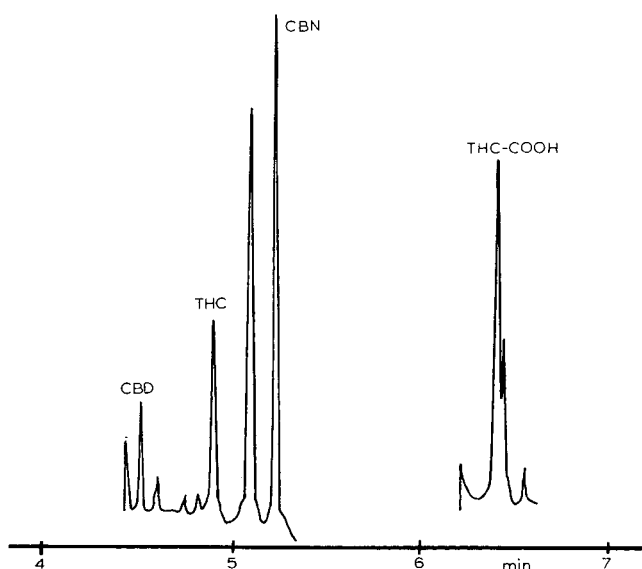
Fig. 5. Nachweis von silyliertem THC, CBN, CBD und THC-COOH im Serum nach Haschisch-vergiftung (Fall D). Die Darstellung erfolgt als Summe der Einzelionen.

produkte oder um sonstige Störsubstanzen gehandelt hat, konnte nich geklärt werden. Eine Zuordnung über die spezifischen Massen anderer Haschischabbauprodukte, die in Lit. 19 angegeben sind, war nicht möglich.

ZUSAMMENFASSUNG

Zum Nachweis einer Einnahme von Haschisch wird in Urin und Blut bzw. Serum auf die Hauptinhaltsstoffe Tetrahydrocannabinol (THC), Cannabidiol und Cannabinol sowie auf das Abbauprodukt von THC, die THC-Carbonsäure, geprüft. Als Vortest wird für den Urin das Emit-st-System verwendet, die Absicherung im Urin und die quantitative THC-Bestimmung im Blut/Serum erfolgt nach Extraktion und Silylierung mit GC—MS mit Einzelionendetektion.

LITERATUR

1 W. Forth, D. Henschler und W. Rummel (Herausgeber), Allgemeine und spezielle Pharmakologie und Toxikologie, Bibliographisches Institut Mannheim, Wien, Zürich, 1975.
2 M.M. Halldin, S. Carlsson, S.L. Kanter, M. Widmann und S. Augurell, Arzneim.-Forsch., 32 (1982) 764—768.
3 M.N. Halldin, L.K.R. Anderson, M. Widmann und L.E. Hollister, Arzneim.-Forsch., 32 (1982) 1135—1138.
4 S.M. Owens, A.J. McBay, H.M. Reisner und M. Perez-Reyes, Clin. Chem., 27 (1981) 619—624.
5 S.J. Gross und J.R. Soares, J. Anal. Toxicol., 2 (1978) 98—100.
6 H.W. Peel und B.J. Perrigo, J. Anal. Toxicol., 5 (1981) 165—167.
7 B. Riesselmann, Dtsch. Apoth. Ztg., 121 (1981) 2078—2082.
8 DFG, Senatskommission für klinisch—toxikologische Analytik, J. Clin. Chem. Clin. Biochem., 20 (1982) 695—696.
9 N.K. McCallum und S.M. Shaw, J. Anal. Toxicol., 5 (1981) 148—149.
10 H.K. Borys und R. Karler, J. Chromatogr., 205 (1981) 303—323.
11 J.A. Winson, D.D. Patel und A.H. Patel, Anal. Chem., 49 (1977) 163—165.
12 J.L. Valentine, P.J. Bryant, P.L. Gutshal, O.H.M. Gan, P.D. Lovegreen, E.D. Thompson und H.C. Niu, J. Pharm. Sci., 66 (1977) 1263—1266.
13 T. Daldrup, U. Matthiesen und T. Krätzschmar, in W. Arnold und K. Püschel (Herausgeber), Symposiumsband Entwicklung und Fortschritte der Forensischen Chemie, Hamburg, 19.—20. März 1982, Verlag Dr. Dieter Helm, Heppenheim, 1982, S. 246—253.
14 J.D. Whiting und W.M. Manders, J. Anal. Toxicol., 6 (1982) 49—52.
15 E.W. Bachmann, A.E. Hofmann und P.G. Waser, J. Chromatogr., 178 (1979) 320—323.
16 D. Rosenthal und D. Brine, J. Forensic Sci., 24 (1979) 282—290.
17 P.L. Williams, A.C. Moffat und L.J. King, J. Chromatogr., 186 (1979) 595—603.
18 J. Breiter, R. Helger und H. Lang, J. Forensic Sci., 7 (1976) 131—135.
19 W. Arnold, in E. Graf und F.R. Preuss (Herausgeber), Gadamers Lehrbuch der chemischen Toxikologie, Band I/2, Verlag Vandenhoeck und Ruprecht, Göttingen, 1979, S. 205—254.
20 K. Harzer und M. Kächele, Beitr. Gerichtl. Med., 37 (1979) 357—361.

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DETERMINATION OF N-MONONITROSOPIPERAZINE AND N,N'-DINITROSOPIPERAZINE IN HUMAN URINE, GASTRIC JUICE AND BLOOD

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SUMMARY

A rapid, selective and sensitive method for the determination of N-mononitrosopiperazine and N,N'-dinitrosopiperazine in human urine, gastric juice and blood has been developed using gas—liquid chromatography with thermal energy analysis. The compounds were isolated by extraction and Extrelut® with dichloromethane or chloroform. The detection limits were between 0.2 and 2 ng/ml.

INTRODUCTION

Piperazine is a widely used anthelmintic drug. It is rapidly nitrosated under in vitro conditions in gastric juice and human saliva to yield N-mononitroso-piperazine (MNPZ) and N,N'-dinitrosopiperazine (DNPZ) [1—5]. In vivo nitrosation of ingested piperazine with nitrite has also been reported [5—7]. Recently we found MNPZ in human gastric juice and urine after ingestion of piperazine alone [8]. DNPZ, and possibly MNPZ, are carcinogenic to laboratory animals [9—11].

Several methods have been developed for the determination of MNPZ and DNPZ in biological fluids. Some of these methods, based on spectro-photometric [4, 12] and thin-layer chromatographic [3] procedures, are only semi-quantitative with a low sensitivity. Gas—liquid chromatographic

(GLC) methods, using nitrogen-specific flame ionisation detection [6, 7], have a higher sensitivity and selectivity. Recently, a high-performance liquid chromatographic method has been reported [5].

However, these methods are not sufficiently sensitive or specific for analysis of low levels of MNPZ and DNPZ. This report describes a rapid, selective and sensitive method for the determination of MNPZ and DNPZ in human urine, gastric juice and blood, using a gas—liquid chromatograph interfaced with a thermal energy analyzer (TEA) detector. The TEA detector exhibits extreme sensitivity and high specificity to N-nitrosamines [13].

EXPERIMENTAL

Reagents

Dichloromethane, chloroform, 2,2,4-trimethylpentane and methanol (all analytical grade) were obtained from E. Merck (Darmstadt, F.R.G.) and were used without further purification. Water was deionized. Kieselguhr (Extrelut®, Merck) was dried overnight at 200°C prior to use and stored at the same temperature; prepacked Extrelut columns were also used. Piperazine syrup (48 mg/ml) was obtained from ACO Läkemedel (Solna, Sweden). All other chemicals were reagent grade or better. All the reagents, organic solvents and deionized water were checked to ensure absence of interfering substances. Trace amounts of MNPZ (3—20 µg/g piperazine) were found in the piperazine syrup.

Standards

A stock solution of MNPZ (100 µg/ml) in 2,2,4-trimethylpentane was obtained from Thermo Electron (Waltham, MA, U.S.A.). A stock solution of DNPZ (100 µg/ml) was prepared by dissolving DNPZ in 2-propanol.

For GLC calibration, working standard solutions (400 ng/ml) of MNPZ in methanol—water (95:5, v/v), and of DNPZ in methanol were prepared. For recovery experiments standard solutions of MNPZ in water or methanol—water (95:5, v/v), and of DNPZ in methanol were prepared.

Stock solutions of MNPZ and of DNPZ were stored at about —15°C, and standard solutions and working solutions of MNPZ and DNPZ at about 4°C.

Apparatus

A Varian 2700 (Palo Alto, CA, U.S.A.) gas chromatograph interfaced with a thermal energy analyzer (TEA, Model 502, Thermo Electron) was used. The GLC furnace was removed from the TEA and connected to the GLC column via a 5.5-cm long glass tube. The glass column (1.8 m × 1.9 mm I.D.) was packed with 15% SE-52 on Chromosorb W HP.

Chromatographic conditions

The operating conditions for GLC—TEA were as follows: column temperature 190°C, injector temperature 240°C, helium flow-rate about 38 ml/min, furnace temperature 475°C, oxygen flow-rate about 5 ml/min, vacuum pressure about 1 mmHg, cold trap temperature —150 to —160°C.

Extraction procedures

Urine. One millilitre of 2 *M* sodium hydroxide was added to 20 ml of urine. If the sample was not worked up at once, 1 ml of sodium sulphamate solution (4 g of sulphamic acid and 3 g of sodium hydroxide per 100 ml of water) was added as a nitrite trap, and 2 ml of 2 *M* sodium hydroxide were added prior to extraction. The mixture was poured on to an Extrelut column (23 cm × 2 cm I.D.), and after 10—15 min the nitrosamines were eluted with 100 ml of dichloromethane. The eluate was evaporated to dryness, and the residue was dissolved in 0.5—1 ml of methanol—water (95:5, v/v). The volume was measured with a 1000-μl Hamilton syringe, and 5-μl aliquots were injected into the gas chromatograph.

Gastric juice. One millilitre of 2 *M* sodium hydroxide was added to 10 ml of gastric juice. The sample was immediately put on a prepacked Extrelut column or a 23-cm long Extrelut column followed by 10 ml of water and analyzed as described above for urine.

Blood. Five millilitres of the blood sample were treated with 15 ml of 0.025% ammonium hydroxide for 10—15 min. The mixture was added to an Extrelut column and the nitrosamines were eluted with 100 ml of chloroform. The eluate was concentrated and analyzed as outlined above.

Recovery studies

To measure recovery, various concentrations of MNPZ and/or DNPZ were added to each body fluid studied, and the samples were extracted as described above. The recovery was determined by comparing peak areas of MNPZ and DNPZ in the sample with peak areas obtained by direct injection of standard solutions.

Calculations

The nitrosamine concentrations were always calculated by the external standard method, using peak area or peak height ratios. A new standard calibration was performed every day.

Artifact formation

Gastric juice. To 10 ml of gastric juice were added, in the following order, 2 ml of 2 *M* sodium hydroxide, 0.5 g of sulphamic acid or ascorbic acid, 1 ml of diluted piperazine syrup (10 mg/ml) and, finally, 1 ml of sodium nitrite (5 mg/ml). In one experiment, sodium hydroxide was added last instead of first to give a pH 12—14. Samples without sulphamic or ascorbic acid were also analyzed for artifact formation. The samples were extracted immediately or after five days' storage at about 5°C, and analyzed as described above.

Urine. To 10 ml of urine containing piperazine (3 mg) and nitrite (22 μg) were added 0.1—2.0 ml of 4% sulphamic acid, 0.1—2.0 ml of sodium sulphamate (4 g of sulphamic acid and 3 g of sodium hydroxide per 100 ml of water), or 200—2000 mg of urea. These samples, and samples without nitrosation inhibitors, were extracted after storing for one day at 5°C and analyzed as outlined above.

RESULTS AND DISCUSSION

Extraction

The use of Extrelut for extraction gave better results than those obtained with earlier described extraction methods [2—7]. Fig. 1 shows a chromatogram of an extract of a gastric juice sample spiked with 28.8 ng/ml MNPZ and 4.8 ng/ml DNPZ. The overall analytical recovery for DNPZ (8—32 ng/ml) was in the range of 80—96% in all media (Table I), while for MNPZ (8—32 ng/ml) the recovery varied between 51 and 78%. The coefficients of variation within the range of levels tested varied from 3.6 to 23.7% for MNPZ and from 0.97 to 8.7% for DNPZ. The poor recovery of MNPZ was probably due to adsorption losses on the Extrelut column.

Artifact formation

When performing analysis of N-nitroso compounds in the ppb range, it is necessary to prevent artifact formation during the analysis [14]. Earlier studies [3, 12, 15] have shown that ascorbic acid, glutathione, ammonium
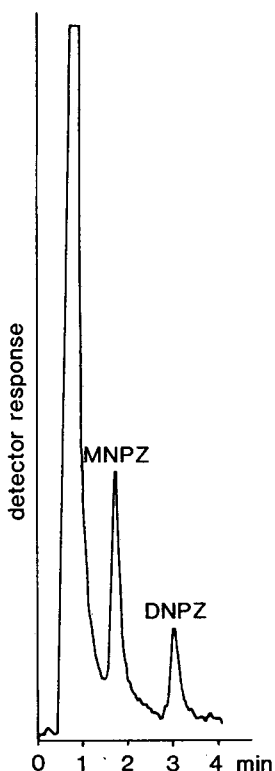
Fig. 1. Chromatogram of an extract of gastric juice spiked with 28.8 ng/ml MNPZ and 4.8 ng/ml DNPZ. ·

TABLE I

RECOVERY OF MNPZ AND DNPZ FROM URINE, GASTRIC JUICE AND BLOOD

Body fluid	Amount added (ng/ml)	No. of determinations		Recovery (%, mean ± S.D.)		Coefficient of variation (%)	
		MNPZ	DNPZ	MNPZ	DNPZ	MNPZ	DNPZ
Urine	8	4	4	63 ± 6.1	94 ± 6.8	9.7	7.2
	16	4	4	74 ± 2.7	93 ± 1.9	3.6	2.0
	32	4	4	75 ± 5.8	96 ± 4.6	7.8	4.8
Gastric juice	8	4	4	66 ± 5.4	92 ± 4.0	8.2	4.4
	16	4	4	73 ± 10.9	84 ± 5.2	15.0	6.2
	32	4	4	78 ± 3.2	80 ± 5.7	4.2	7.1
Blood	8	4	4	52 ± 12.2	88 ± 7.7	23.7	8.7
	16	4	4	51 ± 8.8	85 ± 3.6	17.1	4.2
	32	4	4	63 ± 3.9	86 ± 0.83	6.1	0.97

TABLE II

ARTIFACT FORMATION OF MNPZ AND DNPZ IN ANALYSIS OF GASTRIC JUICE

To 10 ml of gastric juice were added 2 ml of 2 M sodium hydroxide, 0.5 g of sulphamic or ascorbic acid (as nitrosation inhibitor), 1 ml of diluted piperazine syrup (10 mg/ml), and 1 ml of sodium nitrite (5 mg/ml). The samples were extracted immediately or after five days, and analyzed as described in the Experimental section. ND = not detected (< 0.005 μg); tr = trace (0.005—0.015 μg).

Method	Nitrite inhibitor	Time before extraction (days)	Nitrosamine detected* (μg)	
			MNPZ	DNPZ
Sodium hydroxide added first	None	0	tr	ND
		5	tr	ND
	Sulphamic acid	0	tr	0.023
		5	tr	0.046
	Ascorbic acid	0	tr	ND
		5	tr	ND
Sodium hydroxide added last	None	0	3590	715
	Sulphamic acid	0	1.4	ND
	Ascorbic acid	0	1.3	ND

*Not corrected for recovery.

sulphamate and urea inhibit the formation of MNPZ. However, the inhibition is sometimes only partial, depending on the pH. In the analysis of gastric juice, the samples must immediately be made alkaline to block artifact formation (Table II). The presence of sulphamic acid resulted in the formation of measurable amounts of DNPZ in the analysis of gastric juice spiked with piperazine and nitrite. Earlier, ascorbic acid has been reported to increase

TABLE III

ARTIFACT FORMATION OF MNPZ AND DNPZ IN ANALYSIS OF URINE

To 10 ml of urine containing piperazine (3 mg) and sodium nitrite (22 μg) were added sulphamic acid, sodium sulphamate or urea (as nitrosation inhibitor). The samples were extracted and analyzed after one day as described in the Experimental part. ND = not detected (< 0.005 μg); tr = trace (0.005—0.015 μg).

Nitrosation inhibitor	Amount of inhibitor added		Nitrosamine detected* (μg)	
	ml	g	MNPZ	DNPZ
Sulphamic acid (4%)	0	—	tr	ND
	0.1	—	0.020	ND
	0.2	—	0.025	ND
	0.5	—	0.114	ND
	1.0	—	0.273	ND
	2.0	—	0.017	ND
Sodium sulphamate (4%)	0	—	tr	ND
	0.1	—	tr	ND
	0.2	—	tr	ND
	0.5	—	tr	ND
	1.0	—	ND	ND
	2.0	—	tr	ND
Urea	—	0	0.073	ND
	—	0.4	0.065	ND
	—	2.0	0.051	ND

*Not corrected for recovery.

the formation of DNPZ [3]. In the analysis of urine, the nitrosation was effectively blocked by sodium sulphamate (Table III), while sulphamic acid increased the formation of MNPZ. The reason for this is probably the pH change on the addition of sulphamic acid.

In the analysis of blood, an unidentified NO-positive peak occasionally appeared in the chromatogram (Fig. 2). An enhancement of this signal always reduced the MNPZ signal and the recovery of MNPZ. With a high unidentified peak, the recovery of MNPZ was decreased to 6—7%. The DNPZ level was not affected by this unknown peak.

Standards

It has earlier been reported that DNPZ may be unstable if stored in dichloromethane for more than two to three days [3]. We found no decomposition of DNPZ in the solvents used: 2,2,4-trimethylpentane, 2-propanol, dichloromethane, chloroform, methanol, and methanol—water (95:5, v/v). On the other hand, MNPZ was unstable in methanol, but stable in methanol—water (95:5, v/v), water, 2,2,4-trimethylpentane, dichloromethane, and chloroform. In methanol an additional peak with a slightly longer retention time than MNPZ could be observed after storage.

Standard solutions of MNPZ and DNPZ contained two unidentified NO-
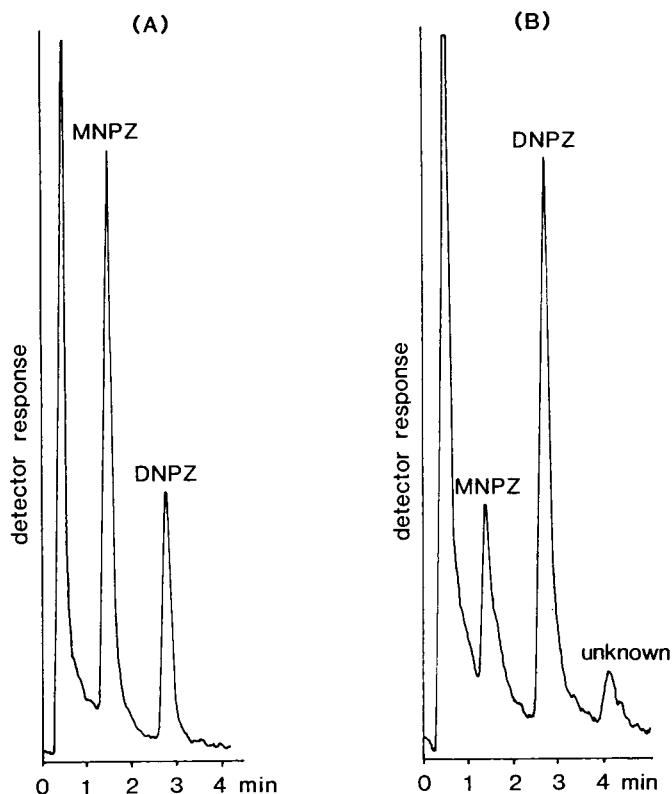
Fig. 2. Chromatograms of extract of blood: (A) spiked with 108 ng/ml MNPZ and 18.0 ng/ml DNPZ; and (B) spiked with 40.0 ng/ml MNPZ and 46.7 ng/ml DNPZ.

positive peaks with retention times about 5 sec longer than MNPZ and DNPZ. These unknown impurities are, in contrast to MNPZ and DNPZ, stable upon ultraviolet radiation.

Detection

In our GLC—TEA system the furnace has been removed from the TEA instrument and connected directly to the GLC column. The short distance between the end of the column and the furnace contributes to the high sensitivity in the analysis of MNPZ and DNPZ.

For GLC calibration and determination of MNPZ, we found that methanol—water (95:5, v/v) was the most suitable solvent. The use of 2,2,4-trimethylpentane gave poor reproducibility at low concentrations, due to partial loss of MNPZ in the syringe.

Good linearity was obtained in the relationship between peak areas and concentrations of MNPZ and DNPZ in standard solutions in the range 5.0—400 ng/ml (Fig. 3). The coefficient of variation was 4.8% for MNPZ (n = 10) and 4.6% for DNPZ (n = 10) at a concentration of 400 ng/ml. To obtain a high sensitivity and a good precision in the GLC calibration and determination, the GLC column was activated by repeated injections of blank
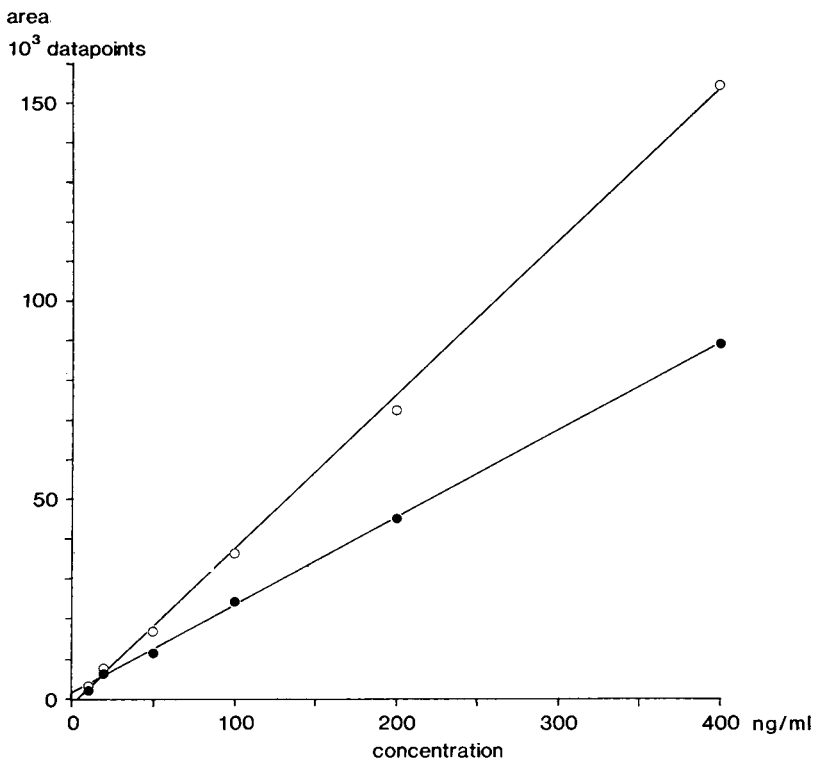
Fig. 3. Standard curves of MNPZ (○) and DNPZ (●) constructed by plotting the peak areas against known concentrations of MNPZ and DNPZ in the range 5.0—400 ng/ml.

urine samples taken through the extraction procedures. One or two 5-μl injections of methanol—water (95:5, v/v) were sometimes required to eliminate memory effects after injections of large amounts of MNPZ and/or DNPZ.

The minimum detectable concentrations of MNPZ and DNPZ in urine were 0.2 and 0.3 ng/ml, in gastric juice 0.4 and 0.5 ng/ml, and in blood 2.0 and 2.0 ng/ml. The signal-to-noise ratio was at least 3:1 for both MNPZ and DNPZ at these concentrations.

Application

The technique was applied to study the in vivo nitrosation of piperazine in the stomach of man [8, 16]. Piperazine syrup was given to fasting volunteers. Urine, gastric juice and blood samples were taken at different times relative to the intake of piperazine. Fig. 4. shows a chromatogram of an extract of a urine sample containing 3.7 ng/ml MNPZ from a volunteer 4 h after the intake of 0.51 g of piperazine and 2.0 g of ascorbic acid. Routine recoveries of MNPZ and DNPZ from urine, gastric juice, and blood in the in vivo nitrosation study, covering a period of about one year, are shown in Table IV. The gastric juice samples were worked up immediately after sampling, and then mailed from Lund to Uppsala for analysis (24 h). The urine (containing sodium sulphamate solution) and blood samples were sent to Uppsala, where the samples were extracted and analyzed.
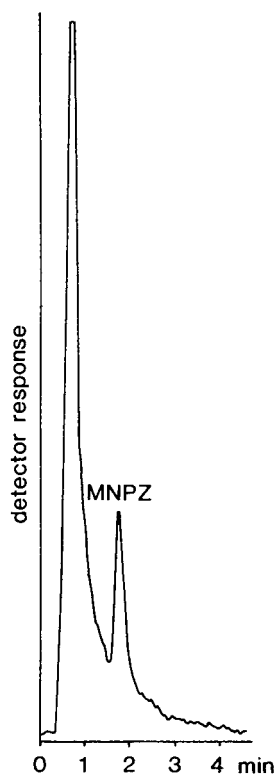
Fig. 4. Chromatogram of an extract of 20 ml of urine containing 3.7 ng/ml MNPZ from a volunteer 4 h after intake of 0.51 g of piperazine and 2.0 g of ascorbic acid.

TABLE IV

EXTRACTION YIELD OF MNPZ AND DNPZ FROM AN APPLICATION OF THE METHOD COVERING A PERIOD OF ABOUT ONE YEAR

Medium	Concentration (ng/ml)	No. of determinations		Recovery (%, mean ± S.D.)	
		MNPZ	DNPZ	MNPZ	DNPZ
Urine	2— 14	17	16	84 ± 12	90 ± 12
	20— 140	22	14	84 ± 16	102 ± 12
	200—1400	4	4	84 ± 8	99 ± 10
Gastric juice	2— 14	4	12	72 ± 17	89 ± 9
	20— 140	6	9	70 ± 16	95 ± 16
	200—1400	7	3	90 ± 10	101 ± 9
Blood	2— 14	10	6	24 ± 8	42 ± 9
	20— 140	11	16	47 ± 12	100 ± 18

REFERENCES

1 S.S. Mirvish, Toxicol. Appl. Pharmacol., 31 (1975) 325.
2 G.S. Rao and G. Krishna, J. Pharm. Sci., 64 (1975) 1579.
3 N.P. Sen and B. Donaldson, in P. Bogovski and E.A. Walker (Editors), N-Nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 103.
4 D. Ziebarth, in P. Bogovski and E.A. Walker (Editors), N-Nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 137.
5 G.S. Rao and D.A. McLennon, J. Anal. Toxicol., 1 (1977) 43.
6 J. Sander, F. Schweinsberg, M. Ladenstein, H. Benzing and S.H. Wahl, Hoppe-Seyler's Z. Physiol. Chem., 354 (1973) 384.
7 J. Sander, J. LaBar, M. Ladenstein and F. Schweinsberg, in P. Bogovski and E.A. Walker (Editors), N-Nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 123.
8 B.T.D. Bellander, L.E. Hagmar and B.-G. Österdahl, Lancet, ii (1981) 372.
9 H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, Z. Krebsforsch., 69 (1967) 103.
10 W. Lijinsky and H.W. Taylor, Cancer Res., 35 (1975) 1270.
11 H. Garcia, L. Keefer, W. Lijinski and C.E.M. Wenyon, Z. Krebsforsch., 74 (1970) 179.
12 D. Ziebarth, Arch. Geschwulstforsch., 51 (1981) 587.
13 D.H. Fine and D.P. Rounbehler, J. Chromatogr., 109 (1975) 271.
14 I.S. Krull, T.Y. Fan and D.H. Fine, Anal. Chem., 50 (1978) 698.
15 S.S. Mirvish, L. Wallcave, M. Eagen and P. Shubik, Science, 177 (1972) 65.
16 B.T.D. Bellander, L.E. Hagmar and B.-G. Österdahl, 8th Int. Meeting on N-Nitroso Compounds: Occurrence and Biological Effects, Banff, Alberta, September 4—9, 1983, IARC, Lyon, in press.

CHROMBIO. 1843

ANALYSIS OF FILTER-PAPER-ABSORBED, FINGER-STICK BLOOD SAMPLES FOR CHLOROQUINE AND ITS MAJOR METABOLITE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

Methodology has been developed to facilitate the collection, transport, and analysis of blood samples in studies of chloroquine absorption and metabolism. The method utilizes high-performance liquid chromatography (HPLC) with fluorescence detection to quantify chloroquine and its major metabolite, desethylchloroquine, in 100-μl quantities of blood collected on filter paper. Detection limits are 5 ng/ml for both analytes. No loss of either analyte occurred from filter-paper-collected blood spots stored over a twelve-weeks' period at room temperature. Filter-paper-collected, finger-stick blood spots give values for each analyte comparable to corresponding determinations on venous, whole-blood samples. The HPLC mobile phase selected has general applicability to the separation of antimalarial drugs. The methodology permits effective assessment of chloroquine prophylaxis compliance and parasite drug resistance in remote, malaria-endemic regions.

INTRODUCTION

Recent advances in the analysis of chloroquine in body fluids have made possible important investigations into its use as an antimalarial and antirheumatic drug [1]. The most sensitive of the methods have utilized high-performance liquid chromatography (HPLC) with fluorescence detection [2,3]. These methods are also sufficiently selective to quantify both chloroquine and its major metabolite, desethylchloroquine. A major deterrent to the field applicability of chloroquine analysis methods has been the requirement for a volume of fresh whole blood, serum, or plasma. Therefore, an analytic method

was developed to accurately and reproducibly determine the concentration of chloroquine and desethylchloroquine in a small sample of blood collected on filter paper from a finger puncture, such as could be collected under field conditions and transported to the analytical laboratory. HPLC analysis with fluorescence detection was employed, because of its sensitivity. Investigations were performed to demonstrate the storage stability of chloroquine and desethylchloroquine in blood-spot samples and the comparability of analytical results between finger-stick and venipuncture samples.

EXPERIMENTAL*

Standards

Chloroquine (Cq) diphosphate was purchased from Sigma (St. Louis, MO, U.S.A.). Before its use as a standard, this material was dried under vacuum over phosphorus pentoxide and the melting point determined using a Fisher-Johns apparatus, calibrated with Fisher TherMetric standards. The melting-point range was 192.1–193.3°C. Desethylchloroquine (DECq) base was supplied by Sterling-Winthrop Research Institute (Rennselaer, NY, U.S.A.). The internal-standard compound, 7-chloro-4-(1'-methyl-4'-isopropylaminobutylamino)-quinoline was provided by Walter Reed Army Institute of Research (Washington, DC, U.S.A.). This compound is the isopropyl analogue of desethylchloroquine and is designated IPA for brevity. Quinine base was formed from commercially available quinine hydrochloride from Merck, Sharp and Dohme (Rahway, NJ, U.S.A.) by extraction from aqueous base using diethyl ether–hexane (1 : 1). The quinine was crystallized from this solvent pair after partial evaporation followed by cooling to room temperature. Amodiaquine was provided by Parke, Davis and Company (Detroit, MI, U.S.A.) and pyrimethamine was from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.).

Reagents and materials

Spectroscopic quality hexane, methyl-*tert.*-butyl ether (MTBE), and methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were glass-distilled. The diethylamine (Aldrich, Milwaukee, WI, U.S.A.) was distilled before use. Human blood used for standards was freshly collected using acid–citrate–dextrose solution (ACD) as an anticoagulant. All other chemicals used were of reagent grade or better. The filter paper used was rectangular (25 mm X 75 mm) cut from sheets of paper (catalogue number 1023-.038, James River Rochester, Rochester, MI, U.S.A.). The 100-μl pipets were Corning catalog number 70995-100 from Corning Glass Works (Corning, NY, U.S.A.).

Equipment

The HPLC apparatus consisted of two Waters Model M-6000 A solvent delivery systems and a Waters Model 660 solvent programmer, coupled to a Waters

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Model U6K loop injector, a Dupont Zorbax Sil column (5—6 μm particle diameter; 25 cm × 4.6 mm), a Varian Vari-chrom variable-wavelength absorbance detector, and a Perkin-Elmer Model 650S fluorescence detector, connected in series. A Varian Model 9176 dual-pen recorder was used to display both an ultraviolet (UV) absorbance trace and a fluorescence trace. Integration of fluorescence peaks was performed using a Varian CDS-111 integrator.

Analysis of filter-paper-collected blood spots

Standards were prepared by spotting chloroquine-free, ACD-anticoagulated blood onto filter paper, cutting out the dried spots, placing each spot in a 57 × 25 mm I.D. glass vial (screwcaps contained PTFE inserts), and adding 50-μl volumes of chloroquine and desethylchloroquine standard solutions to the filter paper in each vial. Sample blood spots on filter paper were similarly cut out and placed in vials. To each vial were added 50 μl of IPA internal-standard solution (1.02 ng/μl in 0.002 *M* hydrochloric acid) and 3 ml of 0.2 *M* hydrochloric acid. The vials were vortexed and allowed to stand for at least 1 h. (For convenience papers could stand in the vials overnight.) The paper in each vial was then macerated using a clean glass rod. Each vial was vortexed and the contents filtered through a plug of silanized glass wool in a Pasteur pipet. The filtrates were collected in 15-ml conical glass centrifuge tubes.

To each of the filtrates were added 3.0 ml of MTBE—hexane (1:1) and 0.5 ml of 5.0 *M* sodium hydroxide. The centrifuge tubes were placed on a Glas-Col Model RD-250 rotator equipped with a Model RD-60 rotator head (Terre Haute, IN, U.S.A.) that inverted the capped tubes at the rate of 30 inversions per min for a period of 15 min. Centrifugation for 5 min was employed as necessary to break any mild emulsions that occurred. Most of each organic layer was transferred to a second centrifuge tube using a Pasteur pipet. The solvent was evaporated by heating on a water bath combined with a gentle flow of dry nitrogen. The samples and standards were reconstituted in 100-μl quantities of freshly prepared mobile phase, and 30-μl quantities of the resulting solutions were injected into the HPLC.

The mobile phase used was hexane—MTBE—methanol—diethylamine (37.25:37.25:25.0:0.5). The flow-rate was 1.0 ml/min (68 bar). The UV detector was set at 340 nm. The UV signal was monitored on the second pen of the dual-pen recorder to provide a check on the quantification of samples that contained high concentrations of chloroquine. For the fluorescence detector, the excitation wavelength was 320 nm and the emission wavelength 380 nm with each slit width set to 5 nm. Fluorescence peak-area-ratio standard curves were calculated for both chloroquine and desethylchloroquine, and the samples were quantified with reference to these curves.

Collection of volunteer blood samples

A researcher traveling to Africa in connection with ongoing malaria studies underwent the recommended chemoprophylactic regimen with chloroquine diphosphate (300 mg/week as base). The researcher, who weighed 190 pounds, volunteered to provide blood samples by venipuncture (collected in heparinized tubes) at intervals during the first two.weeks of his regimen and for a number of weeks after his return, including several weeks subsequent to the last

administration of chloroquine. Additionally, filter-paper-collected, finger-stick blood samples were taken at the same time as certain venipuncture samples. Studies to evaluate the storage-stability of chloroquine in filter-paper-collected blood spots and the comparability of venipuncture and finger-stick samples utilized certain of the samples from this volunteer.

Storage study and venipuncture/finger-stick comparability study

Two 5-ml quantities of blood from the volunteer who was undergoing chloroquine chemoprophylaxis were used in this study — one taken 24 h, the other 10 days after the 25th and final weekly chloroquine dose. The samples were analyzed initially by a procedure in which a 500-μl quantity of each whole-blood sample was placed in a 15-ml centrifuge tube and diluted with 2 ml of demineralized water. The 3.0 ml of MTBE—hexane (1:1) and 0.5 ml of 5 M sodium hydroxide were added, and the analysis was completed as outlined above for the filter-paper, blood-spot method. Standards were processed in the same manner. (Quantities of Cq, DECq, and IPA standards were five-fold those used with blood spots.)

For the blood-spot storage time study, multiple 100-μl pipet applications of the two whole-blood samples were spotted on each of two sheets of filter paper. These were analyzed in duplicate immediately after spotting and at 1, 4, 8, and 12 weeks thereafter; samples were stored at room temperature.

The paired venipuncture and finger-stick samples were analyzed as outlined above and the values compared.

HPLC separation of a mixture of antimalarials

The HPLC system described above was applied to the separation of a mixture of antimalarials containing amodiaquine, pyrimethamine, chloroquine, and quinine in the following respective concentrations in ng/μl: 156, 150, 82, and 398. A 4-μl quantity of the mixture in mobile phase was injected. The mobile phase was hexane—MTBE—methanol—diethylamine (46:46:7.5:0.5). Detection was by UV absorbance at 254 nm at a sensitivity of 0.2 a.u.f.s.

RESULTS AND DISCUSSION

The structures of chloroquine, desethylchloroquine, and the internal-standard compound are shown in Fig. 1. The internal standard is the isopropyl analogue of desethylchloroquine, designated IPA.

Characterization of the method

The filter paper used in this study and recommended for field collections has been used for many years for collecting of blood samples for serological testing [4]. The paper is available in sheets and is generally cut into rectangles of the same dimension as microscope slides (75 × 25 mm). It is sufficiently thick and absorbent that a 100-μl quantity of blood spreads to form a circle of only about 12 mm. Filter papers should be handled by their edges only, and field personnel should take precautions to avoid contamination of the papers with traces of chloroquine from the fingers of persons who may have handled tablets — i.e., patients or the person taking blood samples.
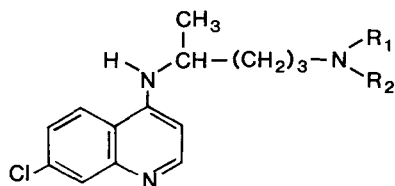
Compound	Abbreviation	R₁	R₂
Chloroquine	Cq	CH_2CH_3	CH_2CH_3
Desethylchloroquine	DECq	CH_2CH_3	H
Internal standard	IPA	$CH(CH_3)_2$	H

Fig. 1. Structures of chloroquine, desethylchloroquine, and the internal-standard compound.

The 100-μl pipets were found to deliver 95 μl of whole blood under the conditions of sampling, i.e., delivery of blood with no subsequent rinsing of the pipet. All blood-spot values were calculated accordingly.

The extraction solvent of MTBE—hexane (1:1) provided a good compromise in polarity, effectively extracting the analytes and internal standard while minimizing the coextraction of potential interfering materials from blood. The use of diethyl ether—hexane mixtures was first explored, but under certain conditions resulted in the transformation of a portion of the chloroquine into a compound giving a peak eluting after chloroquine. The extent of transformation could be minimized by using freshly distilled diethyl ether (lower peroxide levels) and expediting the processing of samples. Since the retention time (t_R) of the transformation product was found to be near that of desethylchloroquine and quite variable depending on methanol concentration and column activity, methyl-*tert.*-butyl ether, with low propensity for peroxide formation, was substituted for diethyl ether. Even though the use of MTBE has eliminated the appearance in the chromatogram of the peak due to the chloroquine transformation product, we now routinely inject a solution containing the product to assure that there is no possibility of its significant formation during sample analysis with subsequent coelution with desethylchloroquine.

A methanol concentration of 25% in the solvent yields a t_R for the transformation product which falls between that of the internal standard and desethylchloroquine. A methanol concentration of 15% results in a t_R for the transformation product which is substantially longer than that for desethylchloroquine. The oxidation product has been identified as the aliphatic amine oxide of chloroquine by independent synthesis, chromatography, and proton nuclear magnetic resonance (NMR) spectrometry [5].

The extraction of the spots using 0.2 *M* hydrochloric acid was found to predispose the samples to the formation of an intractable emulsion when vigorous shaking was used for partitioning from aqueous base into the organic phase. A slow rotation of the samples at 30 inversions per min for a period of 15 min resulted in efficient partitioning and the formation of only a light emulsion which was readily spun down by centrifugation.

Fig. 2 depicts a chromatogram resulting from the analysis of filter-paper
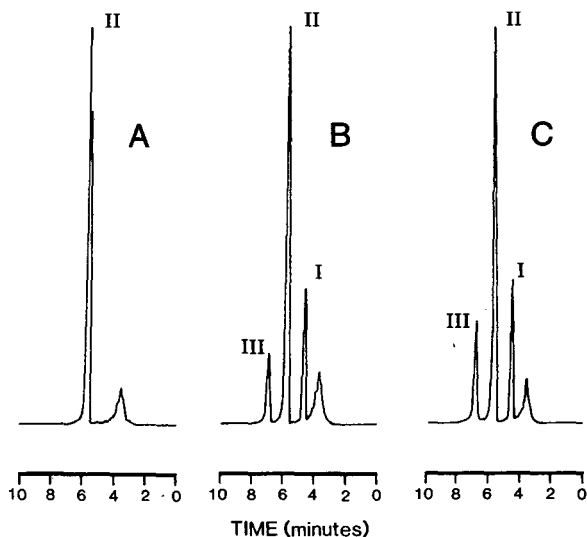
Fig. 2. Chromatograms resulting from the application of the method to: (A) a 100-μl blood-spot blank containing added internal standard alone; (B) a 100-μl blood-spot standard containing 24.6 ng of chloroquine and 12.5 ng of desethylchloroquine; (C) a 100-μl blood-spot sample taken 24 h subsequent to tablet ingestion in the 25th week of a 300-mg/week chemoprophylaxis regimen. Peaks: I = chloroquine, II = internal standard, III = desethylchloroquine.

blood spots. The composition of the mobile phase used was hexane—MTBE—methanol—diethylamine (37.25:37.25:25.0:0.5).

Statistical evaluation of the results of blood-spot filter-paper analysis for both chloroquine and its major metabolite showed that precision was excellent for both analytes (Tables I and II). That for the desethylchloroquine appeared to be somewhat better than for chloroquine, undoubtedly reflecting the former's closer structural similarity to the internal standard. The detection limits is 5 ng/ml for both analytes.

TABLE I

STANDARD-CURVE DATA FOR CHLOROQUINE

Cq added (x, ng)	Cq—internal standard peak-area ratio	Cq calculated* (x', ng)
0.00	0.0000	−0.36
9.85	0.1295	9.94
24.63	0.2986**	23.38
	0.3036	23.78
	0.3150	24.69
	0.3018	23.64
49.25	0.6534	51.60
73.88	0.9169	72.55

*Calculated from the least-squares straight line, $y = mx + b$ ($m = 0.01258$, $b = 0.004516$); $r^2 = 0.9978$; $n = 5$.
**R.S.D. (interstandard, $n = 4$) = 2.35%, R.S.D. (intrastandard, 3 injections) = 1.51%.

TABLE II

STANDARD-CURVE DATA FOR DESETHYLCHLOROQUINE

DECq added (x, ng)	DECq—internal standard peak-area ratio	DECq calculated[*] (x', ng)
0.00	0.0000	0.34
3.12	0.0467	2.83
6.23	0.1043	5.89
12.47	0.2379[**]	13.01
	0.2339	12.80
	0.2323	12.71
	0.2329	12.74
24.93	0.4609	24.89

[*]Calculated from the least-squares straight line, $y = mx + b$ ($m = 0.01878$, $b = -0.006367$); $r^2 = 0.9989$; $n = 5$.
[**]R.S.D. (interstandard, $n = 4$) = 1.07%, R.S.D. (intrastandard, 3 injections) = 0.36%.

To study stability of chloroquine and desethylchloroquine in filter-paper blood spots stored at room temperature, concentrations of both compounds were determined in stored blood samples that had been taken at 24 h and 10 days after administration of the final tablet in a chemoprophylactic regimen. The concentrations of chloroquine and desethylchloroquine at the end of the twelve weeks' period, the longest period tested, appear to be no different from those at the beginning of the storage study (Table III).

TABLE III

STORAGE STABILITY OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN FILTER-PAPER-COLLECTED 100-μl BLOOD SAMPLES FROM A VOLUNTEER UNDERGOING CHEMOPROPHYLAXIS

Sample designation	Time (weeks)	Levels during storage (duplicate spots)			
		Cq (ng/ml)		DECq (ng/ml)	
24 h[*]	Pre-trial analysis[**]	259	267	161	154
	0	277	263	170	173
	1	266	224	139	135
	4	292	286	165	179
	8	267	277	157	149
	12	265	249	170	178
10 days[*]	Pre-trial analysis[**]	65	67	43	45
	0	59	74	44	38
	1	71	63	47	47
	4	57	63	42	47
	8	69	66	42	45
	12	67	65	53	49

[*]The volunteer had been on chemoprophylaxis for 25 weeks. Sample designations refer to the time between administration of the final tablet and sampling.
[**]Determined from 500-μl whole-blood samples processed without absorption on filter paper.

TABLE IV

COMPARISON OF FILTER-PAPER-COLLECTED, FINGER-STICK BLOOD-SPOT VALUES FOR CHLOROQUINE AND DESETHYLCHLOROQUINE WITH THOSE FROM CORRESPONDING VENIPUNCTURE DETERMINATIONS

Sample designation	Cq (ng/ml)		DECq (ng/ml)	
	Venipuncture	Finger-stick	Venipuncture	Finger-stick
W-20, 7 days	123	127	58	64
W-21, 4 h	509	546	182	178
W-21, 24 h	311	282	121	124
W-22, 24 h	249	226	111	104

Venipuncture/finger-stick comparability study

There was close agreement between the analytical results from blood samples collected by venipuncture and corresponding finger-stick blood samples collected on filter papers (Table IV).

General approach to the separation and quantification of antimalarials

We used normal-phase chromatography in our approach to chloroquine analysis, as had Alván et al. [3] previously, because of the selectivity afforded. Our mobile-phase system allows facile adjustment of solvent polarity at lower values of solvent strength than that of Alván et al. [3], permitting application to the separation of less polar antimalarials such as pyrimethamine and amodiaquine. The reservoir for one pump contains hexane—MTBE—diethylamine (49.75:49.75:0.5) while that for the second pump contains methanol—diethylamine (99.50:0.5). The solvents are blended by a solvent programmer, and all analyses are run isocratically. Fig. 3 illustrates the utility of the chromatographic system, depicting a chromatogram run on a mixture of anti-
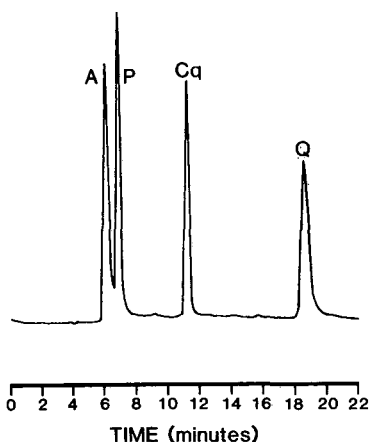
Fig. 3. Chromatogram resulting from the injection of a mixture containing 156 μg/ml of amodiaquine (A), 150 μg/ml of pyrimethamine (P), 82 μg/ml of chloroquine (Cq), and 398 μg/ml of quinine (Q). The mobile phase was hexane—MTBE—methanol—diethylamine (46:46:7.5:0.5), and UV detection was employed at 254 nm and a sensitivity of 0.2 a.u.f.s.

malarials at an intermediate methanol concentration with UV detection at 254 nm. Selection of methanol concentration and UV wavelength allows tailoring of the system to each sample for which the analysis of a specific mixture of antimalarials is to be run. The amodiaquine peak tails somewhat, probably reflecting incomplete attenuation of phenolic acidity by the intra-molecular hydrogen bonding. Pyrimethamine is not completely separated from amodiaquine at this methanol concentration. Sulfadoxine, formulated with pyrimethamine in the antimalarial combination Fansidar®, elutes from the silica column at a methanol concentration of 33%.

Applications of the chloroquine filter-paper, blood-spot method

The method described above makes possible simple and convenient collection and transport of whole-blood samples from the field. Problems associated with collecting venipuncture samples and special handling require-ments of such samples in transit are avoided. These advantages make possible the gathering of data from remote regions of malaria endemicity, allowing, in concert with in vitro anti-malarial susceptibility testing, efficient mapping of the prevalence and spread of chloroquine-resistant *Plasmodium falciparum* malaria.

ACKNOWLEDGEMENT

REFERENCES

1   F.C. Churchill II, D.L. Mount and I.K. Schwartz, J. Chromatogr., 274 (1983) 111.
2   Y. Bergqvist and M. Frisk-Holmberg, J. Chromatogr., 221 (1980) 119.
3   G. Alván, L. Ekman and B. Lindström, J. Chromatogr., 229 (1982) 241.
4   H.M. Matthews, Lab. Manage., 19 (1981) 55.
5   F.C. Churchill and L.C. Patchen, unpublished results.

CHROMBIO. 1847

ANALYSIS OF ERYTHROMYCIN IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and sensitive high-performance liquid chromatographic assay was developed for the quantitative determination of major erythromycin components and their potential metabolites or degradation products in plasma and urine. An ether extract of alkalized plasma sample was chromatographed on a reversed-phase column and the components in the column effluent were monitored by an electrochemical detector. The recovery of the drug from extraction was virtually 100%. The detection limits for erythromycin A in plasma were 5—10 ng/ml and 30 ng/ml using 1 and 0.2 ml of sample, respectively. For urine samples, a simple one-step deproteinization with two volumes of acetonitrile was satisfactory for analysis. The method has been evaluated in plasma and urine from dogs receiving oral or intravenous erythromycin A. The standard curves for potential metabolites or degradation products were not constructed due to the lack of sufficient samples.

INTRODUCTION

Although erythromycin has been widely used for the treatment of various infectious diseases in the last 2—3 decades [1], detailed absorption and disposition kinetics of this antibiotic in the body are only limitedly known to date. This appears to be primarily attributed to the lack of a simple and sensitive method to quantitate the drug and its potential metabolites or degradation products, such as anhydroerythromycin, erythralosamine, and erythromycin enol ether [2].

The novel high-performance liquid chromatographic (HPLC) method of Tsuji [3] based on fluorimetric detection was highly sensitive and capable of separating major erythromycin components from potential metabolites or degradation products. This method apparently has not received wide use,

probably due to the complexity of the procedure and instrumentation involved. For instance, the method required both a pre-column and an analytical column maintained at 70°C, a special system for post-column derivatization, and an on-line extraction before quantitation. About 1 to 3 ml of serum was needed and the sample preparation involved two 5-ml ether extractions followed by evaporation. In addition, its application to urine analysis has not been demonstrated.

The purpose of this paper is to describe a simple and sensitive HPLC method with electrochemical detection* which may be useful in the quantification of major erythromycin components and their potential metabolites or degradation products in both plasma and urine.

EXPERIMENTAL

Materials

U.S.P. erythromycin, des-N-methylerythromycin, erythralosamine and 4″-acetylerythromycin were kindly supplied by Abbott Labs. (North Chicago, IL, U.S.A.). Erythromycins A, B, C and anhydroerythromycin C were generously donated by Upjohn (Kalamazoo, MI, U.S.A.). Erythromycin ethylsuccinate and erythromycin estolate were purchased from Sigma (St. Louis, MO, U.S.A.). Anhydroerythromycin A and erythromycin A enol ether were synthesized from erythromycin A according to the method of Kurath et al. [4]. Sodium acetate, acetonitrile and methanol were of HPLC grade and were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Anhydrous sodium carbonate and glacial acetic acid, also from Fisher Scientific, were of ACS reagent grade. Ethyl ether purchased from Eastman Kodak (Rochester, NY, U.S.A.) was used for extraction.

Stock solutions (1 and 5 mg/ml) of erythromycin A were prepared in acetonitrile. Erythromycin B was similarly dissolved in acetonitrile to make concentrations of 0.01—5 mg/ml. These stock solutions appeared to be stable for at least three months when kept in the freezer.

HPLC instrumentation

The chromatographic system was composed of a solvent delivery pump (ConstaMetric III, Laboratory Data Control, Riviera Beach, FL, U.S.A.), a syringe loading sample injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.), and a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.). The chromatography was carried out at ambient temperature. The column effluent was monitored by using a dual-electrode electrochemical detector (Model 5100A Coulochem™, Environmental Sciences Assoc., Bedford, MA, U.S.A.) in the oxidative screen mode. The applied cell potential of the screen electrode was set at +0.7 V and the sample electrode at +0.9 V. The output from the detector was connected to a 10-mV potentiometric 25-cm recorder (Linear Instruments, Irvine, CA,

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*It appears that the detectability of pure erythromycin by electrochemical detection was first noted by Environmental Sciences Assoc., Inc. and Dr. S.Y. Chu from Abbott Labs., North Chicago.

U.S.A.). The mobile phase was pumped at a flow-rate of 1 ml/min (2000 p.s.i. or 140 bar), and the recorder chart speed was 10 cm/h [5].

Mobile phase preparation

The HPLC mobile phase used in the present study was acetonitrile--methanol--0.2 M sodium acetate (40:5:55), in which the pH of acetate buffer was pre-adjusted to 6.7 with 0.2 M acetic acid. Depending on the performance of a given column, however, the optimal composition of the mobile phase may have to be slightly modified. A proper pH of the acetate buffer was essential to the peak resolution. Furthermore, it was found that satisfactory chromatography may be achieved without the incorporation of methanol in the mobile phase for some columns from the same manufacturer. In order to minimize the background noise and to improve the sensitivity of the detection, it is important to prefilter all the mobile phase components with a 0.22-μm low-extractable membrane. The distilled water used in the mobile phase was purified through the Milli-Q system that contained an Organex-Q^{TM} cartridge (Millipore, Bedford, MA, U.S.A.).

Sample preparation

Aliquots of plasma (0.2 ml) were pipetted into 13 × 100 mm disposable glass culture tubes. The screw cap was lined with a piece of aluminum foil to prevent possible leaching of chemicals and also adsorption of the drug onto the cap. After the addition of internal standard (10 μl of an erythromycin B solution of 0.01—0.1 mg/ml; other internal standards could also be used as will be discussed later), saturated sodium carbonate (20 μl) and ethyl ether (1 ml), each tube was vortexed for 20 sec. Following centrifugation at 800 g for 5 min, 0.75 ml of the diethyl ether layer was transferred to another disposable tube. The diethyl ether layer was then allowed to evaporate at room temperature in a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.) at reduced pressure for 10—15 min. Just prior to the HPLC analysis, the residue was reconstituted with 50 μl of mobile phase and vortexed for 10 sec to facilitate rapid dissolution of the sample. After brief centrifugation, a 20-μl aliquot was injected onto the column.

For urine samples, a 0.1-ml aliquot was transferred to the culture tube. After supplementing with the internal standard (10 μl of an erythromycin B solution of 0.2 mg/ml), the deproteinization was carried out by adding 0.2 ml of acetonitrile, a unique deproteinizing agent first extensively used in this laboratory [6—8 and others]. Following vortex mixing for 10 sec and centrifugation at 800 g for 2 min, about 20 μl of the supernatant was injected into the HPLC.

Standard curves were prepared by spiking blank human plasma with concentrated erythromycin A solution to yield 0.25, 0.5, 1.0, 2.5, and 5 μg/ml. Blank human urine was also supplemented with erythromycin A in concentrations of 2.5, 5, 10, 25, and 50 μg/ml. Due to the lack of sufficient samples, standard curves for erythromycin metabolites or degradation products were not constructed in the present study.

Reproducibility studies

Six replicate analyses of plasma samples at concentrations of 0.5 and 2.5 μg/ml for erythromycin A were performed as described above.

Drug interference studies

Potential interferences of drugs with the assay were studied by injecting aliquots of their stock solutions into the HPLC. The drugs tested included acetaminophen, adriamycin, butaperazine, chlorpheniramine, diazepam, disopyramide, estradiol, furosemide, gentamicin, griseofulvin, lidocaine, naphazoline, oleandomycin, primaquine, pronethalol, propranolol, quinidine·HCl, quinine, rifampicin, theophylline, thiopental, thyroxine, tolbutamide, trifluoroperazine, and trimeprazine.

RESULTS AND DISCUSSION

The electrochemical detector used in the present study is different from those commonly employed in the past in that it has series dual electrodes, which can be set in screen-mode of operation. In this mode, the first electrode was at a potential somewhat lower than the second electrode. The coulometric efficiency of the detector thus decreased background currents and eliminated undesirable components at the first electrode while quantitating erythromycin at the second electrode. In the assay development, the optimal cell potential was first explored using the pure compound dissolved in mobile phase. The resultant hydrodynamic voltammogram for the oxidation of erythromycin A is shown in Fig. 1. Based on this curve, +0.7 and +0.9 V were then chosen for the first and second electrode, respectively.

The high selectivity of the present HPLC method is illustrated by the chromatograms shown in Fig. 2. The peak shapes for most of the individual
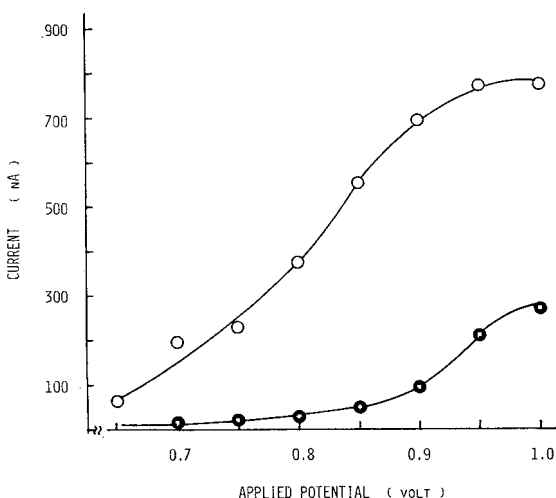
Fig. 1. Hydrodynamic voltammogram for the oxidation of erythromycin A. The lower curve denotes the corresponding background current. The on-column amount injected was 0.2 μg.
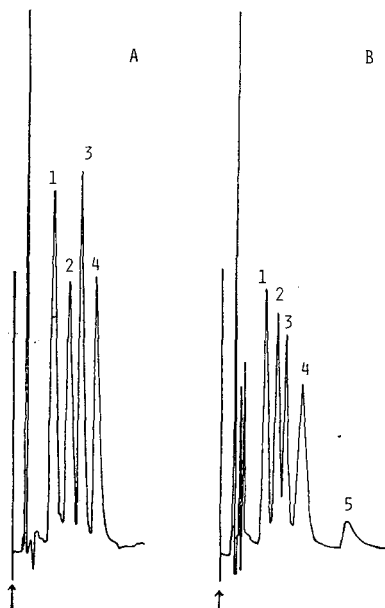
Fig. 2. HPLC chromatograms of various erythromycins, their potential metabolites and degradation products. (A) Peaks: 1 = erythromycin C; 2 = erythromycin A; 3 = anhydroerythromycin A or C; 4 = erythromycin B. (B) Peaks: 1 = des-N-methylerythromycin; 2 = erythromycin A; 3 = erythralosamine; 4 = 4″-acetylerythromycin A; 5 = erythromycin A enol ether. The arrows mark the point of injection.

components of the mixture appear to be symmetrical. In spite of the use of filter element installed before the detector cells, there is no significant peak broadening effect. The retention times for erythromycins A, B, and C were 9.6, 14.2, and 6.9 min, respectively. The retention times of various erythromycins, their potential metabolites and degradation products are listed in Table I. It was found that the powder form of U.S.P. erythromycin from

TABLE I

RETENTION TIMES OF VARIOUS ERYTHROMYCINS AND POTENTIAL METABOLITES OR DEGRADATION PRODUCTS

Compound	Retention time (min)
Erythromycin C	6.90
Des-N-methylerythromycin	7.74
Erythromycin A	9.60
Erythralosamine	11.10
Anhydroerythromycin A	12.36
Anhydroerythromycin C	12.60
4″-Acetylerythromycin A	14.04
Erythromycin B	14.22
Erythromycin ethylsuccinate	19.50
Erythromycin A enol ether	22.08
Erythromycin estolate	22.86

Abbott Labs. or Sigma was essentially free of erythromycin B. Therefore, erythromycin B was chosen as internal standard for this preliminary study. Alternatively, depending on the experimental condition and the availability of the compounds, other erythromycin derivatives such as erythromycin ethylsuccinate or estolate could be used as internal standard.

Typical chromatograms from blank human plasma, and plasma spiked with known concentrations of erythromycins A and B are shown in Fig. 3. No interferences with peaks of the drug and internal standard were found in the plasma samples. As shown in Table II, the standard curve for erythromycin A in plasma is linear over the concentration range studied (0.25—5 μg/ml), which is reflected by the constancy of response factors. Recovery of erythromycin from the extraction was virtually 100%. Excellent reproducibility was obtained for the present HPLC method; the coefficients of variation were 1.5% at 0.5 μg/ml, and 2.3% at 2.5 μg/ml. The intra-day precision was usually between 1.5 and 3.6%, while five replicate analyses in three days gave inter-day precision of 3.9%. As shown in Table II, assay of erythromycin without the use of internal standard may also be satisfactory since the coefficient of variation was only about 5%. To measure peak heights more accurately, it has been recommended that a micrometer [9] and a slower chart speed be used [5].
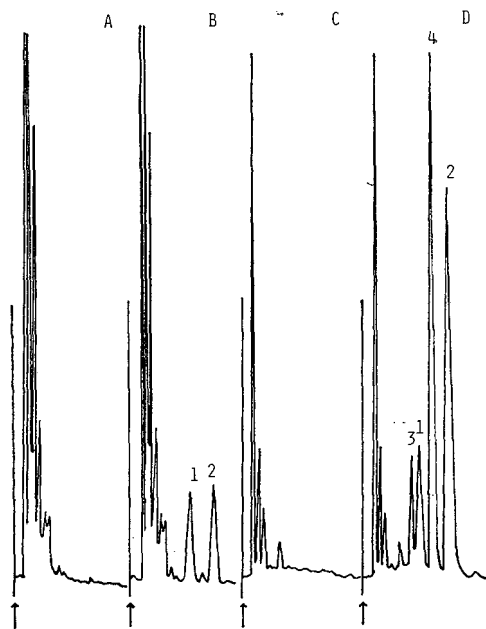
Fig. 3. Chromatograms of extracts from (A) human plasma blank; (B) human plasma spiked with 0.5 μg/ml of erythromycin A and 0.01 mg/ml of internal standard; (C) dog plasma blank; (D) dog plasma collected at 2 h after an oral dose of 500 mg erythromycin A. Concentrations of erythromycin A and internal standard were 1.1 μg/ml and 0.1 mg/ml, respectively. Peaks: 1 = erythromycin A; 2 = internal standard; 3 = unknown product; 4 = anhydroerythromycin A. The arrows mark the point of injection. The detector gain settings were 10 × 10 for A and B, and 50 × 1 for C and D.

TABLE II

RESPONSE FACTORS FOR ERYTHROMYCIN A IN HUMAN PLASMA

Spiked-plasma concentration (μg/ml)	Peak height* (cm)		Response factor I***	Response factor II§
	Erythromycin A	Internal standard**		
0.25	1.30	2.76	5.20	18.84
0.50	2.44	2.60	4.88	18.77
1.0	5.04	2.70	5.04	18.67
2.5	12.96	27.92	5.18	18.57
5.0	28.12	29.71	5.62	18.93
Mean ± S.D.			5.18 ± 0.28	18.76 ± 0.14
Coefficient of variation (%)			5.3	0.75

*Peak heights were measured based on 20-μl injection and normalized gain setting of 10×10.
**The internal standard spiked was 0.01 and 0.1 mg/ml for low (0.25—1.0 μg/ml) and high (2.5—5.0 μg/ml) concentrations of erythromycin A.
***Response factor I = peak height of erythromycin A divided by its concentration.
§ Response factor II = peak height ratio of erythromycin A and internal standard based on unit concentration of each compound.

Because of the coulometric detector design, the background current levels would limit the maximum gain that can be obtained. As a result, the detection limit for a drug was highly dependent on the background noise encountered. Practically speaking, based on a signal-to-noise ratio of 3, the lower limit of quantitation for erythromycin A using 0.2 ml plasma was approximately 30 ng/ml with a detector gain setting of 10×10. However, higher sensitivity could be achieved when 1 ml of plasma was used for extraction. The procedure was carried out as follows. After alkalization with 0.1 ml of saturated sodium carbonate, 2 ml of ethyl ether were added to the plasma sample. Following vortex mixing and centrifugation, the ether layer (about 1.5 ml) was evaporated to dryness. The residue was then reconstituted with 50 μl of mobile phase and centrifuged. An aliquot of 20 μl was injected into the HPLC. In this manner, the detection limit of erythromycin A could be 5—10 ng/ml with a gain setting of 10×10. The sensitivity of the present assay appears to be adequate for drug concentrations encountered clinically. There was no significant increase in the absolute peak height of erythromycin A when the diethyl ether—plasma volume ratio was increased to 4, suggesting a complete recovery of the drug with the volume ratio of 2.

Fig. 3 also shows a chromatogram from the plasma collected 2 h after oral administration of 500 mg erythromycin A to a 12-kg male mongrel dog. It appeared that peaks from two additional compounds were present in all of the samples analyzed. The peak eluting after erythromycin A had a retention time identical to that of anhydroerythromycin A. The other peak, eluting earlier, had a retention time different from those of potential metabolites or degradation compounds tested; its identity is presently under investigation. The plasma concentration—time profile of erythromycin A together with the peak heights of the two metabolites or breakdown products from the dog are depicted in

Fig. 4. The erythromycin levels were found to be 2.1 and 0.3 μg/ml at 1 and 6 h, respectively. It is of interest to note that peaks corresponding to anhydro-erythromycin A and the unknown compound were both present after intravenous administration of erythromycin lactobionate to another dog. Detailed absorption and disposition kinetics of erythromycin in animals and humans are currently being pursued using the assay developed here.
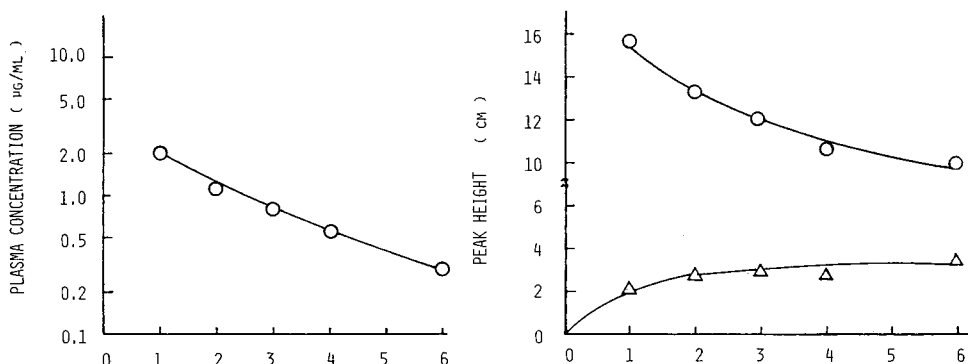
Fig. 4. Plasma concentration profile of erythromycin A (left) and peak heights of two metabolites or degradation products as a function of time (right; O–O, anhydroerythromycin A and △-△, unknown compound) in a dog following an oral dose of 500 mg of erythromycin A.

The pH of the mobile phase was found to have a profound effect on both the retention volume and the resolution of the peaks. In general, the lower the pH, the shorter the retention time, and thus the higher the response. However, when the pH of acetate buffer in the mobile phase was lower than 6.0 a shoulder peak next to erythromycin A would appear; and in addition, the unknown product could not be resolved from the drug. To maximize the performance of the column, therefore, a pH of 6.7 of the buffer was adopted in this study.

One of the advantages of this assay is that a much smaller plasma volume (0.2 ml) can be employed for quantitation as compared with that used (1—3 ml) in the previous report [3]. The extraction procedure is also simpler and easier. Essentially complete recovery was obtained with 1 ml of diethyl ether for 0.2 ml of plasma samples. Several solvents were tested for extraction efficiency, including benzene, toluene, chloroform, ethyl acetate, hexane and diethyl ether. Among these, chloroform and diethyl ether offered the greatest recovery and the least interference. Diethyl ether was selected because of the easy handling of diethyl ether layer which was on the top of the aqueous solution. It has to be pointed out that alkalization of plasma samples by sodium carbonate was critical to the high extraction efficiency. The extraction procedure used in this study appeared somewhat similar to that employed earlier [3].

The results of interference studies showed that none of the drugs tested interfered with the analysis of erythromycin.

Since the drug concentrations in urine samples are usually much higher than found in plasma, a simple one-step deproteinization method was sufficient for electrochemical detection. Fig. 5 shows chromatograms from blank human urine, urine spiked with known concentrations of erythromycins A and B, together with that from dog's urine collected after receiving erythromycin A. A similar peak pattern was observed in the dog's urine and plasma. The response factor data for erythromycin A in urine are shown in Table III. Linearity was found within the concentration range 2.5—50 μg/ml. The coefficient of variation of response factors was found to be 5.0% in the absence of internal standard, indicating the adequacy of performing the assay without an internal standard.
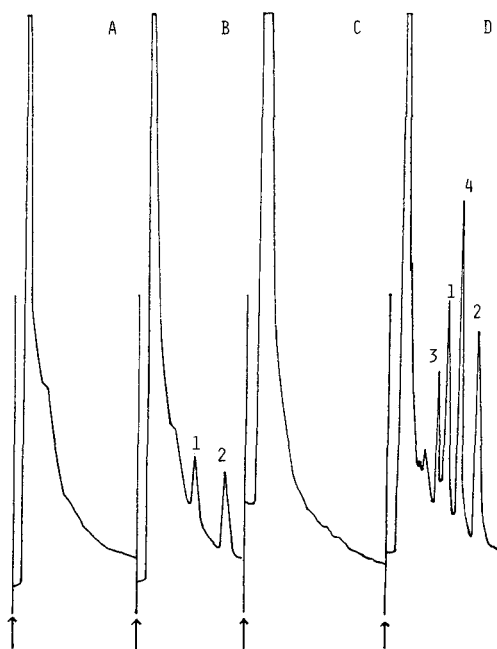
Fig. 5. Chromatograms from (A) human urine blank; (B) human urine spiked with 10 μg/ml of erythromycin A and 0.2 mg/ml of internal standard; (C) dog urine blank; (D) dog urine collected up to 6 h after an oral dose of 500 mg erythromycin A. Concentrations of erythromycin A and internal standard were 0.33 and 5 mg/ml, respectively. Peaks: 1, erythromycin A; 2, internal standard; 3, unknown product; 4, anhydroerythromycin A. The arrows mark the point of injection. The detector gain settings were 50 × 1 for A and B, and 5 × 1 for C and D.

With a daily or frequent use of electrochemical detector, it is often advised that the HPLC system be maintained in operation condition to reduce the background currents and to enhance detector stability. Long-term exposure of bonded silica columns to a high-pH mobile phase, however, may significantly shorten the column life. In order to preserve optimum column performance, the column was removed from the system when not in use (e.g. overnight),

TABLE III

RESPONSE FACTORS FOR ERYTHROMYCIN A IN HUMAN URINE

Spiked-urine concentration (μg/ml)	Peak height* (cm)		Response factor I	Response factor II
	Erythromycin A	Internal standard**		
2.5	0.45	2.37	0.180	15.13
5.0	0.91	2.41	0.182	15.04
10.0	1.91	2.46	0.191	15.53
25.0	4.92	2.48	0.197	15.87
50.0	10.10	2.58	0.202	15.66
Mean ± S.D.			0.190 ± 0.009	15.45 ± 0.35
Coefficient of variation (%)			5.0	2.28

*Peak heights were measured based on 20-μl injection and the gain setting was 50 × 1.
**The concentration of internal standard spiked was 0.2 mg/ml.

while the chromatograph was maintained with the mobile phase recirculating at a rate of 0.2 ml/min, and also with the electrode activated. The column was then flushed with 20 void volumes (about 30 ml) of water and methanol prior to storage. Just before the start of the assay, the column was first flushed with 30 ml of water and then equilibrated with mobile phase at a flow-rate of 1 ml/min for 0.5 h. The system could be quickly restored to operating conditions by reconnection of the pre-equilibrated column to the chromatograph.

REFERENCES

1 M.A. Sande and G.L. Mandell, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, p. 1222.
2 K. Tsuji and J.F. Goetz, J. Chromatogr., 147 (1978) 359.
3 K. Tsuji, J. Chromatogr., 158 (1978) 337.
4 P. Kurath, P.H. Jones, R.S. Egan and T.J. Perun, Experientia, 27 (1970) 362.
5 W.L. Chiou, Clin. Chem., 25 (1979) 197.
6 W.L. Chiou, M.A.F. Gadalla and G.W. Peng, J. Pharm. Sci., 67 (1978) 182.
7 G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith and W.L. Chiou, Clin. Chem., 23 (1977) 1838.
8 R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 146 (1978) 121.
9 N.K. Athanikar, G.W. Peng, R.L. Nation, S.-M. Huang and W.L. Chiou, J. Chromatogr., 162 (1979) 367.

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THE ANALYSIS OF [14C]CLOFIBRIC ACID GLUCURONIDE AND [14C]CLOFIBRIC ACID IN PLASMA AND URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method has been developed for the quantitation of [14C]clofibric acid glucuronide and [14C]clofibric acid using conventional scintillation counting. The assay has a linear relationship between the added and observed ratios of clofibric acid glucuronide : clofibric acid in the range of 0.001—0.6 for plasma and 0.5—100 for urine, and is able to quantitate previously unmeasurable concentrations of clofibric acid glucuronide in plasma.

INTRODUCTION

We have previously reported a high-performance liquid chromatographic (HPLC) method for clofibric acid glucuronide (CFAG) which does not require hydrolysis to clofibric acid (CFA) [1]. This method, which uses the ultraviolet (UV) absorbance of CFAG as the means of detection, is insufficiently sensitive to measure plasma concentrations of CFAG in man [2]. In order to overcome this difficulty the previously described chromatographic method has been modified to allow detection of [14C]CFAG and [14C]CFA with scintillation counting which results in greatly increased sensitivity. The method has been applied to an animal model of CFA disposition.

EXPERIMENTAL

Biosynthesis and purification of [14C]clofibric acid glucuronide
   The methods used for the preparation and purification of [14C]CFAG ([14C]clofibric acid glucuronide) are a modification of those previously

described for the preparation of unlabelled CFAG [1]. Each of two male hooded Wistar rats received 925 kBq of [1-^{14}C]p-chlorophenoxy-2-methyl-propionic acid ([^{14}C]CFA) of specific activity 1290 MBq/mmol in 2.5 ml of 0.9% sodium chloride solution by intraperitoneal injection. Each rat received a further 5 ml of water by mouth immediately after the dose was given and was placed in a metabolic cage which allowed for the separate collection of urine and faeces. The urine container of each cage contained 15 ml of 0.5 M glycine buffer, pH 2.5, which was kept cold by contact with ice. Urine was collected for 16 h after the dose. The pooled urine was extracted twice with an equal volume of ice cold ethyl acetate and the combined ethyl acetate extracts were reduced to dryness at room temperature and reduced pressure in an evacuated centrifuge (Speed Vac Concentrator, Servant Instruments, NJ, U.S.A.). The residue was reconstituted in 3.5 ml of ice cold mobile phase (0.01 M citrate buffer, pH 2.5—acetonitrile, 65:35). Chromatographic purification of [^{14}C]-CFAG was carried out with 0.02-ml aliquots of the mobile phase containing [^{14}C]CFAG using the chromatographic system and conditions previously described [1] but with the above mobile phase.

Pooled fractions (2.5—3.0 min) containing the [^{14}C]CFAG were assayed for [^{14}C]CFA and [^{14}C]CFAG to determine their radiochemical purity and used as such for the preparation of calibration curves. Samples of [^{14}C]CFAG to be used in animal studies or for characterization by hydrolysis with β-glucuronidase were reduced to dryness over approximately 6 h by freeze-drying (Edwards High Vacuum Model EF3, Sussex, U.K.). [^{14}C]CFAG prepared by freeze-drying and that in mobile phase was stored at −20°C until used.

The freeze-dried material was dissolved in ice cold 0.9% sodium chloride solution ($\simeq 5 \cdot 10^7$ dpm/ml) and two 0.05-ml aliquots were diluted with 0.45 ml of 0.1 M acetate buffer, pH 5.0, and incubated at 37°C for 2 h with approximately 500 Fishman units of β-glucuronidase (type H, Sigma, St. Louis, MO, U.S.A.). The pH of the solution was adjusted to 2.5 with 0.5 M buffer, pH 2.5, and assayed for [^{14}C]CFA and [^{14}C]CFAG.

Stability studies

Solutions of [^{14}C]CFAG were stored in separate glass tubes at −20°C which were then thawed and assayed for [^{14}C]CFAG and [^{14}C]CFA content over a period of six weeks. [^{14}C]CFAG solution (0.2 ml containing $4 \cdot 10^4$ dpm) was added to 2 ml of fresh rabbit plasma and incubated at 37°C. Plasma samples (0.2 ml) were taken at 1, 2.5, 5, 7.5, 10, 15, 20, 30, 45 and 60 min, after the addition and assayed for [^{14}C]CFAG in order to determine the stability of the glucuronide in fresh rabbit plasma.

Sample preparation

A schematic representation of the procedure is shown in Fig. 1. Each sample is split and an aliquot is counted in order to determine the total ^{14}C-labelled content in each sample which consists of [^{14}C]CFA and [^{14}C]CFAG. A second aliquot is extracted to determine the proportion in each sample of [^{14}C]CFA and [^{14}C]CFAG. The absolute radioactivity of CFA and CFAG is calculated as

$$CFA \ (dpm/ml) \ = \ T \times \ \frac{CFA}{CFA + CFAG} \qquad (1)$$

and

$$CFAG \ (dpm/ml) \ = \ T \times \ \frac{CFAG}{CFAG + CFA} \qquad (2)$$

where T is the total radioactivity in dpm/ml and CFA and CFAG are the disintegration rates of the chromatographic fractions corresponding to CFA and CFAG, respectively. Plasma or urine samples (0.2—0.5 ml) were placed in 4.5-ml capacity glass tubes containing 0.5 ml of ice cold 0.5 M glycine buffer, pH 2.5, and 1 ml cold ethyl acetate. All subsequent manipulations, with the exception of the ethyl acetate evaporation, were carried out either with the tubes in ice or at 4°C. The tubes were mixed by slow tumbling for 10 min and the ethyl acetate, separated by centrifugation at 1500 g for 3 min was transferred to a fresh tube and the process was repeated twice with fresh 1-ml portions of ethyl acetate. The combined ethyl acetate extracts were reduced to dryness over a period of approximately 20 min using the evacuated centrifuge, and the tube was rinsed with 0.1 ml of ice-cold mobile phase and kept on ice
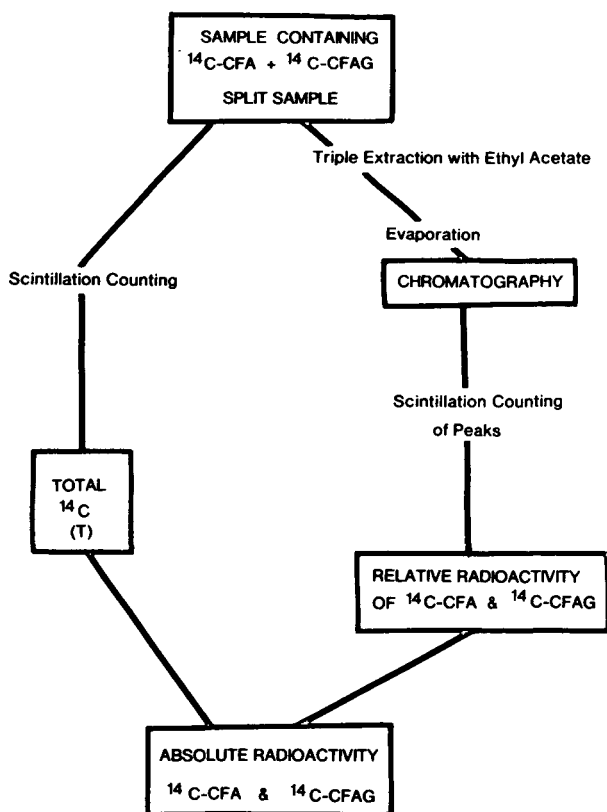
Fig. 1. A schematic representation of the sample preparation, chromatography and quantitation methods used in the essay.

until chromatographed. The entire content of each tube was injected into the high-performance liquid chromatograph (Waters, Model 6000A) via a Waters Model U6K injector (Waters Assoc., MA, U.S.A.). A 10-μm particle size reversed-phase column (Waters μBondapak C_{18}) was used with a mobile phase of 0.01 M citrate buffer, pH 2.5—acetonitrile (65:35). Eluent fractions corresponding to [^{14}C]CFAG and [^{14}C]CFA were collected into 25-ml capacity glass scintillation vials via a short length of stainless-steel tubing fitted to the column.

Scintillent, 15 ml (PCS, Amersham, IL, U.S.A.) was added to each tube and the count rates were determined in a scintillation counter (Model 6892, Searle Analytic, IL, U.S.A.). The count rates were converted to disintegration rates using the external standard method for quench correction. The disintegration rate of a 0.05—0.1-ml aliquot of biological fluid was also determined in a manner described above to obtain the total ^{14}C-labelled content of the sample.

Calibration

Calibration curves in plasma were prepared by adding known quantities of [^{14}C]CFAG (450—1000 dpm) and [^{14}C]CFA to plasma to produce ratios of [^{14}C]CFAG:[^{14}C]CFA in the range of 0.001—0.6. A similar procedure was used in urine but the range of ratios of [^{14}C]CFAG:[^{14}C]CFA was from 0.5—100. These samples were taken through the analysis and the observed ratios compared with those added to plasma or urine. Control samples which did not contain added ^{14}C were taken through the analytical procedure in order to determine background count rates which were subtracted from those of [^{14}C]CFA and [^{14}C]CFAG containing samples.

RESULTS AND DISCUSSION

Biosynthesis of [^{14}C]CFAG

[^{14}C]CFAG was prepared on two separate occasions. The percent of [^{14}C]CFA dose recovered in urine over 16 h was 92% and 71% on the first and second occasion, respectively. Direct injection of 0.01-ml aliquots under the chromatographic conditions described, revealed two fractions containing ^{14}C at 2.5—3 min and 6.5—7.5 min, the first corresponding to the retention time of authentic unlabelled CFAG under these conditions [1] and the second corresponding to the retention time of authentic [^{14}C]CFA.

In the first preparation, 77% of the urinary radioactivity was in the [^{14}C]CFAG fraction and 23% in the [^{14}C]CFA fraction, the corresponding values for the second preparation being 92% and 8%, respectively. When urine was directly injected, 1-min fractions collected for 30 min after the injection did not show any radioactive peaks greater than 1% of the total ^{14}C, other than those due to [^{14}C]CFA and [^{14}C]CFAG. After ethyl acetate extraction there was a mean of 5.2% of the urinary ^{14}C-activity remaining in the urine, which probably reflects the difficulty of complete removal of the ethyl acetate from a large volume of urine rather than the non-extraction of ^{14}C from the urine. The fractions from 2.5—3 min resulting from the injection of the ethyl acetate extract were collected and pooled. Subsequent chromatography of these pooled fractions showed that the first preparation had greater than 99% of the

[14]C in the CFAG fraction, the remainder being CFA. The second preparation had 97.4% of the ^{14}C in the CFAG fraction. Aliquots of the fractions corresponding to [^{14}C]CFAG were reduced to dryness using the same conditions for each batch. Reanalysis of the first batch reconstituted in 0.9% sodium chloride solution showed that greater than 99% of the ^{14}C was associated with the CFAG fraction but in the second batch 85% of the ^{14}C was in the CFAG fraction, the remainder being in the CFA fraction. This reduction in radiochemical purity during solvent evaporation occurred in spite of apparently identical conditions being used on each occasion.

Analysis of the first batch indicated that all but 5% of the ^{14}C in the CFAG fraction before hydrolysis was associated with the CFA fraction after hydrolysis with β-glucuronidase and no other peaks were detected. Under the same conditions but in the absence of β-glucuronidase approximately 16% of ^{14}C in the CFAG fraction was associated with CFA representing non-enzymatic hydrolysis. In the second batch, 12.5% of the ^{14}C remained in the CFAG fractions after β-glucuronidase treatment. The activity associated with the [^{14}C]CFAG fraction but which was non-hydrolysable by β-glucuronidase may be due to rearrangement to non β-glucuronidase hydrolysable forms of the glucuronide in vivo or in vitro as previously reported for CFAG [2,3]. Taken together these data indicate that ^{14}C in the 2.5—3-min fraction is [^{14}C]CFAG, in that its retention time corresponds to authentic CFAG [1] and it is hydrolysed to a single product, CFA, by β-glucuronidase at a rate which exceeds non-enzymatic hydrolysis under the same conditions.

Stability studies

The ratio of [^{14}C]CFAG to CFA of the CFAG samples in 0.5 M glycine buffer, pH 2.5, at −20°C did not alter over a period of six weeks. Freeze-dried material stored at −20°C in anhydrous conditions also did not alter in the ratio of CFAG to CFA over this period.

In order to use the method in biological samples it was necessary to obtain an estimate of the hydrolysis rate of CFAG in biological fluids. Fig. 2 shows the loss of CFAG in fresh rabbit plasma at 37°C, the half-life for this process being 23 min. For this reason, in subsequent experiments with rabbits, blood samples were withdrawn and plasma was frozen in pH 2.5 buffer within 90 sec.

Analytical method

The approach outlined in Fig. 1 is valid only when all of the ^{14}C-labelled content in the biological fluid is due to CFA or CFAG. In preliminary experiments with plasma from rabbits administered [^{14}C]CFA, greater than 99% of the activity was removed by triple extraction with ethyl acetate and examination of this extract showed that this activity was associated only with CFA and CFAG. Similarly, when urine from rabbits administered [^{14}C]CFA was directly injected into the chromatographic system and 1-min fractions were collected for 30 min, radioactivity was found only in the 2.5—3 min and 6.5—7 min fractions, corresponding to CFAG and CFA respectively. It is unlikely that CFA would be converted to a lipophilic metabolite eluted substantially after CFA on a reversed-phase column. These findings are consistant with those of Caldwell et al. [4] who reported that rabbits administered [^{14}C]CFA excrete
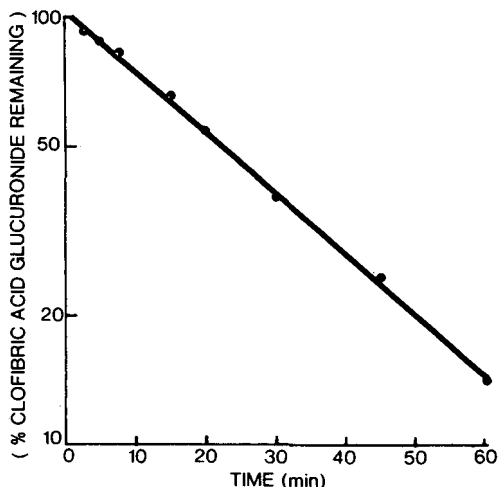
Fig. 2. The hydrolysis rate of CFAG (percent remaining, log. scale) in fresh rabbit plasma at 37°C. The half-life of this process is 23 min.

urinary ^{14}C, 9% as [^{14}C]CFA and 91% as CFAG. The approach outlined in Fig. 1 would not be valid in species such as dog or cat in which a substantial fraction of the dose is excreted as a taurine conjugate, unless the peak corresponding to this compound was separated and counted.

The sample preparation and chromatography present many opportunities for selective loss of CFA or CFAG and thus in order to validate the method it is necessary to demonstrate a correlation between the ratios of CFAG:CFA added to biological fluids and those estimated by the assay. Because of the extremely small ratios of CFAG to CFA anticipated in plasma (see Fig. 3) and the large ratios expected in urine, it was also important to ensure that there was efficient chromatographic separation of the two peaks with minimal carry over of radioactivity between them. The retention times of approximately 2.7 and 7 min for CFAG and CFA respectively meet these conditions, the column eluent between the 2.5—3 min fraction and the 6.5—7 min fraction falling to background values.

Because of the very wide range of values the regression equation for the relationship between the ratios of [^{14}C]CFAG to [^{14}C]CFA added to plasma and those estimated by the assay has been performed on the logarithms of the ratios in order to increase the weight of the lower values. If no weighting were used this would result in the low ratios making an insignificant contribution to the best fit regression equation. The regression equation in plasma is $y = 0.002 + 1.003x$ where y is the logarithm of the observed ratio and x is the logarithm of the added ratio, $r^2 = 0.9966$, $n = 35$ which indicates that the added and observed ratios in plasma are highly correlated with a slope of 1. The corresponding equation for urine is $y = -0.023 + 1.014x$, $r^2 = 0.991$, $n = 26$. These relationships indicate that in plasma over a range of CFAG:CFA ratios of the 0.001—0.6 and in urine over the range of 0.5—100 the method is able to accurately quantitate the concentrations of [^{14}C]CFAG and [^{14}C]CFA in biological fluids.
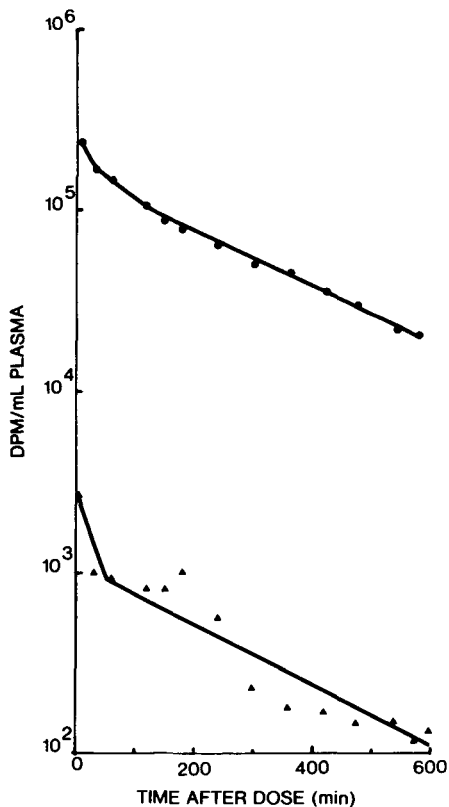
Fig. 3. The plasma concentration—time profiles of CFA (o—o) and CFAG (▲—▲) in a rabbit administered 1.85 MBq of [^{14}C]CFA.

The application of the method is illustrated in Fig. 3 which shows the plasma concentration—time profiles for CFAG and CFA resulting from the administration of 1.85 MBq of [^{14}C]CFA to a rabbit. The mean ratio in plasma of CFAG to CFA in this animal is 0.005.

The present method enables the quantitation of CFAG without prior hydrolysis to CFA and thus offers advantages over methods employing enzymatic or chemical hydrolysis in that it is more selective and sensitive [1]. The use of [^{14}C]CFA in conjunction with scintillation counting has increased the sensitivity of the method 100-fold relative to the previously described method using UV absorbance detection [1] and the only practical limitation to further increases in sensitivity is the specific activity of [^{14}C]CFA and its cost. The present method has allowed the quantitation of circulating plasma concentrations of [^{14}C]CFAG resulting from [^{14}C]CFA administration in an animal model which was not possible with the previous method based on UV absorbance detection. The application of the method to human studies would necessitate an evaluation of the ethical problems arising from the administration of radioisotopes to man.

ACKNOWLEDGEMENT

REFERENCES

1 J.R. Veenendaal and P.J. Meffin, J. Chromatogr., 223 (1981) 147.
2 J.R. Veenendaal, P.M. Brooks and P.J. Meffin, Clin. Pharmacol. Ther., 29 (1981) 351.
3 C.E. Hignite, C. Tschanz, S. Lemons, H. Wiese, D.L. Azarnoff and D.H. Huffman, Life Sci., 28 (1981) 2077.
4 J. Caldwell, T.S. Emudainughe and R.L. Smith, Brit. J. Pharmacol., 66 (1979) 421P.

CHROMBIO. 1818

REVERSED-PHASE CHROMATOGRAPHY OF FENFLUMIZOLE, A NEW POTENTIAL ANTI-INFLAMMATORY AGENT, AND ITS APPLICATION IN PHARMACOKINETIC STUDIES IN RAT, DOG AND MAN

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SUMMARY

Two high-performance liquid chromatographic methods for quantitation of fenflumizole in biological fluids are described. Fenflumizole is a new agent with demonstrable anti-inflammatory properties in laboratory rodents. The major difference between the two methods lies in the utilization of fluorescence detection of the column eluate in assay II as compared to the less selective ultraviolet—visible detection used in assay I. Assay II appeared suitable for monitoring metabolites in addition to the parent compound. The quantitation limit of plasma fenflumizole was below 10 ng/ml for assay I and approximately 100 pg/ml for assay II. The reproducibility within a plasma concentration range of 50—150 ng/ml was below 7% (standard deviation) in both assays. Fenflumizole was found to be subject to photodecomposition. The impact of this on the analytical performance was evaluated and precautionary measures were assessed. The reliability of the analytical methods is examined and their applicability in in vivo studies conducted in rat, dog, and man.

INTRODUCTION

Fenflumizole, 2-(2,4-difluorophenyl)-4,5-bis(4-methoxyphenyl)imidazole, is a potential anti-inflammatory agent of the non-steroidal type, structurally belonging to the series of substituted triarylimidazoles (Fig. 1) [1,2]. It has displayed analgesic and antipyretic properties similar to those of other non-steroidal anti-inflammatory drugs, but with fewer gastrointestinal side-effects [2].

This paper describes two rapid, accurate, and analytical high-performance liquid chromatographic (HPLC) procedures for the determination of fenflumizole in biological fluids. The validity of the methods is demonstrated by the determination of fenflumizole plasma levels after single administrations to rats, a beagle dog, and a human volunteer.
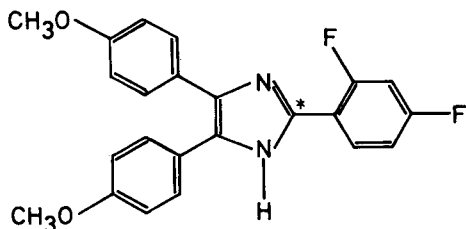
Fig. 1. Chemical structure of fenflumizole, a potential anti-inflammatory agent. Position of radioactive labelling at carbon-2 in the imidazole nucleus is shown by an asterisk.

EXPERIMENTAL

Reagents and materials

Fenflumizole was synthesized and supplied by the Synthesis Department of Dumex Ltd. [^{14}C]Fenflumizole was purchased from the Huntingdon Research Centre and was labelled at carbon-2 in the imidazole nucleus (Fig. 1). Acetonitrile was of HPLC grade, triethylamine of synthetic grade and all other chemicals were of analytical grade.

Instrumentation and analytical conditions

The liquid chromatograph consisted of a Waters Assoc. Model 6000 A constant flow pump, a Waters U6K injector, either a Waters Model 440 UV detector or a Kontron spectrofluorometer SFM-23 LC and a Hewlett-Packard Model 3380 integrator. The fluorometer was equipped with a slit width of 4 nm and a 150-W xenon lamp with a wavelength range of 200—800 nm. A Knauer stainless steel chromatographic column, 250 mm × 4 mm I.D. (Dr. Herbert Knauer, Taunus, F.R.G.), was packed with 5-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.) using a slurry technique. A Beckman liquid scintillation counter LS 150 was used for determining extraction yields.

The mobile phase for the analysis of fenflumizole was a mixture of a 50 mM aqueous solution of triethylamine and acetonitrile (23:77). Chromatography was done at room temperature and the flow-rate was 1 ml/min. Under these conditions the capacity ratio of fenflumizole was 6.7 and the number of theoretical plates was calculated to be approximately 6000.

Analytical procedure for assay I

A 5-ml volume of the extraction medium was added to 1 ml of an aqueous sample (e.g. plasma, urine) and the mixture was rotated for 5 min. The extraction medium was a 1:1 mixture of n-hexane—dichloromethane. After centrifugation 4 ml of the organic supernatant were transferred to a clean tube and dried under a stream of nitrogen gas at ambient temperature. The residue was redissolved in 100 μl of the mobile phase, and an aliquot of 40 μl was injected onto the HPLC column. The absorbance at 280 nm (filter) of the eluate was monitored with a UV detector (Fig. 2).

Calibration standards within a range of 0—200 ng/ml were obtained by adding known amounts of fenflumizole to drug-free plasma. Ethanol was used

as solvent for the stock solution of fenflumizole. It was stored in the dark at 4°C when not in use because light-induced decomposition was an important factor (see below).

Analytical procedure for assay II

Owing to the expected presence of more polar metabolites in plasma and urine in relation to the parent compound, the extraction mixture was replaced by diethyl ether and UV detection was changed to fluorescence detection (Fig. 3). In all other respects the assays were similar. By recording the spectra, the excitation and emission maxima were found to be 316 nm and 410 nm. This was achieved by filling the flow cell with a standard solution of fenflumizole and scanning in situ.

Quantitation

A calibration curve was obtained by analysing 1-ml drug-free samples to which were added 0—200 ng of fenflumizole. A linear relationship between the detector response and the fenflumizole concentration was observed. From a computerized plot of the standard peak areas versus concentrations the actual concentrations of the samples were found. Regression coefficients and ordinate intercepts were obtainable by means of linear regression (method of least squares).

RESULTS AND DISCUSSION

Conditions for chromatography and extraction

Initially, an unbuffered mobile phase (methanol—water, 90:10) was used. However, continued use of this solvent failed to give reproducible retentions. A buffered solvent (acetonitrile—phosphate buffer, pH 3.0, 80:20) was therefore prepared. This helped the retention performance, but was inconvenient for routine use because it necessitated frequent regeneration of the chromatographic system, independent of the strength of the buffer. A 77:23 mixture of acetonitrile and 0.05 M triethylammonium acetate (pH 4.0) was finally chosen as a standard eluent. Separation with this solvent proved to be excellent and has subsequently been used in all analyses of biological samples.

The extraction procedure was closely linked to the choice of detection principle. When using absorbance at 280 nm (see Fig. 2) as detection method, fenflumizole was chromatographically separated from endogenous biological constituents after extraction with n-hexane—dichloromethane (1:1). However, it was necessary to consider the possibility that metabolites might occur in the effluents. With this in mind the technique was further improved by the use of a fluorimetric detector and a less restricting extraction with diethyl ether.

Figs. 2 and 3 show typical elution tracings from the alternative analytical methods. The UV detector leaves a poorly resolved crowding at the beginning of the chromatogram which does not fluoresce at the conditions specified.

The extraction recoveries were estimated by employing ^{14}C-labelled fenflumizole. For plasma the fenflumizole recoveries were found to be 84% in assay I and only 72% in assay II. The extractions from urine were fully

effective in both assays. These results were confirmed by liquid chromatography by ratioing the slopes of the standard curves of direct and processed standards. A time-dependent extraction yield was found for plasma when the tubes were rotated for less than 5 min.
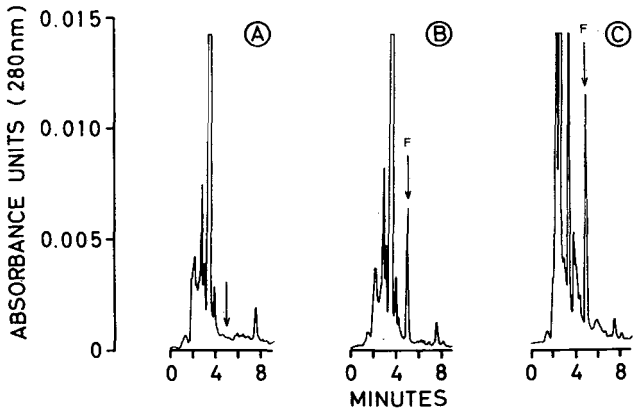
Fig. 2. HPLC UV traces of fenflumizole (F) in (A) blank plasma, (B) a plasma standard of 50 ng/ml, and (C) a plasma sample of approximately 100 ng/ml. Chart speed was 0.5 cm/min and injection volume 40 μl.
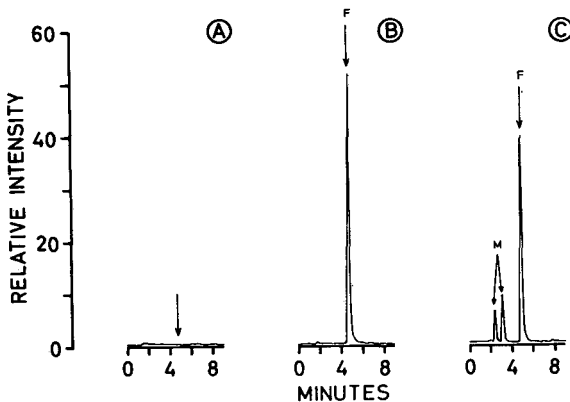
Fig. 3. HPLC fluorescence traces of fenflumizole (F) in (A) blank plasma, (B) a plasma standard of 50 ng/ml, and (C) a plasma sample of approximately 40 ng/ml; two metabolites (M) are visible. Chart speed was 0.5 cm/min and injection volume 40 μl.

Reliability and characteristics of the analytical methods

The analytical variation was evaluated by means of the precision and accuracy. The variation of the slope and the ordinate intercept of the standard curve and its deviation from linearity provided an estimate of the accuracy, and the precision was determined by including control samples in each analytical series (Table I). These were prepared by adding plasma to a known amount of fenflumizole. After mixing, volumes of 1 ml were stored at −20°C until analysis.

TABLE I

ESTIMATION OF ANALYTICAL VARIATION

Comparison of two analytical methods for determining fenflumizole concentrations (ng/ml) in plasma as indicated by ten consecutive analytical series over six weeks. Slope and ordinate intercepts were calculated according to the equation $Y = a + bX$ (linear regression). Analytical variation was estimated with the aid of quality control samples (pool I and pool II), which were assayed along with normal plasma samples. Figures given are means ($\pm$ S.D.).

Parameter		UV detection (assay I)	Fluorimetric detection (assay II)
Ordinate intercept	a	2.74 ($\pm$ 0.92)	−3.68 ($\pm$ 1.26)
Slope	b	0.315 ($\pm$ 0.05)	9.65 ($\pm$ 0.79)
Correlation coefficient	r	0.997 ($\pm$ 0.07)	0.999 ($\pm$ 0.04)
Inter-assay analytical variation	Pool I	–	47.6 ($\pm$ 3.3)
	Pool II	144.7 ($\pm$ 9.7)	145.3 ($\pm$ 8.1)

The comparative data in Table I show acceptable parameters for both methods. The fluorimetric detection method with its inherent high sensitivity demonstrates a correlation coefficient and a precision (defined as the reciprocal of the variance, $1/S.D.^2$) that are superior to those obtained with the UV detector. The reproducibility measured as the relative standard deviation is estimated to be below 7% in all assays.

In the assays described the quantitation limit was found to be below 10 ng/ml for assay I and approximately 100 pg/ml for assay II. The latter was limited primarily by the onset of a destructive photodecomposition within the flow cell caused by too intense excitation of the fluorophore.

Two other aspects were considered when judging the magnitude of laboratory error in the analytical method. First, storage of plasma pool samples at −20°C was found not to cause measurable changes of the fenflumizole content over at least eighteen months. Second, repetitive freeze—thaw cycles of plasma and urine up to three times left the sample unchanged with regard to nanogram-range concentrations of fenflumizole.

With regard to the plasma and serum analyses, fluorimetric detection in assay II did not require haemolysis-free samples, unlike the 280 nm absorbance measurement in assay I.

Photodecomposition

As with several other polyarylimidazoles, fenflumizole exhibits instability when exposed to certain wavelengths of light [1].

From the outset, daylight caused deterioration in stock solutions up to the mg/ml concentration range, and sunlight rapidly made this worse. As a rule the specimens were handled in subdued light or protected from light by the use of brownish-coloured or tinfoiled glass tubes. By applying this rule, the longevity of stock ethanol solutions of fenflumizole was found to be at least five days. Experiments on the photolytic properties of fenflumizole are in progress and will be reported.

Probe studies of plasma kinetics in the rat, beagle dog and normal man

The validity of the assays was assessed by determining plasma concentrations of fenflumizole in in vivo probe studies. Male Sprague-Dawley rats and a beagle dog were given single intravenous doses of 3 and 10 mg/kg body weight (Fig. 4), and a human male volunteer ingested fenflumizole in single doses of 0.1, 2 and 5 mg/kg body weight (Fig. 5). Although the determination of plasma fenflumizole was feasible with both assays, assay II appeared superior because the metabolite peaks were also of interest. A preliminary finding common to these studies was the rapid occurrence (within 1 h of administration) of two metabolite peaks both of higher polarity than the parent compound (Fig. 3). The terminal elimination half-lives (above 24 h) were estimated to be about 13 h for the rats, 37 h for the beagle dog and 55 h for the male human volunteer.
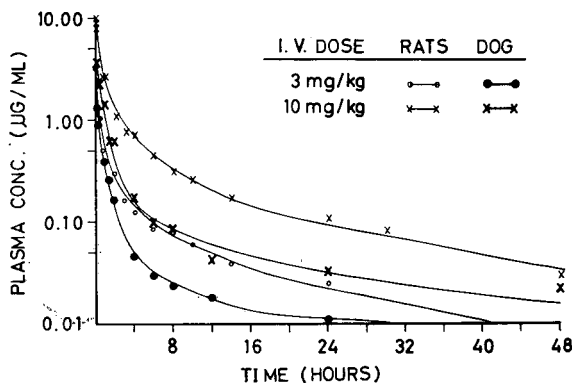
Fig. 4. Plasma concentration—time curves of fenflumizole for Sprague-Dawley rats and one beagle dog after the doses indicated. Each point on the curves for the rats is the mean of three animals, which were killed at this time.
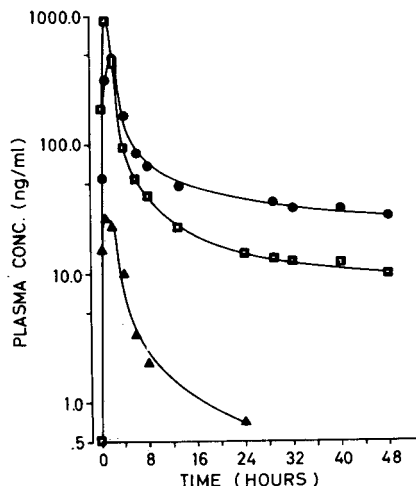
Fig. 5. Plasma concentration—time curves of fenflumizole for a male volunteer after ingestion of (▲) 0.1 mg/kg, (■) 2 mg/kg, and (●) 5 mg/kg.

CONCLUSIONS

Two assays have been developed to quantitate fenflumizole and metabolites in biological fluids. Fenflumizole is a potential non-steroidal anti-inflammatory agent, recently developed. The assays combine adequate sensitivity with appropriate precision and accuracy. The sample processing is limited to one extraction step. Precautions must be taken against light owing to photolability.

The validity of the analytical assays has been demonstrated in probe pharmacokinetic studies in rat, beagle dog and normal man. In forthcoming papers these applications will be discussed in detail.

REFERENCES

1 J.G. Lombardino and E.H. Wiseman, J. Med. Chem., 17 (1974) 1182.
2 T. Corell and G. Hasselmann, Acta Pharmacol. Toxicol., (1983) in press.

CHROMBIO. 1845

DETERMINATION OF QUINIDINE IN SERUM BY SPECTROFLUOROMETRY, LIQUID CHROMATOGRAPHY AND FLUORESCENCE SCANNING THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Quinidine is determined in serum by direct and extraction spectrofluorometry, by reflectance fluorescence scanning thin-layer chromatography (TLC), and by high-performance liquid chromatography (HPLC). Least-squares analyses of patients' sera ($n = 62$) analyzed first by direct fluorometry (x) and then HPLC (y) gave a slope of 0.52, an y-intercept of −0.40, a standard error of estimate of 0.65, and a correlation coefficient of 0.83. Comparison of patients' sera ($n = 59$) determined by extraction fluorometry (x) and then HPLC (y) gave a slope of 0.998, an y-intercept of −0.175, a standard error of estimate of 0.30, and a correlation coefficient of 0.96. Comparison of patients' sera ($n = 36$) by HPLC (x) and then reflectance fluorescence scanning TLC (y) gave a slope of 0.837, an y-intercept of 0.152, and a correlation coefficient of 0.94. Methaqualone and oxazepam interfere with HPLC. Within-run precision is 1.6, 1.0, 5.2 and 3.0% by direct fluorometry, extraction fluorometry, TLC and HPLC while between-run precision is 5, 3.5, 9 and 6.0%, respectively.

INTRODUCTION

Quinidine, a naturally occurring alkaloid, is the dextrorotatory isomer of quinine. It acts as a myocardial depressant by depressing excitability, contractibility, and conduction velocity, and is used to treat cardiac arrhythmias. Quinidine has a narrow therapeutic range [1—7] and knowledge of the quinidine serum concentration is therefore required for effective therapy.

Analytical methods for quinidine determinations are characterized by a combination of sample-preparation techniques and of instrumentation required for detection and quantitation. Some of the first methods were developed to measure the naturally occurring fluorescence of quinidine and they were very simple and sensitive. An example is the protein precipitation—fluorescence method of Brodie and Udenfriend [8]. Because of its simplicity, this method is non-selective. Quinidine metabolites are not separated or distinguished from quinidine in the sample and other drugs that fluoresce will interfere with this fluorometric analysis. The therapeutic range is 4—8 mg/l [1].

In attempts to improve the analytical selectivity of quinidine methods, various extraction techniques and solvents to preferentially remove the parent drug from the metabolites have been developed. In most of these methods the sample is made alkaline and then extracted with an organic solvent. Generally, these methods employed benzene [2,9,10], ethylene dichloride [9,11], chloroform [8,13], toluene [14] and an amylalcohol—benzene mixture [12,15]. In some studies, the organic extract was acidified with trichloroacetic acid [1,11] whereas in others, the organic extract was re-extracted with acid and subsequently the acidic solution was measured spectrofluorometrically [9,10,14,16]. The most commonly used extraction procedure is the double-extraction method of Cramer and Isaksson [9]. Many interferents and the more polar metabolites of quinidine are eliminated by this double-extraction procedure [9,10]. The therapeutic range is 2—5 mg/l when determined by this method.

Although the selectivity for quinidine analysis is improved by the single- and double-extraction procedures, there are still some disadvantages. Dihydroquinidine and quinidine are not separated and the extraction steps increase the overall length of time for completion of the analysis. Nevertheless, the acquired selectivity outweighs the disadvantage of the increased time for analysis. A double-extraction method is also more applicable to urine samples.

A large group of quinidine methods are based on some type of chromatographic technique. Examples of these types of methods are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and gas chromatography—mass spectrometry (GC—MS). For TLC, the sample may either be directly applied (spotted) on the TLC plate [17,18] or the sample may be extracted and the concentrated extract be applied to the TLC plate [12,19]. Quantitation is done by scraping off the separated compounds of interest from the adsorbent. The concentrated extracts are then measured for quinidine with a fluorometer [3,12,15,20—22]. An alternative to extracting the TLC plate is to directly scan the plate while monitoring either fluorescence [17,18,23] or ultraviolet absorption [19,24].

TLC methods for quinidine are selective and sensitive, with the precision of some of the methods being similar to HPLC. Dihydroquinidine and quinidine metabolites are separated from quinidine by TLC. A major disadvantage to TLC in the past was the lack of an appropriate instrument for detection and quantitation without having to remove the compound from the TLC plate. Our TLC method for quinidine uses a dual-channel reflectance fluorescence scanning spectrodensitometer and circumvents the previous difficulties.

The requirement for more sensitive and selective analytical methods for

quinidine has produced a variety of HPLC methods that use fluorescence [5,25—30] and ultraviolet [31—41] detection. Some of these methods provide the separation and quantitation of quinidine, dihydroquinidine, and metabolites such as 2′-quinidinone, 3-hydroxyquinidine, quinidine-N-oxide and O-desmethylquinidine [5,25,27,28,30,42,43]. A method based on the direct injection of the sample without any type of extraction has been reported [32].

A limited number of GC methods for quinidine have been developed [13,15, 23,44,45,46]. They all require a flame ionization detector except one [15] which used a nitrogen-selective detector. Because of the poor detectability of quinidine by GC the parent drug is usually derivatized either as the trimethylsilyl derivative [13,23] or the methyl derivative [44—46]. Quinidine and dihydroquinidine could not be distinguished by one of the methods [46].

Several attempts have been made to determine quinidine by MS or GC—MS. One method required isobutane chemical ionization [47]. In this method quinidine is extracted with benzene from alkalinized plasma and the extract is evaporated to dryness. The residue is then put into the mass spectrometer by direct insertion probe. Another technique is methane chemical ionization [19]. Although GC—MS methods are sensitive and specific, the instrumentation is very expensive and requires experienced personnel for its operation and maintenance. For the present, these methods can only be used for research studies. The most recently developed analytical method for quinidine is enzyme immunoassay [48,49]. A comparison of EMIT* (enzyme multiplied immunoassay technique) with the fluorescence method of Cramer and Isaksson [9] and HPLC [27,50] indicates that the EMIT method is not specific for quinidine [49], but the method is acceptable for clinical use.

Quinidine therapeutic levels will vary depending on which one of the above analytical methods is used for quantitation. For example, the reported therapeutic range is 4—8 mg/l when determined by the non-specific fluorometric methods [1,6,9,10]. As the methods become more selective, the range narrows from 2.3 to 5 mg/l [2,51]. In a study in which HPLC was used the levels were 50% of that found in the direct fluorometric methods [34]. Those methods that do not separate quinidine from dihydroquinidine may still be useful because both compounds have similar pharmacokinetics and activities [3,52,53]. Regardless of the final method selected for quinidine determinations, the resulting therapeutic range should be a guide to the physician and will have to be interpreted in conjunction with clinical signs and symptoms of the patient.

Quinidine concentrations above 10 mg/l are associated with toxic effects [1,54—56]. About one-third of the treated patients will have an immediate adverse intolerance to quinidine and therapy will have to be altered. Precautions should be taken when patients with congestive heart failure and impaired renal function are treated with quinidine because of the persistence of elevated serum levels of quinidine [57] and its metabolites [2]. As concentrations of quinidine in plasma go above 2 mg/l, the QRS complex** widens progressively. This change is useful for the monitoring of patients. A 25%

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*EMIT is the trade name for the enzymatic immunoassay system marketed by SYVA, Palo Alto, CA, U.S.A.
**QRS complex is the ventricular electrical activity of the heart.

increase of QRS complex is a cause for concern and with a 50% increase, the dosage should be immediately reduced. The most common adverse reactions to quinidine are nausea, vomiting and diarrhea [55,56,58]. Cinchonism, which includes tinnitus, loss of hearing, slight blurring of vision and gastrointestinal disturbances, is caused by an overdose of quinidine. If toxicity is severe enough, confusion, delirium and psychosis may occur.

Pharmacokinetic data of quinidine and its metabolites indicate that a two-compartment open model best describes the pharmacokinetics of quinidine [20,52]. The major route of metabolism appears to be in the liver since renal excretion of intact drug accounts for only 10—20% of the given dose which is 10—20 mg/kg d [14,20,21,26]. The distribution half-life is about 6—12 min and the elimination half-life is about 4—8 h [2,20,21,26,59,60]. The elimination half-life does not appear to vary in patients with congestive heart failure [2,26] or poor renal function [2,61]. The apparent volume of ditribution is about 3 l/kg and the central-compartment volume is 0.9 l/kg [14,20,26]. The total body clearance is about 4.5 ml/min/kg with great variation being observed among patients [6,20,26,50,60]. Some of the metabolites of quinidine that have been identified are 3-hydroxyquinidine [62], 2'-quinidinone [13,62,63], O-desmethylquinidine [4,13,25,62,64], and quinidine-N-oxide [6,36]. The major end-products of quinidine metabolism are 3-hydroxyquinidine and 2'-quinidinone. About 1—2% of quinidine goes to O-desmethylquinidine and is excreted in the urine [4]. Studies have revealed that some of the metabolites have anti-arrhythmic activity but definitive human data is questionable [25,27,50].

With the acceptance of newer analytical methodology for quinidine, investigators are re-evaluating the pharmacokinetic data, the interaction of quinidine and other drugs such as digoxin [65—69], digitoxin [70,71], propranolol [72], and phenytoin [73], and the effect of other drugs in the presence of quinidine [40,74].

In this study four analytical methods for quinidine are compared. Two of the methods, the fluorometric ones, are currently used in our laboratories. The HPLC method is a recently developed method by our laboratory and the fluorescence scanning—TLC method that uses a spectrodensitometer with dual-channel reflectance fluorescence capabilities was developed during this investigation.

MATERIALS AND METHODS

Apparatus

A Model J4-8963 ellipsoidal condensing system spectrofluorometer and a Model J10-280 photomultiplier microphotometer equipped with a 1P21 photomultiplier tube (all from American Instrument, Silver Spring, MD, U.S.A.) were used. A Model SD 3000 scanning spectrodensitometer equipped with a QPM 30 quartz prism monochromator, an SDA-335 reflection mode assembly and a Model SDC 300 density computer (all from Schoeffel Instrument Division, Kratos, Westwood, NJ, U.S.A.) were used for the fluorescence TLC measurements.

For high-performance liquid chromatography (HPLC) we used a Model

6000-A solvent-delivery system, Model 600 solvent programmer, Model 440 fixed-wavelength absorbance detector (254 nm), Model 450 variable-wavelength absorbance detector, Model U6K universal liquid chromatograph injector (all from Waters Assoc., Milford, MA, U.S.A.) and a Model B5217-1 dual-pen recorder (Houston Instrument Division, Bausch and Lomb, Austin, TX, U.S.A.). HPLC separations were done on a prepacked 10-μm particle size μ-Bondapak C_{18} (300 × 3.9 mm I.D.) reversed-phase column from Waters Assoc.

TLC separations were done on 20 × 20 cm, Type LK5D, precoated silica gel plates with a preadsorbent area (Whatman, Clifton, NJ, U.S.A.). Tanks were equilibrated with the solvent prior to use.

Reagents

All solutions were prepared in glass-distilled de-ionized water from analytical or spectral grade reagents and solvents (Burdick and Jackson, Muskegon, MI, U.S.A.) unless otherwise stated.

Metaphosphoric acid, 20% (w/v): dissolve 20 g of metaphosphoric acid in 80 ml of water. Sulfuric acid, 0.05 M: dilute 2.8 ml of concentrated sulfuric acid to 1 l with water. Sulfuric acid in methanol, 0.05 M: dilute 0.28 ml of concentrated sulfuric acid in 40 ml of water, then dilute to 100 ml with methanol. Sulfuric acid, 10% (v/v): mix 10 ml of concentrated sulfuric acid with 90 ml of water. Sodium hydroxide, 0.5 M: dissolve 20 g of sodium hydroxide pellets in 1 l of water. Isoamyl alcohol in n-heptane, 1.5% (v/v): dissolve 1.5 ml of isoamyl alcohol in 100 ml of n-heptane (HPLC grade). Other solutions were: diethyl ether (anhydrous), methanol (HPLC grade), ethyl acetate, ethanol (absolute), 1-butanol, and ammonium hydroxide (concentrated).

HPLC mobile phase: 1-octanesulfonic acid 0.005 M in methanol—water (60:40, v/v). Dissolve 2.16 g of 1-octanesulfonic acid sodium salt (Eastman Organic Chemicals, Eastman Kodak, Rochester, NY, U.S.A.) in 800 ml of water and then filter through a Type HA Millipore filter (Millipore, Bedford, MA, U.S.A.). Dilute the filtered solution to 2 l with methanol previously filtered through a Type LS filter, also from Millipore. Adjust the pH to 3.5 with 0.05 M sulfuric acid in methanol.

TLC developing solvent: ethyl acetate—absolute ethanol—1-butanol—concentrated ammonium hydroxide (56:28:4:0.5, v/v).

Standards

Quinidine sulfate (Sigma, St. Louis, MO, U.S.A.) and loxapine succinate (Lederle Laboratory Division, American Cyanamid, Pearl River, NY, U.S.A.) were used as the salts; however, all concentrations are expressed as the free base.

Quinidine stock standard, 100 mg/l: dissolve 115 mg of quinidine sulfate in 1 l of 0.05 M sulfuric acid. Quinidine working standards, 2, 4, and 8 mg/l: dilute 2, 4, and 8 ml of quinidine stock standard to 100 ml with water. These aqueous standards were used to reconstitute unassayed Normal Serum Control (Ortho Diagnostic Systems, Raritan, NJ, U.S.A.). Loxapine stock standard, 1 g/l: dissolve 34 mg of loxapine in 25 ml of ethanol. Loxapine (internal

standard), 15 mg/l: dilute 1.5 ml of loxapine stock solution to 100 ml with water.

Operating conditions

For spectrofluorometric analysis, the excitation and the emission wavelengths were 360 and 450 nm, respectively. The ellipsoidal condensing system slit width was 3 mm and all other slit widths were 2 mm. Sensitivity setting was between 90 and 100.

For TLC fluorescence scanning, the excitation and the emission wavelengths were 364 and 440 nm, respectively. The QPM monochromator slit was 0.5 mm. The spectrodensitometer was set in the reflectance mode. The TLC plates were scanned at 10 cm/min and the chart speed was set at 10.2 cm/min.

For HPLC analysis, the fixed and the variable wavelengths were 254 and 330 nm, respectively. The mobile phase was set at a flow-rate of 1.5 ml/min and the chart speed was set at 0.25 cm/min.

Procedures

Direct precipitation method. Metaphosphoric acid is added to diluted serum to precipitate the serum proteins and the fluorescence of the supernatant is read in a spectrofluorometer.

To a 50-ml glass-stoppered centrifuge tube were added 0.5 ml of serum, 19.5 ml of water, and 5.0 ml of 20% metaphosphoric acid. The mixture was shaken on a mechanical shaker (Eberbach Model 6000) for 15 min, then centrifuged for 30 min and the fluorescence of the supernatant was measured with a spectrofluorometer. The fluorescence of the patients' sera, the control sera, and the quinidine serum standards were corrected by the subtraction of the fluorescence of a serum blank which was taken through the procedure.

Extraction method. Quinidine is extracted from alkaline serum into an organic solvent and then back extracted into sulfuric acid. The fluorescence of the acidic extract is measured spectrofluorometrically. A micro-aliquot of the extract is submitted to TLC separation and fluorescence is measured with a TLC fluorescence scanner. A second aliquot of the acidic extract is made basic and then extracted with an organic solvent which is evaporated. The residue is redissolved in acidic methanol and analyzed by HPLC.

To a 50-mL glass-stoppered centrifuge tube were added 1.0 ml of serum, 2.0 ml of 0.5 M sodium hydroxide, and 15 ml of 1.5% isoamyl alcohol in n-heptane. The mixture was shaken on a mechanical shaker for 5 min and then centrifuged for 5 min. A 10-ml aliquot of the organic layer was transferred to a 15-ml screw-capped centrifuge tube containing 4.0 ml of 0.05 M sulfuric acid and the mixture was shaken on a mechanical shaker for 5 min. After 5 min of centrifugation, the organic layer was discarded and the fluorescence of the acidic aqueous phase was measured with a spectrofluorometer (single-extraction method). This acidic aqueous phase was also submitted to TLC and HPLC analyses.

A 3.0-ml aliquot of the acidic aqueous phase was transferred to 1 15-ml screw-capped centrifuge tube to which 2.0 ml of 0.5 M sodium hydroxide, 0.2 ml of 15 mg/l loxapine internal standard, and 10 ml of ethyl ether were added. The mixture was shaken on a mechanical shaker (low speed) for 2 min

and then centrifuged for 2 min. An 8-ml aliquot of the ether layer was transferred to a 12-ml screw-capped test tube and evaporated to dryness at 40°C. The residue was redissolved in 50 μl of 0.05 M sulfuric acid in methanol and a 25-μl aliquot was submitted to HPLC analysis.

A 20-μl aliquot of the acidic aqueous phase was spotted on a silica gel TLC plate and developed to 10 cm in a mixture of ethyl acetate—ethanol—1-butanol—ammonium hydroxide (56:28:4:0.5, v/v). The plate was then sprayed lightly with 10% sulfuric acid and heated to 70°C for 5 min. TLC—fluorescence scans of the plate were made with the spectrodensitometer set in the fluorescence reflectance mode.

For all three analyses, spectrofluorometry, TLC—fluorescence scanning and HPLC, standard curves were obtained by analyzing serum standards containing known amounts of quinidine and in the case of HPLC, a known amount of loxapine internal standard. For each set of patients' sera that were analyzed, a serum blank, serum controls and three quinidine serum standards (2, 4 and 8 mg/l) were analyzed. The results of the serum standards were used to draw the standard curve as a check on linearity. However, quinidine concentrations of the patients' sera were not obtained from the curve, but rather by using the nearest serum standard which was usually the 2 mg/l serum standard.

For HPLC analysis the ratios of the peak heights of quinidine to that of loxapine (the internal standard) were used to calculate quinidine concentrations. For the TLC fluorescence analysis, the peak heights for quinidine in the patients' sera were compared with those of the serum standards, and in the spectrofluorometric analysis the relative fluorescence intensities were compared after correcting for background interferences.

RESULTS

Spectrofluorometric analysis

Direct precipitation method. When the relative fluorescence intensity of quinidine serum standards, 3—6 mg/l, were plotted against concentration the resulting line had a slope of 1.33, an y-intercept of 0.02, a standard error of estimate (S_{yx}) of 0.008 and a correlation coefficient of 1.0. Within-run precision at the 3 mg/l concentration ($n=5$) averaged 1.6% while between-run precision averaged 5% ($n=29$).

Single-extraction method. Duplicate quinidine serum standards were determined by the single-organic extraction—fluorescence method using isoamyl alcohol in n-heptane. When the relative fluorescence intensity of quinidine serum standards were plotted against concentration (0.4—10 mg/l), the resulting line had a slope of 3.48, an y-intercept of 0.051, a standard error of estimate of 0.15, and a correlation coefficient of 0.999 ($n=15$).

The daily, within-run, and the day-to-day, between-run, variability of the single-organic extraction—fluorescence method was 1% ($n=6$) and 3.5% ($n=18$) at the 3 mg/l concentration. The absolute percent recovery was 59 ± 1% while the relative percent recovery using serum standards was 100 ± 1% ($n=8$).

124
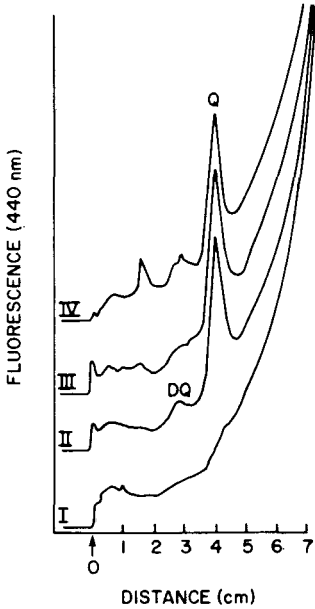
Fig. 1. TLC fluorescence scans of a serum blank (I), a serum standard with 2 mg/l of quinidine (II), a serum control with 2.1 mg/l of quinidine (III) and a patient's serum with 1.5 mg/l of quinidine (IV). Arrow marks origin of TLC plate. Q = quinidine, DQ = dihydro-quinidine.
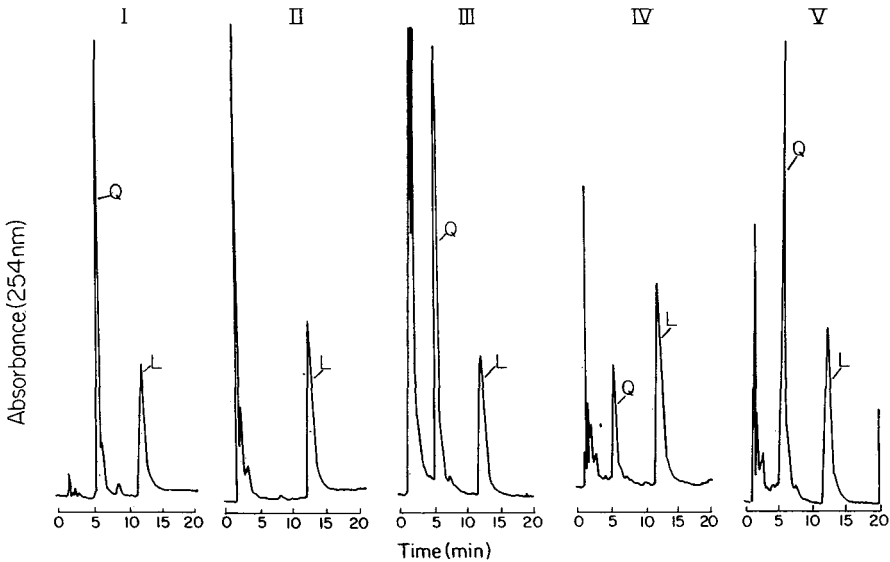
Fig. 2. Liquid chromatograms of a pure 0.1 g/l mixture of quinidine (Q) and loxapine (L) (I), a serum blank (II), a serum standard with 3 mg/l of quinidine (III), a patient's serum with 0.6 mg/l of quinidine (IV) and a patient's serum with 2.7 mg/l of quinidine (V). Q = quinidine, L = loxapine internal standard (0.8 mg/l). Conditions: C-18 reversed-phase column; mobile phase, methanol—water (60:40, v/v) with 5 mmol octanesulfonic acid; flow-rate, 1.5 ml/min; 25 μl injected.

TLC fluorescence scanning

TLC fluorescence scans of a serum blank, a quinidine serum standard, a serum control, and a patient's serum are shown in Fig. 1. R_F values are 0.46 and 0.59 for dihydroquinidine and quinidine, respectively.

When peak heights of quinidine serum standards, subjected to single organic extraction and then thin-layer chromatography followed by fluorescence reflectance scanning, were plotted against concentration (0.4—10 mg/l), the resulting line had a slope of 10.0, an y-intercept of −3.75, a standard error of estimate of 3.25, and a correlation coefficient of 0.995 ($n=14$).

The daily, within-run, precision of the TLC—fluorescence method ($n=5$) was 5.2% while the day-to-day, between-run, variability ($n=7$) was 9.2% at the 3 mg/l concentration. The relative percent recovery using serum standards was 95 ± 14% ($n=5$).

HPLC analysis

High-performance liquid chromatograms at 254 nm of a pure mixture, a serum blank, a serum standard and two patients' sera are given in Fig. 2. Similar results were observed at 330 nm. Retention times are 0.49 and 0.55 for quinidine and dihydroquinidine, relative to loxapine, 13.0 min.

Peak height ratios of quinidine to that of loxapine (the internal standard) were calculated for quinidine serum standards subjected to the double-organic extraction HPLC method using n-heptane isoamyl alcohol and then ethyl ether. When peak height ratios determined at 254 nm were plotted against the concentration of quinidine serum standards (0.8—10 mg/l), the resulting line had a slope of 0.98, an y-intercept of 0.38, a standard error of estimate of 0.27, and a correlation coefficient of 0.996 ($n=6$). If peak height ratios measured at 330 nm were used, then the resulting line had a slope of 0.81, an y-intercept of 0.74, a standard error of estimate of 0.38, and a correlation coefficient of 0.987 ($n=5$).

The daily and day-to-day precision of the double-organic extraction HPLC method as determined by using a 3 mg/l serum control was 3% ($n=6$), 6% ($n=17$) and 4% ($n=5$), 5% ($n=15$) at 254 and 330 nm, respectively. The relative percent recovery using serum standards was 100 ± 7% ($n=6$).

Interference studies

Blank serum samples, toxicology serum controls (Hyland Diagnostics, Deerfield, IL, U.S.A.), and eighteen serum samples from patients who were not being treated with quinidine were analyzed in the same manner as the serum standards and sera from quinidine-treated patients. The HPLC analysis of one patient's serum (quinidine untreated) gave a peak where quinidine was usually found when detected at 254 nm. The peak was equivalent to 2.3 mg/l of quinidine. However, when calculations were made from the same peak detected at 330 nm, the peak was equivalent to 0.9 mg/l of quinidine. The use of two wavelengths to check for HPLC interference is therefore very important. The following drugs interfere with quinidine by HPLC: oxazepam, methaqualone, nordiazepam and p-chlorodisopyramide. The following drugs will not interfere by HPLC, TLC spectrofluorometry, and EMIT: procainamide, salicylate, diazepam, sulfanilamide, ethchlorvynol, meprobamate, chlordiazepoxide,

TABLE I

STATISTICAL ANALYSIS OF THE COMPARISON BETWEEN QUINIDINE METHODS

PPT—Fluores = protein precipitation—fluorometric method; SE—Fluores = single-organic extraction—fluorometric method; DE—HPLC = double-organic extraction—HPLC method; SE—TLC Fluores = single-organic extraction—TLC fluorescence scanning method; EMIT = enzyme multiplied immunoassay technique.

Method (x) vs. method (y)	n	Regression equation ($y = bx + a$)	Standard error of estimate (S_{yx})	Correlation coefficient (r)
PPT—Fluores vs. SE—Fluores	92	$y = 0.58x - 0.31$	0.65	0.87
PPT—Fluores vs. DE—HPLC (254 nm)	62	$y = 0.52x - 0.40$	0.65	0.83
PPT—Fluores vs. SE—TLC Fluores	36	$y = 0.389x - 0.088$	0.595	0.74
SE—Fluores vs. DE—HPLC (254 nm)	59	$y = 0.998x - 0.175$	0.30	0.96
SE—Fluores vs. SE—TLC Fluores	36	$y = 0.887x - 0.034$	0.396	0.90
DE—HPLC vs. SE—TLC Fluores	36	$y = 0.837x + 0.152$	0.294	0.94
EMIT vs. DE—HPLC (254 nm)	17	$y = 0.90x - 0.27$	0.49	0.95
SE—Fluores vs. EMIT	17	$y = 1.15x - 0.072$	0.28	0.98

glutethimide, hydantoin, propoxyphene, amobarbital, phenobarbital, secobarbital, pentobarbital, flurazepam, amitriptyline and doxepin. The best solution to interference problems is to use another technique for analysis at the end of the sample preparation step as previously described [75].

Comparison of methods

Sera of patients receiving quinidine were analyzed by the four methods and the levels of quinidine were compared. Least-squares analysis was used to calculate the slope, y-intercept, standard error of estimate, S_{yx}, and correlation coefficient for each pair of methods. A summary of the statistical analysis is given in Table I. Included in Table I are also the results of patients' analysis using the enzymatic immunoassay technique (EMIT). Excellent agreement was observed between the fluorescence extraction method and HPLC, TLC, and EMIT (correlation coefficients, r, of 0.96, 0.90 and 0.98). Comparison of patients' sera between HPLC and TLC, and EMIT and HPLC, gave also a high correlation ($r = 0.94$ and 0.95).

DISCUSSION

In this study a comparison was made of four different analytical methods for the determination of quinidine in serum. The methods varied in the preparation

of the sample for analysis and in the analytical instrumentation for the detection and quantitation of quinidine. Three of the methods, HPLC, TLC and extraction fluorescence required two or more liquid—liquid extractions whereas the fourth method required the serum sample to be deproteinated with metaphosphoric acid and the resulting supernatant to be analyzed by spectro-fluorometry. Traditionally, this method is called the direct fluorometric method of Brodie and Udenfriend [8], but in this report an abbreviated identification (PPT—Fluores method) is used. Quinidine levels determined by this method are known as Q_P values.

Two of the quinidine methods were identical in sample preparation, but the instrumentation which was used for quantitation was different. Quinidine was extracted from alkalinized serum with an organic solvent and then re-extracted into acid. If the acidic solution was analyzed by spectrofluorometry, then the procedure is called the single-organic extraction—fluorometric (SE—Fluores) method and the quinidine concentrations obtained are known as Q_E values. In the literature, this method is often called the double-extraction—fluorometric method of Cramer and Isaksson [9]. The original method used benzene as the extraction organic solvent. Because benzene is a substance posing a potential occupational carcinogenic risk and is currently regulated by the Occupational Safety and Health Administration (OSHA), the SE—Fluores method in this study employed isoamyl alcohol in n-heptane as an alternative to the carcinogen benzene. Isoamyl in n-heptane has not been previously used as an extraction solvent for quinidine. In order to ascertain its analytical accuracy as an extraction solvent, we used two chromatographic techniques, TLC and HPLC after the extraction procedure to measure quinidine concentrations in serum.

If the above acidic solution was subjected to thin-layer chromatography and the quinidine on the TLC plate was measured by a spectrodensitometer in the reflectance fluorescence mode, then the procedure is identified as the single-extraction—TLC fluorescence scanning (SE—TLC Fluores) method. Quinidine levels obtained by this method are referred to as Q_{TLC} values.

The fourth method in this study was an extension of the single-organic extraction method previously described. An aliquot of the resulting acidic solution from those procedures was made basic and extracted with diethyl ether. The extract was concentrated and analyzed by HPLC with ultraviolet absorbance detection. In this report, this procedure is known as the double-organic extraction—HPLC (DE—HPLC) method and its quinidine concentrations are given as Q_{HPLC} values. Lastly, we compared our method to the EMIT system since it is a commonly used technique for quinidine analysis.

Precision was estimated for three of the methods based on the statistical analysis of the concentration of quinidine in a serum control. The mean, standard deviation, and coefficient of variation (C.V.) were calculated for each of three daily (within-run) and day-to-day (between-run) determinations on five and seventeen identical serum controls, respectively. For the SE—Fluores method, the daily C.V. was in a range of 0.7—1.7% whereas the day-to-day C.V. was 2.2%. The DE—HPLC method gave a daily C.V. range of 3.0—6.0% and a day-to-day C.V. of 6.0%. For the SE—TLC Fluor method the daily C.V. was in a range of 5.2—8.9% and the day-to-day C.V. was 9.2%. These results

indicated that the SE—Fluores method gives the least variation of all the analytical methods studied.

Quinidine concentrations in the sera of patients receiving quinidine therapy were determined simultaneously by the four methods described in this report and EMIT. The results obtained were evaluated by the least-squares linear regression method (Table I). The statistical analysis included the calculation of a slope (b), an y-intercept (a), a standard error of estimate (S_{yx}) and a correlation coefficient (r). The PPT—Fluores method when compared with the three extraction methods gave correlation coefficients between 0.74 and 0.87. Good agreement, reflected by a correlation coefficient of 0.96 and a slope of 0.99, was noted between the SE—Fluores and DE—HPLC methods. A correlation coefficient of 0.94 and standard error of estimate of 0.294 were associated with the comparison between the DE—HPLC and SE—TLC Fluores methods. Consistently high correlations were observed between EMIT and our extraction procedure: 0.95 and 0.98 (Table I).

The above results confirm that our extraction fluorescence procedure with isoamyl alcohol n-heptane gives comparable results to HPLC, EMIT and TLC. As expected, a poorer correlation was observed between the extraction procedure and the direct precipitation method since the later measures quinidine and its metabolites. What is interesting, however, is the fact that a significant correlation of 0.87 was observed between the non-selective precipitation method and the extraction procedure, which suggests that in patients with normal renal function the concentration of quinidine metabolites present in serum are constant. The high correlation between the extraction fluorescence method and HPLC, 0.96 or TLC, 0.90, confirms that the extraction procedure extracts primarily quinidine and dihydroquinidine and none of the other metabolites. The R_F values for the isolated quinidine and dihydroquinidine by TLC fluorescence scanning were 0.59 and 0.46, respectively. The retention times for quinidine and dihydroquinidine by HPLC were 6.4 and 7.2, respectively. Using n-heptane and isoamyl alcohol as an extraction solvent therefore eliminates interference from metabolites and serves as a selective solvent for the analysis of quinidine in serum. The use of HPLC and TLC after the extraction procedure does not add any specificity to the procedure except for the separation of quinidine from dihydroquinidine.

Although fluorometry, TLC, and HPLC have been used to measure quinidine, this study has incorporated several improvements. The double-organic extraction methods will leave behind 3-hydroxyquinidine and other metabolites, but not dihydroquinidine. The use of isoamyl alcohol in n-heptane as an extracting solvent is not as biohazardous as benzene. The use of loxapine as an internal standard and 1-octanesulfonic acid in a methanol—water mixture as a mobile phase in the HPLC analysis, and the use of the TLC conditions with reflectance fluorescence scanning to quantitate quinidine have not been previously reported.

In the past, quantitative TLC analysis for quinidine has been limited because of a lack of appropriate instrumentation, the use of involved sample preparation steps, and difficulties in the standardization of the results. Some of the early problems were caused by the coating materials and the plates. Poor reproducibility occurred due to non-uniform plate thickness and the varying

consistency of the coating materials. After TLC separation, the absorbent had to be scraped from the plate, the compounds of interest had to be eluted from the absorbent, and, finally, the eluant was subjected to spectrophotometry for quantitation. This technique was time-consuming and required a large sample size. With the advent of scanning densitometers, the ultraviolet absorbance or density was measured directly from the plate without removal of the chromatographed compounds from the plate.

In this study, a TLC analysis for quinidine was formulated that surmounted these obstacles. The chromatographic aspect of the analysis was improved by the use of precoated silica gel plates with a preadsorbent area and nineteen channels premarked on the plate. Development time for 10 cm was 30 min. The preadsorbent allows the developing solvent to extract and concentrate the applied sample and thus presents the sample to the silica gel adsorbent layer as a uniform, concentrated band. Improved quantitation was possible because the scanning densitometer consists of photomultipliers in the reference channel as well as the sample channel that intercept emitted light from the surface of the TLC plate being scanned. This interception takes place at 45° to the normal angle of incidence of the exciting radiation. This type of instrument configuration, TLC reflectance spectrofluorescence scanning, will minimize any differences on the TLC plate and any variation that occurs with the lamp power supply or the characteristics of the lamp itself. The above instrumentation and TLC plates resulted in an inexpensive, sensitive, selective and precise procedure for the determination of quinidine in serum.

The choice of which method to use for the determination of quinidine in serum should be determined on the basis of the particular analytical need; sample size, turn around time, equipment required, costs, personnel requirements, and likely interferents. For example, the spectrofluorometric precipitation method is simple, fast and does not require sophisticated instrumentation, but is non-specific. Even with a single or double organic extraction, quinidine and dihydroquinidine are not separated. On the other hand, the HPLC analysis which is more selective, is time-consuming, expensive, requires sophisticated instrumentation, but does separate quinidine from dihydroquinidine and other interferents. The double extraction affords a cleaner sample to analyze than the protein precipitation method. The TLC method has advantages over both previously described methods. Quantitative TLC is not unreasonably long or involved; the spectrodensitometer is moderately priced and simple to operate. Quinidine is separated from the dihydroquinidine and other interferents. The selection of both excitation and emission wavelengths gives improved selectivity whereas fluorescence gives an increased sensitivity. In TLC each sample is separated on new absorbent, but in HPLC the column is used many times with the expectation that all of the excess endogenous materials can be removed. The ability to remove the developing solvent before scanning the TLC plate eliminates the incompatibility that may be found between the mobile phase and the ultraviolet detector of an HPLC system. TLC analysis time can be reduced by scanning only the area of interest on the TLC plate. TLC scanning is not without some disadvantages such as the small range of linear correlation between sample concentration and detection signal. Also, 10 cm may not be enough distance to separate interfering substances from the compound of interest.

Interference studies showed that various basic drugs will not interfere with the present procedures (TLC, EMIT and spectrofluorometry), except for oxazepam, methaqualone, nordiazepam and *p*-chlorodisopyramide which will interfere with quinidine by HPLC. A scheme to the analysis of quinidine in serum from patients on quinidine therapy may initially include the determination of the drug by a protein precipitation fluorometric method. If the result is above the therapeutic range of 4—8 mg/l, then one may wish to consider an alternative method such as the single-organic extraction method followed by spectrofluorometry, although the TLC procedure is warranted. If the new result is still high, then the double organic extraction and HPLC analysis may be in order before making a decision to reduce the therapeutic dose in a patient. In our experience the direct precipitation method and the organic extraction procedure are simple to perform and give more useful information on parent drug and metabolite concentration than either procedure by itself. One would rarely have to go beyond these two procedures for monitoring of quinidine therapy.

ACKNOWLEDGEMENT

REFERENCES

1 M. Sokolow and A.L. Edgar, Circulation, 1 (1950) 570.
2 K.M. Kessler, D.T. Lowenthal, H. Warner, T. Gibson, W. Briggs and M. Reidenberg, N. Engl. J. Med., 290 (1974) 706.
3 C.T. Ueda, B.J. Williamson and B.S. Dzindzio, Res. Commun. Chem. Pathol. Pharmacol., 14 (1976) 215.
4 D.E. Drayer, D.T. Lowenthal, K.M. Restivo, A. Schwartz, C.E. Cook and M.M. Reidenberg, Clin. Pharmacol. Ther., 24 (1978) 31.
5 N. Weidner, J.H. Ladenson, L. Larson, G. Kessler and J.M. McDonald, Clin. Chim. Acta, 91 (1979) 7.
6 T.W. Guentert, R.A. Upton, N.H.G. Holford and S. Riegelman, J. Pharmacokinet. Biopharm., 7 (1979) 303.
7 H.R. Ochs, D.J. Greenblatt and E. Woo, Clin. Pharmacokinet., 5 (1980) 150.
8 B.B. Brodie and S. Udenfriend, J. Pharmacol. Exp. Ther., 78 (1943) 154.
9 G. Cramer and B. Isaksson, Scand. J. Clin. Lab. Invest., 15 (1963) 553.
10 G. Härtel and A. Harjanne, Clin. Chim. Acta, 23 (1969) 289.
11 A.L. Edgar and M. Sokolow, J. Lab. Clin. Med., 36 (1950) 478.
12 G. Härtel and A. Korhonen, J. Chromatogr., 37 (1968) 70.
13 K.H. Palmer, B. Martin, B. Baggett and M.E. Wall, Biochem. Pharmacol., 18 (1969) 1845.
14 D.J. Greenblatt, H.J. Pfeifer, H.R. Ochs, K. Franke, D.S. MacLaughlin, T.W. Smith and J. Koch-Weser, J. Pharmacol. Exp. Ther., 202 (1977) 365.
15 M.A. Moulin and H. Kinsun, Clin. Chim. Acta, 75 (1977) 491.
16 T. Huynh-Ngoc and G. Sirois, J. Pharm. Sci., 66 (1977) 591.
17 J.M. Steyn and H.K.L. Hundt, J. Chromatogr., 111 (1975) 463.
18 J. Christiansen, J. Chromatogr., 123 (1976) 57.
19 B. Wesley-Hadzija and A.M. Mattocks, J. Chromatogr., 144 (1977) 223.
20 C.T. Ueda, D.S. Hirschfeld, M.M. Rowland, B.J. Williamson and B.S. Dzindzio, Clin. Pharmacol. Ther., 19 (1976) 30.
21 C.T. Ueda, B.J. Williamson and B.S. Dzindzio, Clin. Pharmacol. Ther., 20 (1976) 260.
22 C.T. Ueda, B.E. Ballard and M. Rowland, J. Pharmacol. Exp. Ther., 200 (1977) 459.

23 E. Smith, S. Barkan, B. Ross and M. Maienthal, J. Pharm. Sci., 62 (1973) 1151.
24 K.Y. Lee, D. Nurok, A. Zlatkis and A. Karmen, J. Chromatogr., 158 (1978) 403.
25 D.E. Drayer, C.E. Cook and M.M. Reidenberg, Clin. Res., 24 (1976) 623A.
26 K.A. Conrad, B.L. Molk and C.A. Chidsey, Circulation, 55 (1977) 1.
27 D.E. Drayer, K. Restivo and M.M. Reidenberg, J. Lab. Clin. Med., 90 (1977) 816.
28 B.J. Kline, V.A. Turner and W.H. Barr, Anal. Chem., 51 (1979) 449.
29 S. Sved, I.J. McGilveray and N. Beaudoin, J. Chromatogr., 145 (1978) 437.
30 P.A. Reece and M. Peikert, J. Chromatogr., 181 (1980) 207.
31 W.G. Crouthamel, B. Kowarski and P.K. Narang, Clin. Chem., 23 (1977) 2030.
32 J.L. Powers and W. Sadee, Clin. Chem., 24 (1978) 299.
33 T.W. Guentert and S. Riegelman, Clin. Chem., 24 (1978) 2065.
34 J.L. Powers and W. Sadee, Clin. Chem., 24 (1978) 1007.
35 M.R. Bonora, T.W. Guentert, R.A. Upton and S. Riegelman, Clin. Chim. Acta, 91 (1979) 277.
36 T.W. Guentert, P.E. Coates, R.A. Upton, D.L. Combs and S. Riegelman, J. Chromatogr., 162 (1979) 59.
37 R.E. Kates, D.W. McKennon and T.J. Comstock, J. Pharm. Sci., 67 (1978) 269.
38 J.G. Flood, G.N. Bowers and R.B. McComb, Clin. Chem., 26 (1980) 197.
39 M.G. Horning, A.A. Taylor, E.C. Horning and S.E. Barrow, Fed. Proc. Fed. Amer. Soc. Exp. Biol., 39 (1980) 307.
40 J.T. Ahokas, C. Davies and P.J. Ravenscroft, J. Chromatogr., 183 (1980) 65.
41 J. Pao and J.A.F. de Silva, J. Chromatogr., 221 (1980) 97.
42 W.G. Crouthamel, B. Kowarski and P.K. Narang, Clin. Chem., 24 (1978) 1863.
43 T.W. Guentert, A. Rakhit, R.A. Upton and S. Riegelman, J. Chromatogr., 183 (1980) 514.
44 K.K. Midha and C. Charette, J. Pharm. Sci., 63 (1974) 1244.
45 D.H. Huffman and C.E. Hignite, Clin. Chem., 22 (1976) 810.
46 J.L. Valentine, P. Driscoll, E.L. Hamburg and E.D. Thompson, J. Pharm. Sci., 65 (1976) 96.
47 W.A. Garland, W.F. Trager and S.D. Nelson, Biomed. Mass Spectrom., 1 (1974) 124.
48 C. Collins, M. Hu, C. Crowl, D.S. Kabakoff and P. Singh, Clin. Chem., 25 (1979) 1093.
49 D.E. Drayer, B. Lorenzo and M.M. Reidenberg, Clin. Chem., 27 (1981) 308.
50 D.E. Drayer, M. Hughes, B. Lorenzo and M.M. Reidenberg, Clin. Pharmacol. Ther., 27 (1980) 72.
51 N.J. Carliner, M.L. Fisher, W.G. Crouthamel, P.K. Narang and G.D. Plotnick, Amer. Heart J., 100 (1980) 483.
52 C.T. Ueda and B.S. Dzindzio, Eur. J. Clin. Pharmacol., 16 (1979) 101.
53 C.T. Ueda, Biochem. Pharmacol., 29 (1980) 737.
54 C.F. Quenzer and D.R. Hardy, J. Anal. Tox., 4 (1980) 54.
55 A. Goth, Medical Pharmacology: Principles and Concepts, Mosby, St. Louis, MO, 9th ed., 1979, pp. 397—401.
56 J.T. Bigger and B.F. Hoffman, in A. Goodman-Gilman, L.S. Goodman and A. Gilman (Editors), The pharmacological Basis of Therapeutics, MacMillan, New York, 6th ed., 1980, pp. 768—774.
57 S. Bellet, L.R. Roman and A. Boza, Amer. J. Cardiol., 27 (1971) 368.
58 E. Woo and D.J. Greenblatt, Clin. Pharmacol. Ther., 27 (1980) 188.
59 H.R. Ochs, E. Grube, D.J. Greenblatt, E. Woo and G. Bodem, Amer. Heart J., 99 (1980) 468.
60 G. Sirois and M. Eshaque, Biopharm. Drug Disposition, 1 (1980) 167.
61 W.P. Swisher, H.G. Wedell, J.T.O. Cheng, G.C. Sutton and D.C. Sutton, Amer. Heart J., 47 (1954) 449.
62 F.I. Carroll, D. Smith and M.E. Wall, J. Med. Chem., 17 (1974) 985.
63 B.B. Brodie, J.E. Baer and L.C. Craig, J. Biol. Chem., 188 (1951) 567.
64 C.T. Ueda and J.G. Nickols, J. Pharm. Sci., 69 (1980) 1400.
65 D.S. Rich and L.P. Jeffrey, J. Amer. Med. Assoc., 242 (1979) 1850.
66 R. Dahlquist, G. Ejvinsson and K. Schenck-Gustafsson, N. Eng. Med., 301 (1979) 727.
67 E.B. Leahey, Jr., Ann. Intern. Med., 93 (1980) 775.

68 T. Chen and H.S. Friedman, J. Amer. Med. Assoc., 224 (1980) 665.
69 T.P. Gibson and H.A. Nelson, J. Lab. Clin. Med., 95 (1980) 417.
70 P.E. Fenster, J.R. Powell, P.E. Graves, K.A. Conrad, W.P. Hager, S. Goldman and F.I. Marcus, Ann. Intern. Med., 93 (1980) 698.
71 H.R. Ochs, J. Pabst, D.J. Greenblatt and H.J. Dengler, N. Engl. J. Med., 303 (1980) 672.
72 P. Fenster, D. Perrier, M. Mayersohn and F.I. Marcus, Clin. Pharmacol. Ther., 27 (1980) 450.
73 P. Jaillon and R.E. Kates, J. Pharm. Exp. Ther., 213 (1980) 33.
74 M.K. Poker, S. Chen and L.J. Marton, Ther. Drug Monitoring, 3 (1981) 91.
75 J. Vasiliades, C. Owens and F. Ragusa, Clin. Chem., 25 (1979) 1900.

CHROMBIO. 1819

Note

Volatile carboxylic acid profiling in physiological fluids

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Many inborn errors of metabolism are characterized by an increase in the concentrations of various carboxylic acids in blood and urine. For example, the metabolic disorders propionic acidemia [1], methylmalonic aciduria [2,3], maple syrup urine disease [4], isovaleric acidemia [4] and glutaric aciduria type II [5,6] show increased amounts of volatile carboxylic acids in blood or urine. Procedures for the profiling and/or quantitation of these carboxylic acids require their isolation from blood or urine by vacuum distillation [6], extraction [7,8], silicic acid absorption [9] or ion-exchange chromatography [10] prior to gas chromatographic (GC) or gas chromatographic—mass spectrometric (GC—MS) analysis. These procedures are time-consuming and may result in losses of the more volatile acids. We have developed a rapid procedure for the profiling of volatile carboxylic acids in blood and urine that involves neither extraction or derivatization of the carboxylic acids prior to GC analysis.

EXPERIMENTAL

Materials

All reference carboxylic acids were purchased either from Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.) either as the free acid or as the sodium salt and were used as received. The 10% SP-1200—1% H_3PO_4 on 80—100 Chromosorb W AW (1.83 m × 2 mm I.D.) and the 10% SP-1000—1% H_3PO_4 on 100—200 Chromosorb W AW (1.83 m × 2 mm I.D.) glass GC columns were purchased from Supelco (Bellefonte, PA, U.S.A.). A Varian 3700 gas chromatograph (Palo Alto, CA, U.S.A.) with a flame ionization detector was used for the volatile carboxylic acid profiling. The profiles were recorded on a Varian 9176 recorder.

Analytical procedure

GC columns were conditioned at 190°C with a nitrogen flow-rate of 30 ml/min. Periodic injection of a 1% formic acid solution was performed until a stable baseline resulted (1—2 days).

Urine profiling. To 1.00 ml of urine were added 10 μl of a 0.05 mmol/ml trimethylacetic acid (internal standard) [9] solution. The sample was acidified by the addition of 3—5 drops of 1 *M* hydrochloric acid. A 2-μl aliquot was injected onto the SP=1200 column. Column temperature, 90°C for 4 min then 5°/min to 200°C; injector temperature, 230°C; detector temperature, 230°C; nitrogen flow-rate, 30 ml/min.

Urine samples exhibiting an abnormal profile were analyzed on the SP-1000 column. A 1-μl aliquot of the sample was injected onto the SP-1000 column. Column temperature, 150°C; injector temperature, 230°C; detector temperature, 230°C; nitrogen flow-rate, 30 ml/min.

Peak identification was by the peak relative retention time (RRT) on each column and peak enhancement when the urine sample was spiked with the authentic reference compound.

Between sample injections, 10 μl of a 1% formic acid solution were injected onto the column to remove ghost peaks.

RESULTS AND DISCUSSION

Blood or urine samples are profiled initially on an SP-1200 column [11] using a temperature gradient. Volatile carboxylic acids that can be separated in this manner are shown in Fig. 1A. Table I lists the retention time and the RRT (relative to the internal standard) of each carboxylic acid. Butanoic acid appears as a shoulder on the internal standard peak (RRT slightly greater than 1.0) while 2-methylbutanoic and 3-methylbutanoic acids are not separated.

In Fig. 1B is shown the profile of an abnormal urine sample (due to valproic acid therapy) on the SP-1200 column. At this sensitivity, the profile of a normal sample would show only the internal standard peak. Abnormal samples detected by the first method are then analyzed isothermally (150°C) on an SP-1000 GC column [11] which permits resolution of a greater number of volatile carboxylic acids than can be separated under the previous conditions. These separations are shown in Fig. 2. In Table II are listed the retention time and RRT (relative to the internal standard) of each carboxylic acid on the SP-1000 column.

Fig. 3A shows the urinary volatile carboxylic acid profile from an infant with methylmalonic aciduria and Fig. 3B shows the plasma volatile carboxylic acid profile from the same infant. Under the GC conditions of the profiling procedure, methylmalonic acid quantitatively undergoes a thermal decarboxylation to propanoic acid [12]. Confirmation that the large propanoic acid peak in these profiles was due to methylmalonic acid and not propanoic acid was provided by a positive (purple) reaction of the urine and plasma samples with tetrazotized *o*-dianisidine (Fast Blue B) [13]. Methylmalonic acid reacts with tetrazotized *o*-dianisidine while propanoic acid does not react.

Without access to a gas chromatograph—mass spectrometer we are not able to confirm the identity of each peak in the chromatogram. Thermally labile
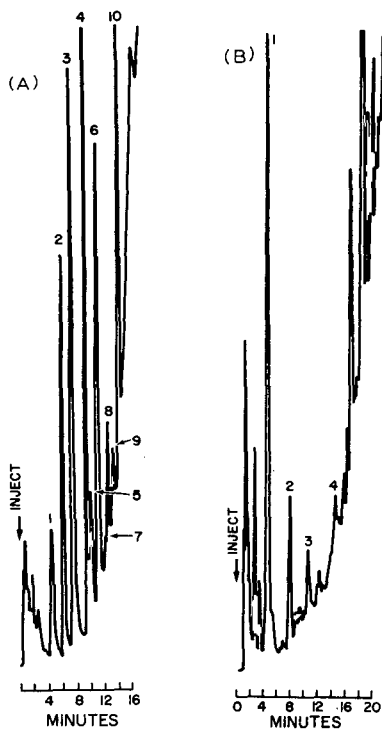
Fig. 1. A, Profile of the standard volatile carboxylic acid mixture on the SP-1200 column using a temperature program: 90°C for 4 min then 5°C/min to 200°C. Peaks: 1 = propanoic acid; 2 = isobutyric acid; 3 = internal standard, trimethylacetic acid; 4 = 2-methylbutanoic and 3-methylbutanoic acids; 5 = crotonic acid; 6 = pentanoic acid; 7 = β-methylcrotonic acid; 8 = tiglic acid; 9 = isocaproic acid; 10 = hexanoic acid. The concentration of each acid was 3 nmol/ml. B, Profile of an abnormal urine sample on the SP-1200 column (temperature program: 90°C for 4 min then 5°C/min to 200°C). Peaks: 1 = 3-heptanone (from valproic acid); 2 = internal standard, trimethylacetic acid; 3 = pentanoic acid; 4 = hexanoic acid and several unidentified peaks.

TABLE I

RETENTION TIMES (t_R) AND RRT OF THE VOLATILE CARBOXYLIC ACIDS ON THE SP-1200 COLUMN (90—200°C TEMPERATURE GRADIENT)

Organic acid	t_R (min)	RRT
Propanoic	4.0	0.58
Isobutyric	5.8	0.81
Internal standard	6.6— 7.2	1.00
Butanoic	6.6— 7.2	1.00
2-Methylbutanoic	8.6— 9.2	1.28—1.30
3-Methylbutanoic	8.6— 9.2	1.28—1.30
Crotonic	9.0—10.4	1.36—1.39
Pentanoic	10.2—11.0	1.53—1.55
β-Methylcrotonic	11.4—12.2	1.69—1.73
Tiglic	11.8—12.6	1.75—1.79
Isocaproic	12.6—13.2	1.83—1.91
Hexanoic	13.2—14.2	1.97—2.00
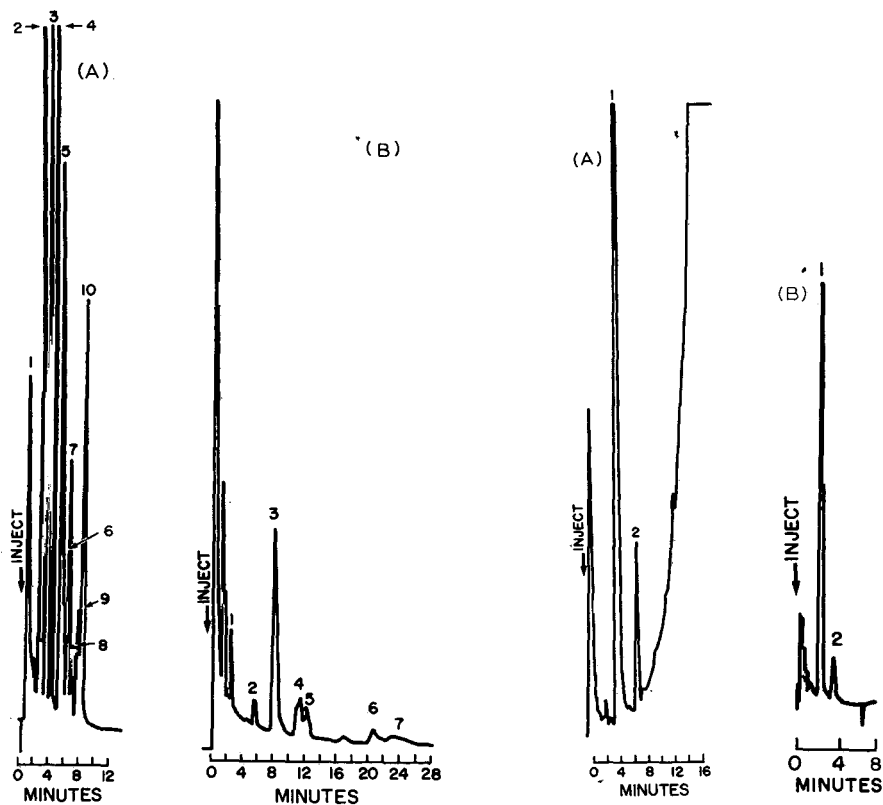
Fig. 2. A, Profile of the standard volatile carboxylic acid mixture on the SP-1000 column isothermal at 150°C. Peaks: 1 = propanoic and isobutyric acids; 2 = internal standard, trimethylacetic acid; 3 = butanoic acid; 4 = 2-methylbutanoic and 3-methylbutanoic acids; 5 = crotonic acid; 6 = pentanoic acid; 7 = β-methylcrotonic acid; 8 = isocaproic acid; 9 = tiglic acid; 10 = hexanoic acid. The concentration of each acid was 3 nmol/ml. B, Profile of the standard volatile α-ketocarboxylic acid mixture isothermally (150°C) on the SP-1000 column. Peaks: 1 = internal standard; 2 = pyruvic acid; 3 = α-ketoisovaleric acid and α-keto-butyric acid; 4 = α-ketovaleric acid and α-keto-β-methylvaleric acid; 5 = α-ketoisocaproic acid; 6 = succinic acid; and 7 = lactic acid. The concentration of each acid was 0.4 μmol/ml.

Fig. 3. A, Profile of a urine sample from an infant who had methylmalonic aciduria (SP-1200 column). Peaks: 1 = propanoic acid (11.0 μmol/ml) from the thermal decarboxylation of methylmalonic acid; 2 = internal standard, trimethylacetic acid. B, Profile of a plasma sample from an infant who had methylmalonic aciduria (SP-1200 column). Peaks: 1 = propanoic acid (17.4 μmol/ml) from the thermal decarboxylation of methylmalonic acid; 2 = internal standard, trimethylacetic acid. Attenuation $16 \cdot 10^{-11}$

carboxylic acids may decompose under these chromatographic conditions, thus altering peak identification. Each carboxylic acid, however, did afford a single peak with a reproducible relative retention time when analyzed by GC. Definitive identification of each peak should be determined by GC—MS.

TABLE II

RETENTION TIMES (t_R) AND RRT OF THE VOLATILE CARBOXYLIC ACIDS ON THE SP-1000 COLUMN (ISOTHERMAL AT 150°C)

Carboxylic acid	t_R (min)	RRT
Propanoic	2.0—2.2	0.83—0.92
Isobutyric	7.0—2.2	0.83—0.92
Internal standard	2.4	1.00
Butanoic	7.5—3.0	1.25
2-Methylbutanoic	3.6—3.8	1.50—1.58
3-Methylbutanoic	3.6—3.8	1.50—1.58
Crotonic	4.6—4.8	1.92—2.00
Pentanoic	5.0—5.4	2.08—2.25
β-Methylcrotonic	5.8—6.0	2.42—2.50
Isocaproic	6.4	2.67
Tiglic	7.2	3.00
Hexanoic	7.6	3.17
Pyruvic	4.6—4.8	1.92—2.00
α-Ketoisovaleric	8.0	3.08
α-Ketobutyric	8.0	3.08
α-Keto-β-methylvaleric	11.2	4.31
α-Ketovaleric	11.2	4.31
α-Ketoisocaproic	12.4	4.77
α-Ketocaproic	17.0	6.54
Succinic	20.6	7.92
Lactic	23.4	9.00

Ghost peaks [11] can be a problem on both the SP-1200 and the SP-1000 columns. We were able to minimize the appearance of ghost peaks in our profiles by the injection of 10 μl of a 1% formic acid solution onto the column at the end of each profile.

With these procedures, the average column lifetime is 6 months for the SP-1200 column and 8—12 months for the SP-1000 column. Over the course of daily use, the retention time of the volatile acids on the SP-1200 column steadily decreases over the 6-months lifetime of the column. For example, the retention time for the internal standard on a new column was 6.6 min, after 3 months of use the internal standard retention time was 5.4 min and after 5 months the retention time was 4.0 min. The resolution of the volatile acids was not changed over the column lifetime. There is a similar but less rapid change in the volatile acid retention times over the lifetime of the SP-1000 column.

We have found that this profiling procedure permits the rapid identification of abnormal volatile fatty acid concentrations in the plasma and urine of ill newborns suspected of having inborn errors of metabolism. Recently this method permitted positive identification of methylmalonic aciduria in an infant only 30 min after the sample was received in the laboratory. Since early institution of therapy is crucial for a favorable outcome in these circumstances, we recommend this method for early identification of the volatile carboxylic acids in body fluids.

REFERENCES

1　F.A. Hommes, J.R.G. Kuipers, S.D. Elema, S.F. Jansen and J.H.P. Jonxis, Pediatr. Res., 2 (1968) 519.
2　U.G. Oberholzer, B. Leven, E.A. Burgess and W.F. Young, Arch. Dis. Child., 42 (1967) 492.
3　O. Stokke, L. Elkjarn, K.R. Norum, J. Steen-Johnson and S. Halvorsen, Scand. J. Clin. Lab. Invest., 20 (1967) 313.
4　J. Dancis and M. Levitz, in J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw-Hill Book Company, New York, 3rd ed., 1972, p. 426.
5　H. Przyrembel, V. Wendel, K. Becker, H.G. Bremer, L. Bruinvis, D. Ketting and S.K. Wadman, Clin. Chim. Acta, 66 (1976) 227.
6　L. Sweetman, W.L. Nyhan, D.A. Trauner, A. Merritt and M. Sengh, J. Pediatr., 96 (1980) 1020.
7　J.S. Whitehead, Y.S. Kim and R. Prizont, Clin. Chim. Acta, 72 (1976) 315.
8　B. McArthur and A.P. Sarnaik, Clin. Chim., 28 (1982) 1983.
9　C. Bachmann, J.-P. Colombo and S. Beruter, Clin. Chim. Acta, 92 (1979) 153.
10　S.C. Gates, N. Dendramis and C.C. Sweeley, Clin. Chem., 24 (1978) 1674.
11　A. Kuksis, Sep. Pur. Meth., 6 (1977) 353.
12　E.P. Frenkel and R.L. Kitchens, J. Lab. Clin. Med., 85 (1975) 487.
13　C. Auray-Blais, R. Gizuere, D. Paradis and B. Lemieux, Clin. Biochem., 12 (1979) 43.

CHROMBIO. 1820

Note

The measurement of methylamines in biological material using a gas chromatographic head space gas technique

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The three methylamines, methylamine, dimethylamine and trimethylamine, are produced by bacterial action in the gut and are excreted in the urine and faeces after some modification. The complete details of this process have yet to be elucidated. To facilitate the further study of these amines a simple method for their detection is desirable.

Various methods have been described for the measurement of methylamines by gas chromatography (GC) and many different column packings have been tried, e.g. Carbopak B [1], Chromosorb 103 [2] and Pennwalt 223 [3]. Some have involved derivatisation of the amine and others the use of a precolumn. All these methods are unsatisfactory in some way for the detection of the three amines in biological material.

Trimethylamine has also been assayed in the urine of subjects with trimethylaminuria. Marks et al. [4] used a column of 10% free fatty acid phase and 5% potassium hydroxide on 80—100 mesh Chromosorb W and injected urine directly onto the column. However, there is no mention of the analysis of methylamine and dimethylamine.

Dunn et al. [5] have described a system for the detection of amines in the ultrafiltrate of biological fluids using a packing of 10% amine 220 and 10% potassium hydroxide on 80—100 mesh Chromosorb W. This method does not separate methylamine and dimethylamine.

Kuwata et al. [6] have shown that a simple column packed with Chromosorb 102 treated with 5% trimethylchlorosilane and coated with potassium hydroxide can separate aqueous mixtures of all three amines at low concentration.

Head space analysis combined with GC can be used to analyse volatile substances which have become extracted from the general medium. Using a

head space analysis technique a method was developed for the detection of methylamine, dimethylamine and trimethylamine in biological material, using isopropylamine as an internal standard.

EXPERIMENTAL

Standards

Standard solutions of methylamine, dimethylamine, trimethylamine and isopropylamine were made from their hydrochloride salts (BDH, Poole, U.K.). Aliquots of a stock solution of 1 mg/ml were neutralized and diluted to the required concentrations.

Samples

Urine, faeces and rat gut contents have been used as biological samples. These were collected into 1 *M* hydrochloric acid to trap the amines as hydrochloride salts. The amines were extracted from faeces and gut contents by mixing them 4—6 times with 5-ml aliquots of 1 *M* hydrochloric acid for 30 sec followed by centrifugation at 1600 *g* for 5 min. The supernatants from these washings were pooled. Acidified urine was used without further modification. All samples were stored at −20°C until analysed.

Head space analysis

Aliquots of 1 ml (for dimethylamine and trimethylamine) or 5 ml (for methylamine) of the sample or standard were placed in 2 cm × 4 cm screw-top bottles with rubber seals and pierced metal caps. Samples previously acidified with hydrochloric acid were neutralized with potassium hydroxide (10 *M*). Isopropylamine hydrochloride was added as an internal standard; 20 μl of 10 *N* ammonia and 2 g of potassium carbonate per ml of sample were added. The containers were heated in a water bath at 50°C for 40 min.

The vapour above the liquid was sampled with a gas-tight syringe and injected directly onto the column.

Chromatography

A Carlo Erba 4200 Fractovap gas chromatograph fitted with a Carlo Erba NPD 40 nitrogen detector was used for the chromatography. The column was packed with Chromosorb 102 coated with 5% trimethylchlorosilane and 5% potassium hydroxide (Chromatography Supplies, Wirral, U.K.). The working conditions were: nitrogen carrier gas flow-rate, 50 ml/min; injector temperature, 160°C; column temperature, 160°C (isothermal), detector temperature, 200°C; detector hydrogen and air pressures, 0.8 kg/cm^2; sample size, 0.5 ml.

RESULTS AND DISCUSSION

Fig. 1a and b shows the chromatogram of the vapour collected from the head space of a 1-ml and a 5-ml incubation of a 1 μg/ml standard. The retention times of each peak (mean ± standard deviation (S.D.) for seven repeated injections) are: ammonia, 0.64 ± 0.02 min; methylamine, 1.16 ± 0.02
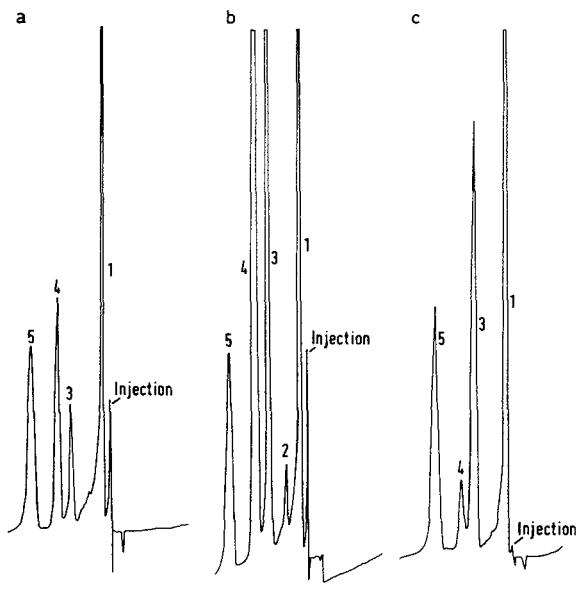
Fig. 1. Chromatogram of the head space above (a) a 1-ml incubation and (b) a 5-ml incubation of a standard solution containing methylamine, dimethylamine and trimethylamine each at a concentration of 1 μg/ml and (c) a 1-ml incubation of human urine. Peaks: 1 = ammonia; 2 = methylamine; 3 = dimethylamine; 4 = trimethylamine; 5 = isopropylamine.

min; dimethylamine, 1.73 ± 0.03 min; trimethylamine, 2.23 ± 0.03 min; isopropylamine, 3.16 ± 0.02 min.

A straight-line calibration graph is obtained for standards in the concentration range 0.25—200 μg/ml with correlation coefficients between 0.99 and 1.00. Fig. 2 shows a calibration graph in the range 0.5—30 μg/ml for dimethylamine. Each point shows the mean ± S.D. for five incubations and injections of that concentration. Similar calibration graphs were obtained for methylamine and trimethylamine.

A larger sample is required for methylamine since it is more soluble in water and less readily vaporised.

Recoveries of 100—102% were obtained on adding the pure amines to urine.

The detection limit for methylamine is 0.25 μg/ml (limited by its solubility), while that for dimethylamine and trimethylamine is considerably lower (ca. 0.01 μg/ml). This is an improvement on the method described by Dunn et al. [5] and is probably due to the use of a nitrogen detector.

Potassium carbonate and ammonia are necessary to drive the very soluble amines out of solution. Potassium carbonate is a non-reactive, very soluble alkaline salt and is thus suited to this purpose. The amount of ammonia added is critical since higher concentrations interfere with the methylamine peak and also cause the resolution and response of other peaks to deteriorate.

Direct injection of aqueous solution of standards and samples as used by Kuwata et al. [6] was also tried. The resulting water peak could not be separated from the methylamine peak. The water also caused a negative peak
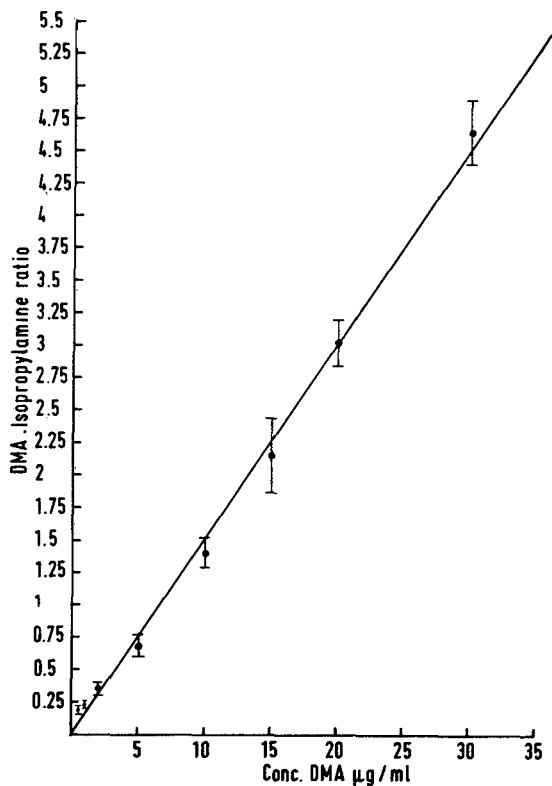
Fig. 2. A calibration graph for dimethylamine. Each point shows the mean ± S.D. for five separate incubations and injections.

at high sensitivities which interfered with the dimethylamine peak. Head space analysis overcame these problems.

Fig. 1c shows a chromatogram of the vapour sampled from the head space over a 1-ml sample of human urine. Chromatograms of similar quality were obtained for extracted rat faeces and rat gut contents. The repeated analysis (seven separate incubations of a single human urine sample) gave a mean ± S.D. value of 18.3 ± 1.8 μg/ml for dimethylamine, 1.7 ± 0.3 μg/ml for trimethylamine and 1.8 ± 0.2 μg/ml for methylamine.

The amounts of amines found in rat urine, rat faeces and various parts of the rat gut are shown in Table I. These rats had been fed Rat Maintenance Diet No. 1 supplied by Special Diet Services (Witham, U.K.) which contains 2.4% fat, 13.6% protein, 18.6% dietary fibre, 38% starch, 10.4% sugars and an adequate supply of minerals and vitamins.

A simple method has been developed for the detection of aliphatic amines in biological materials which is both more sensitive and gives greater resolution than the ultrafiltration technique of Dunn et al. [5]. Use of head space samples avoids the possibility of interference from other water soluble, biological substances in the chromatography.

TABLE I

AMOUNTS (μg) OF METHYLAMINES FOUND IN RAT URINE, RAT FAECES AND VARIOUS PARTS OF THE RAT GUT

Values are mean ± S.D. for eight rats.

	Trimethylamine	Dimethylamine	Methylamine
Rat urine 24 h	57 ± 27	410 ± 200	84 ± 35
Rat faeces 24 h	11.7 ± 9.8	9.8 ± 4.8	14.1 ± 14.4
Rat upper small bowel	0.9 ± 0.6	3.7 ± 0.4	trace
Rat lower small bowel	2.5 ± 1.5	8.7 ± 4.4	trace
Rat caecum	11.8 ± 6.1	4.4 ± 0.4	35.4 ± 11.6
Rat colon	8.7 ± 5.4	6.0 ± 1.7	24 ± 11.1

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REFERENCES

1 A. Di Corcia, R. Samperi and C. Severini, J. Chromatogr., 170 (1979) 325.
2 C.E. Andre and A.R. Mosier, Anal. Chem., 45 (1973) 1971.
3 M. Dalene, L. Mathiasson and J.Å. Jönsson, J. Chromatogr., 207 (1981) 37.
4 R. Marks, M.W. Greaves, C. Prottey and P.J. Hartop, Brit. J. Dermatol., 96 (1977) 399.
5 S.R. Dunn, M.L. Simenhoff and L.G. Wessen, Jr., Anal. Chem., 48 (1976) 41.
6 K. Kuwata, Y. Yamazaki and M. Uebori, Anal. Chem., 52 (1980) 1980.

CHROMBIO. 1826

Note

High-performance liquid chromatographic determination of oestrogens in human urine

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Much research has been done to improve methods for oestrogen determination in body fluids [1—3]. The results of this analysis are very important in clinical chemistry for the information that can be obtained during pregnancy. In the last months of pregnancy the oestriol concentration should rise rapidly, while low levels, or a decrease in its excretion, are indicative of malfunctions in the regular progress of the pregnancy.

It would consequently be very advantageous to have quantitative results in a fast and simple way. For routine analysis the urine can be frequently collected and the oestrogen concentration monitored daily.

Among the various methods used for oestrogen determination, colorimetry and spectrometry are not very specific, radioimmunoassay is very sensitive but difficulties are encountered in the use of radioactive compounds ·(e.g. only authorised laboratories can handle these compounds). Gas chromatography has been successfully used, but normally only after derivative formation, and consequently the sample treatment is more complicated and time-consuming. High-performance liquid chromatography (HPLC) has been advantageously introduced for oestrogen analysis in urine by adsorption or in the reversed-phase mode [4—6] and with selective detection method [7].

In this paper we describe the HPLC determination of oestrogen in urine using a simplified procedure for sample treatment. Various mobile phases have been investigated for an optimum HPLC separation and the results obtained for urine samples before and after enzymatic hydrolysis are reported.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Series 2 liquid chromatograph was used with a variable-wavelength detector, LC 55, and a scanning system to record the spectra

of the eluted peaks. Two columns were used (25 × 4.6 mm) packed in our laboratory with derivatized silica LiChrosorb RP-18 or DIOL, 10 μm (Merck, Darmstadt, F.R.G.). The first column is normally used for routine analysis, the second one in only a few cases for confirmation of oestrogen peaks. By running the same sample in both the columns of different polarity or also by comparing the ultraviolet spectra of the chromatographic peak with .the reference compounds, identification of the oestrogen peak and purity are established. All the solvents used (acetonitrile, methanol, isopropanol, hexane, and tetrahydrofuran) were for HPLC (Carlo Erba, Milan, Italy). The flow-rate was 1.5 ml/min. The steroids were detected at 280 nm. Sample solutions and standards were injected (50—100 μl) with a Rheodyne valve using a 175-μl loop.

Procedure

The oestrogens in urine are partly present in the free form but mainly as glucuronide and sulphate conjugates. To determine the total concentration the sample is hydrolyzed; 5 ml of urine are mixed with 100 μl of β-glucuronidase—arylsulphatase (Merck) and 2 ml of 0.1 M acetate buffer at pH 5.5 and incubated for 24 h at 4°C.

To determine the free oestrogens, 20 ml of urine are used as such. In both cases the sample is centrifuged and passed through a Sep-Pak C_{18} cartridge (Waters Assoc.) previously washed with methanol and subsequently with water. Only after this treatment do new cartridges show reproducible retention. After urine passage the Sep-Pak is washed with 10 ml of water and the oestrogens are recovered by elution with 2 ml of methanol. The solvent is

5 ml urine   20 ml urine

enzyme hydrolysis

centrifuge

Sep-Pak C_{18} cartridge

wash with water

elution with methanol

evaporation under nitrogen

add 1 ml carbonate buffer pH 10.2

extract with ether

evaporation under nitrogen

dissolve with 100 μl of methanol

inject into chromatograph
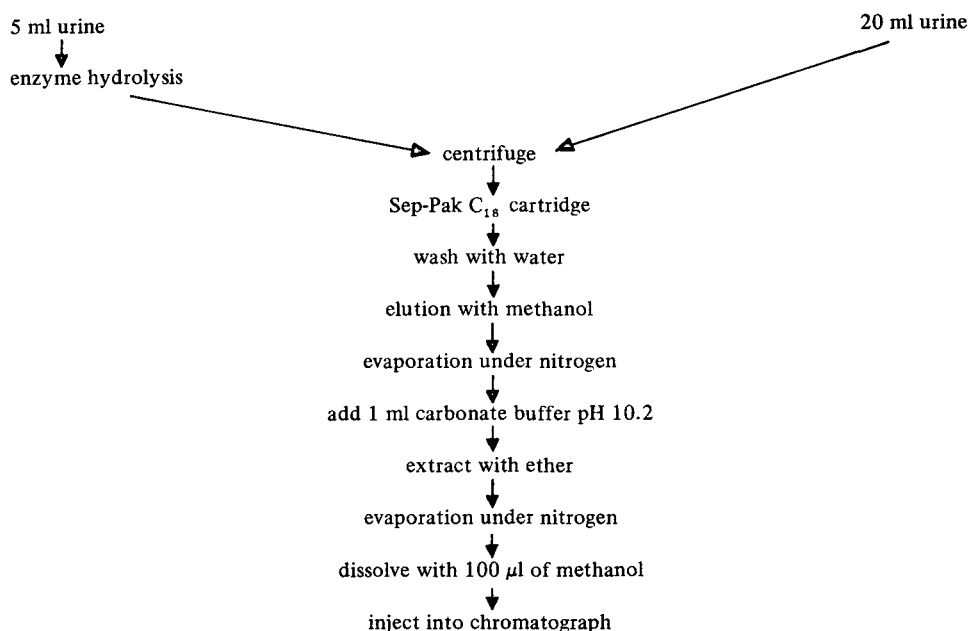
Fig. 1. Flow diagram of the oestrogen extraction from urine, and sample treatment.

evaporated under nitrogen and the residue is dissolved in 1 ml of 0.1 M carbonate buffer at pH 10.2 and about 0.1 g of sodium sulphate is added. The steroids are extracted with ether (3 × 1 ml) and the ether extracts are washed with 1 ml of water, dried over sodium sulphate and evaporated under nitrogen. The residue is dissolved in 100 μl of methanol and injected into the chromatograph. This extraction procedure is outlined in Fig. 1.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of a standard mixture of oestriol, oestradiol and oestrone, with the RP C_{18} column. The first part of the chromatogram is run in isocratic conditions (acetonitrile—water, 23:77) and, after the elution of oestriol, with a gradient up to 73% of acetonitrile in water at 3% per min.
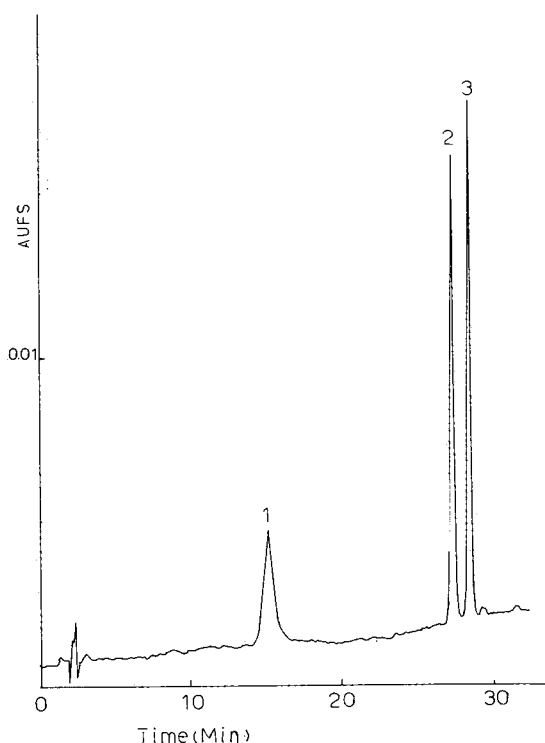
Fig. 2. Chromatogram of the oestrogen mixture on RP-18 column (10 μm, 25 × 4.2 mm). Flow-rate = 1.5 ml/min. Mobile phase: 23% acetonitrile in water for 15 min and then programmed to 77% acetonitrile in water at 3% per min. Peaks: 1 = oestriol, 2 = oestradiol, 3 = oestrone.

Because of the large difference in polarity between oestriol and the other oestrogens it is advisable to use the gradient. Table I shows the values of capacity ratios and the separation factors with various solvents. The percentage of water is regulated in order to have comparable k' values for the last peak (oestrone). The best separation of oestrone and oestradiol is achieved

TABLE I

SEPARATION FACTORS (α) AND CAPACITY FACTORS (k') OF OESTROGENS WITH DIFFERENT MOBILE PHASES (RP-18 COLUMN)

Mobile phase		k'			α Oestrone/ oestradiol
		Oestriol	Oestradiol	Oestrone	
Methanol—water	45:55	2.2	13.5	15.5	1.1
Acetonitrile—water	35:65	1.0	9.9	15.8	1.6
Tetrahydrofuran—water	35:65	2.3	10.3	13.5	1.3
Methyl acetate—2-propanol—water	25:13:62	1.7	9.6	13.6	1.4
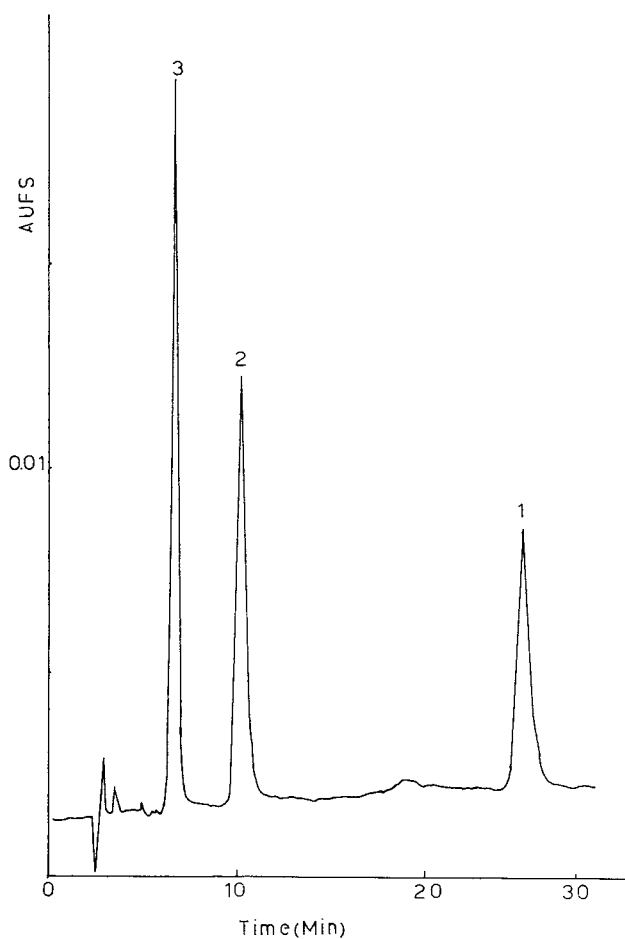
Fig. 3. Chromatogram of the oestrogen mixture on DIOL column (10 μm, 25 × 4.2 mm). Flow-rate = 1 ml/min. Mobile phase: n-hexane—isopropanol (9:1) for 10 min and then programmed to 30% of isopropanol in n-hexane at 1% per min. Peaks as in Fig. 2.

with acetonitrile—water. With the DIOL column, as shown from the chromatogram of Fig. 3, the elution order is completely changed and this fact can be usefully employed as a confirmatory test.

By using a Sep-Pak C_{18} cartridge a very fast and simple extraction of free and conjugated oestrogens is obtained, without the formation of emulsion which usually occurs with solvent extraction. The sample clean-up is performed by partition of the oestrogens between ether and a buffer solution at pH 10.2. Under these conditions oestrogens can be extracted from ether while many impurities (acidic and phenolic compounds) remain in the water phase. Recoveries of 85—90% were obtained from urine spiked with the oestrogens and extracted according to the full procedure reported in Fig. 1 for conjugated steroids.

Figs. 4 and 5 show the chromatograms obtained from a urine sample before and after enzymatic hydrolysis.

Under the working conditions described, many polar constituents are eluted before oestriol and do not interfere with the oestrogen determination. Table II shows the oestrogen concentration of different samples. Sample 1 is the
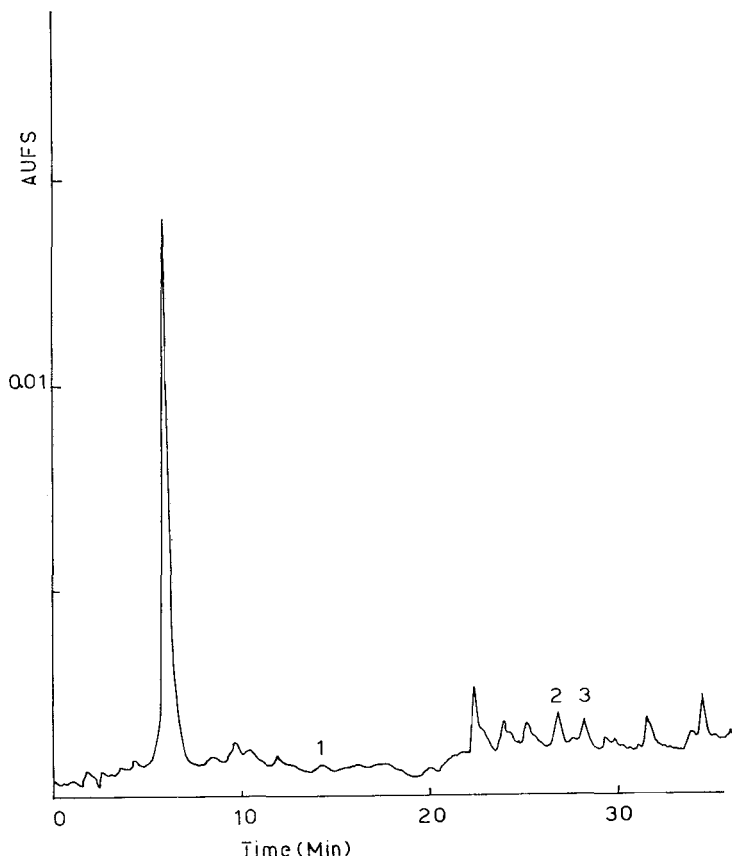
Fig. 4. Chromatogram of an extract of a non-pregnancy urine. Conditions and peaks as in Fig. 2.
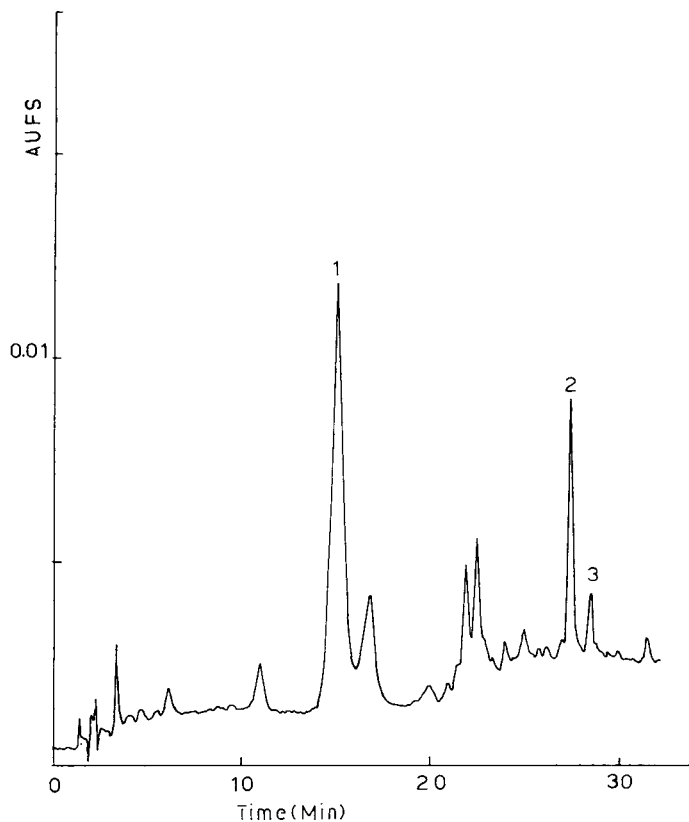
Fig. 5. Chromatogram of an extract from a pregnancy urine after hydrolysis. Conditions and peaks as in Fig. 2.

TABLE II

HPLC DETERMINATION OF OESTROGEN CONCENTRATION IN URINE

Sample No.	Months of pregnancy	Oestriol (mg/l)	Oestradiol (mg/l)	Oestrone (mg/l)
1	0	0.2	0.2	0.4
2	9	25.0	3.8	0.8
3	9	11.4	0.2	0.2
3	9	11.4	0.3	0.3
4	9	22.0	3.0	0.9
4	9	21.8	3.0	0.7
4	9	21.5	3.5	0.7
4	9	21.6	3.5	0.8
4	9*	0.06	0.05	0.04
5	4	2.5	0.3	0.3
6	8	7.2	1.1	1.3

*Not hydrolyzed.

150

urine of a non-pregnant woman and, as expected, low levels of oestrogens are observed. To show the reproducibility of the method the values obtained from duplicate analysis of sample 3, and from four analyses of sample 4 are reported. These analyses were repeated on different portions of the same urine sample. Sample 4 was also analyzed without hydrolysis and, as observed by other authors [5], the concentration of oestrogens in the free form is very small compared with the concentration of the conjugated compounds. The oestriol concentration rises sharply in the last period of pregnancy and is a very useful indicator of the patient's situation. Samples 2, 3 and 4 are from different subjects at the end of pregnancy, and consequently there are natural differences in the oestriol concentrations, but all of them show high values of oestriol, while the oestradiol and oestrone concentrations are at lower levels.

By HPLC many routine determinations of oestrogens can be carried out for clinical analysis with a simple and efficient extraction procedure and sample treatment.

ACKNOWLEDGEMENT

REFEFENCES

1 M.P. Kautsky (Editor), Steroid Analysis by HPLC: Recent Applications, Marcel Dekker, Basel, 1981, Ch. 5.
2 G. Keravis, M. Lafosse and M.H. Durand, Chromatographia, 10 (1977) 678.
3 J.-T. Lin and E. Heftmann, J. Chromatogr., 212 (1981) 239.
4 R.J. Dolphin, J. Chromatogr., 83 (1973) 421.
5 R.J. Dolphin and P.J. Pergande, J. Chromatogr., 143 (1977) 267.
6 W. Slikker, Jr., G.W. Lipe and G.D. Newport, J. Chromatogr., 224 (1981) 205.
7 Z.K. Shihabi, J. Scaro and B.F. Thomas, J. Chromatogr.. 224 (1981) 99.

CHROMBIO. 1835

Note

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Extraction and separation of androstenedione from products of aromatase assays on micro-columns of magnesium oxide

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Transformations of steroid hormones, particularly the aromatization of androgens, are being examined with increasing frequency in central neural tissues [e.g. ref. 1].

While many extraction procedures are available, two commonly encountered problems are low recoveries of steroids and the presence of unwanted lipid which must usually be removed before further analysis. Solvent and phenolic partitioning have been widely used to extract lipid contaminants and unreacted substrate in aromatase reactions [2].

The procedure described, in conjunction with a modification of the magnesium oxide micro-column method of Kawahara et al. [3], gives greater recoveries of steroids with less contaminating lipid, and permits rapid and efficient separation of unreacted substrate from products of aromatase assays with neural tissue, cultured cell suspensions and microsomal fractions of human placenta.

MATERIALS AND METHODS

Water was glass-distilled. All solvents were redistilled before use. Unlabelled steroids were obtained from Sigma (St. Louis, MO, U.S.A.). The following radiolabelled steroids were obtained from Amersham (Australia): [4-^{14}C]-androstenedione (58 mCi/mmol), [1,2,6,7-^{3}H]-testosterone (83 Ci/mmol), [2,4,6,7-^{3}H]-oestrone (92 Ci/mmol), [2,4,6,7-^{3}H]-17β-oestradiol (92 Ci/mmol) and [2,4,6,9-^{3}H]-oestriol (92 Ci/mmol)*. These were checked for homogeneity

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*Androstenedione = 4-androsten-3,17-dione; testosterone = 17β-hydroxy-4-androsten-3-one; oestrone = 3-hydroxy-1,3,5[10]-oestratrien-17-one; 17β-oestradiol = 1,3,5[10]-oestratriene-3,17β-diol; oestriol = 1,3,5[10]-oestratriene-3,16α,17β-triol.

before use by thin-layer chromatography in the following systems by volume; benzene—toluene—methanol (9 : 1 : 1) and diethyl ether—benzene (2 : 1).

Magnesium oxide (light) was obtained from BDH (Poole, U.K.) and was washed extensively with water to remove fines, then heated overnight at 180°C before use.

Fresh lamb brains were obtained from Westfield Freezing Works (Auckland, New Zealand). The hypothalami were excised and homogenised with an Ultra Turrax instrument at full speed for 15 sec in phosphate buffered saline (PBS): 10 mM sodium phosphate—0.15 M sodium chloride, pH 7.4 (1 : 3, w/v).

Androstenedione and oestrone were chosen as representative C_{19}- and C_{18}-steroids respectively and were used in recovery experiments. Labelled steroids were added to 25-ml conical flasks and the solvent evaporated under a fine stream of oxygen-free nitrogen. Just prior to the addition of homogenate, the steroids were redissolved in 4 drops of absolute ethanol. A 5-ml sample of homogenate was added, mixed and immediately frozen in a mixture of dry-ice and ethanol. After thawing, 25 μg each of unlabelled androstenedione and oestrone, in 0.5 ml ethyl acetate was added and the mixture extracted with 4 volumes of 2,2,4-trimethylpentane (isooctane)—ethyl acetate (93 : 7, v/v) four times. Extracts were dried under reduced pressure, the volume made to 1 ml with ethyl acetate and duplicate samples taken for liquid scintillation counting. A sample was also taken for total lipid estimation [4].

The micro-column method of Kawahara et al. [3] was modified to allow separation of androstenedione from products of aromatization, with a single change of solvent. Disposable plastic insulin syringes (1 ml) were used as columns and were plugged with a small piece of glass wool. A slurry of magnesium oxide and isooctane was poured and allowed to settle by gravity to a height of 3 cm. Approximately 5 ml isooctane was then run through the column to ensure uniform packing. Samples were dissolved in 50—75 μl ethyl-methylketone to which was added 0.4 ml isooctane and the sample was layered onto the column. Sample tubes were then rinsed with 0.15 ml isooctane twice and both washes were applied to the column. Elution was begun with 10 ml isooctane—ethyl acetate (9 : 1, v/v) then with 6 ml ethyl acetate—methanol (8 : 2, v/v); each fraction was collected separately. Fractions were dried under nitrogen, made up to 1 ml with ethyl acetate and samples were taken in duplicate for liquid scintillation counting.

For phenolic partitioning, steroids were extracted from neural tissue homogenates, partitioned against sodium hydroxide and re-extracted with diethyl ether, essentially as described by Ryan [2].

Scintillation counting was performed using a Packard Tricarb 3200 Liquid Scintillation Spectrometer, with scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis(5-phenyloxazolyl-2)benzene in toluene. Results were analysed according to the equations of Coghlan et al. [5] for double-label experiments.

RESULTS AND DISCUSSION

On the basis of experiments with androstenedione and oestrone, the extraction procedure described was compared to three previously published

TABLE I

COMPARISON OF METHODS FOR EXTRACTION OF [^{14}C]-ANDROSTENEDIONE AND [^{3}H]-OESTRONE FROM NEURAL TISSUE HOMOGENATES

Steroids were extracted from lamb brain homogenates by each of the techniques described, dried under reduced pressure, made up to 1 ml ethyl acetate and samples taken for liquid scintillation counting and total lipid estimation.

Extraction method [ref.]	Steroid recovered (%) (± S.D.)*		Lipid extracted (mg) (± S.D.)*
	[^{14}C]-Androstenedione	[^{3}H]-Oestrone	
5 vol. Chloroform, three times [2]	63.85 (1.8)	64.4 (2.5)	27.2 (1.7)
5 vol. Water saturated ethyl acetate, three times [8]	82.5 (4.4)	87.9 (3.7)	37.5 (0.9)
5 vol. Diethyl ether, three times [9]	75.9 (3.1)	88 (2.4)	34.9 (2.5)
4 vol. Isooctane—ethyl acetate (93:7, v/v) four times	96.9 (2.2)	96.3 (4.8)	7.9 (0.6)

*S.D. = standard deviation (n = 3).

methods with respect to recovery of steroids and the amount of contaminating lipid in the extract. These data are presented in Table I.

Extraction of steroids from homogenates of neural tissue with isooctane—ethyl acetate gives significantly higher recoveries, with much less lipid, about 25% of that found with the other procedures. In addition, two further advantages are apparent: (a) the solvent did not readily form emulsions with the aqueous phase, thereby obviating the need for centrifugation which is frequently necessary where ethyl acetate is used and (b) the immiscibility of the organic and aqueous phases makes it unnecessary to dry organic extracts over such compounds as anhydrous sodium sulfate [6]. The method has been applied by the authors to the extraction of steroids from aromatase assays using cell suspensions and human placental microsomal preparations. With [4-^{14}C]-androstenedione as substrate, the average recovery of label was 92 ± 4% (n = 29).

When the various extracts were applied to micro-columns of magnesium oxide only the isooctane—ethyl acetate extracts could be eluted, in contrast to the other extracts which caused the columns to clog.

Table II compares total recoveries and separation of [^{14}C]-androstenedione and [^{3}H]-oestrone after extraction from brain homogenates with isooctane—ethyl acetate, followed by either magnesium oxide micro-column chromatography or phenolic partitioning. The results indicate that both total recovery and overlap of steroids in the various fractions are more efficient with magnesium oxide micro-columns than with phenolic partitioning. It is worthwhile noting that the magnesium oxide micro-column method requires only 2—3 h to complete, using much less solvent and fewer manipulations than phenolic partitioning.

TABLE II

SEPARATION OF [^{14}C]-ANDROSTENEDIONE AND [^{3}H]-OESTRONE EXTRACTED FROM BRAIN HOMOGENATES BY MAGNESIUM OXIDE MICRO-COLUMNS AND PHENOLIC PARTITIONING

Steroids were extracted and magnesium oxide micro-columns run as described under Materials and methods. Phenolic partitioning of steroids was performed according to the method of Ryan [2].

Technique	Steroid recovered (%) ($\pm$ S.D.)*	
	[^{14}C]-Androstenedione	[^{3}H]-Oestrone
Magnesium oxide micro-column:		
Isooctane—ethyl acetate	91.7 (2.2)	2.8 (0.5)
Ethyl acetate—methanol	4.2 (0.8)	86.8 (3.0)
Phenolic partitioning:		
Androgen fraction	89.8 (1.2)	6.4 (4.6)
Phenolic fraction	1.2 (1.0)	75.2 (1.5)

*S.D. = Standard deviation ($n = 4$).

As the authors are concerned with enzymic transformations of androstenedione by various cell lines (unpublished data), it was of interest to test the suitability of the magnesium oxide micro-column method for the separation of unreacted androstenedione from testosterone and oestrogens other than oestrone. For this purpose, 25 μg each of androstenedione, testosterone, oestrone, 17β-oestradiol and oestriol were added to test tubes containing ^{14}C-labelled androstenedione and tritiated testosterone, oestrone, 17β-oestradiol and oestriol. Columns were run and fractions analysed for total [^{14}C] and [^{3}H]; these data are presented in Table III.

The results show that this method offers advantages for two main reasons: (a) it allows rapid and efficient separation of unreacted substrate from products, which is especially important in systems where conversion is low, making subsequent characterization of products by chromatography or recrystallization easier, and (b) it allows retention of products which might

TABLE III

SEPARATION OF [^{14}C]-ANDROSTENEDIONE FROM [^{3}H]-TESTOSTERONE, [^{3}H]-OESTRONE, [^{3}H]-17β-OESTRADIOL AND [^{3}H]-OESTRIOL ON MAGNESIUM OXIDE MICRO-COLUMNS

A 25-μg amount each of unlabelled androstenedione, testosterone, oestrone, 17β-oestradiol and oestriol was added to test tubes containing [^{14}C]-androstenedione (99,500 dpm) and [^{3}H]-testosterone, [^{3}H]-oestrone, [^{3}H]-17β-oestradiol and [^{3}H]-oestriol (total [^{3}H] = 572,900 dpm). Columns were run and results analysed as described under Materials and methods and are presented as total [^{14}C] and [^{3}H] recovered.

Column fraction	[^{14}C] Recovered (%) ($\pm$ S.D.)*	[^{3}H] Recovered (%) ($\pm$ S.D.)*
Isooctane—ethyl acetate	96.3 (4.8)	2.4 (0.3)
Ethyl acetate—methanol	5.6 (0.8)	98.3 (4.5)

*S.D. = Standard deviation ($n = 6$).

otherwise be lost when liquid—liquid partition systems are employed. In addition, the method as described results in an increase in recovery of steroids of approximately 20% over the original method of Kawahara et al. [3] and from 10—20% over the Sephadex LH-20 micro-column method of Gips et al. [7].

The authors believe the two procedures described allow more efficient extraction and separation of C_{19}- and C_{18}-steroids from experiments using neural tissues, where samples have a high lipid content.

REFERENCES

1 F. Naftolin, K.J. Ryan, I.J. Davies, V.V. Reddy, F. Flores, Z. Petro and M. Kuhn, Recent Prog. Horm. Res., 31 (1975) 295.
2 K.J. Ryan, J. Biol. Chem., 234 (1959) 268.
3 C. Kawahara, X. Kozbur, S. Geduld and L. Parker, Anal. Biochem., 102 (1980) 310.
4 R.D. Ellefson and W.T. Caraway, in N.W. Tietz (Editor), Fundamentals of Clinical Chemistry, Saunders, Philadelphia, London, Toronto, 1976, p. 492.
5 J.P. Coghlan, M. Wintour and B.A. Scoggins, Aust. J. Exp. Biol. Med. Sci., 44 (1966) 639.
6 W. Gibb and J.-C. Lavoie, Steroids, 36 (1980) 507.
7 H. Gips, K. Korte, B. Meinecke and P. Bailer, J. Chromatogr., 193 (1980) 322.
8 I.E. Bush, The Chromatography of Steroids, Pergamon, Oxford, London, New York, Paris, 1961, p. 192.
9 G.V. Callard, Z. Petro and K.J. Ryan, Brain Res., 202 (1980) 117.

CHROMBIO. 1832

Note

A simple, rapid and sensitive method for the determination of rat serum uric acid by reversed-phase high-performance liquid chromatography with electrochemical detection

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The biochemical and clinical importance of uric acid (UA) in gout and several other disease states has been discussed [1]. UA in serum is routinely determined either by a spectrophotometric procedure with phosphotungstate [2], or by adaptation of the enzymic method with uricase [3]. These techniques have problems as discussed elsewhere [4]. High-performance liquid chromatography (HPLC) is now an accepted technique as a reference method for the determination of UA. HPLC methods employing ion-exchange columns coupled with electrochemical detection (ED) [4] or ultraviolet detection (UVD) [5] have been proposed. Recently, reversed-phase liquid chromatography with UVD has been studied [6, 7]. However, there has been no report on a method for the determination of UA by reversed-phase high-performance liquid chromatography (RP-HPLC) with ED.

In this note, we describe a simple, rapid and sensitive method for the determination of UA in rat serum by RP-HPLC—ED.

MATERIALS AND METHODS

A JASCO-HPLC, Model Trirotar III with a 25 cm × 4.6 mm I.D. stainless-steel column packed with Fine Sil C_{18} (particle size, 10 μm; JASCO, Tokyo, Japan), coupled to an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Japan) was employed in this study. The flow-rate was 0.5 ml/min. The column temperature was always kept at 35°C. The mobile phase was 0.2 M phosphate buffer (KH_2PO_4—H_3PO_4, pH 2.0). Unless otherwise stated, 10 μl of each sample solution were injected into the RP-HPLC—ED system.

UA was purchased from Wako Pure Chemicals, Tokyo, Japan. Any other chemicals used in this study were the same as used in previous reports [8—10].

All buffers and aqueous solutions were prepared in glass-distilled deionized water.

An aliquot (0.05 ml) of 0.05 M sodium hydroxide solution was dropped onto 10 mg of UA, and 9.95 ml of 0.1 M hydrochloric acid were then added to the dissolved UA solution. The stock solution thus prepared was stored at 4°C. Appropriate dilution of the stock solution with 0.1 M hydrochloric acid solution was done just before use.

Whole rat blood was collected after decapitation; the serum was obtained by centrifugation for 15 min at 4000 g and stored at −80°C until use. A 1.0-ml volume of 2% sulphosalicylic acid (SSA) solution was added to 1.0 ml of rat serum. The mixture was frozen at −80°C and then thawed at room temperature. The mixture was centrifuged at 3000 g for 20 min. The supernatant was injected into the chromatographic system employed in this study. The concentration of UA in rat serum pool was ascertained by the standard addition technique following the procedure of Pachla et al. [4].

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of UA in aqueous solution containing only UA as an electrochemically active component except for (a) peak component(s) near the void volume. In this case, the electrochemical detector was set at +800 mV vs. the silver—silver chloride reference electrode. Fig.
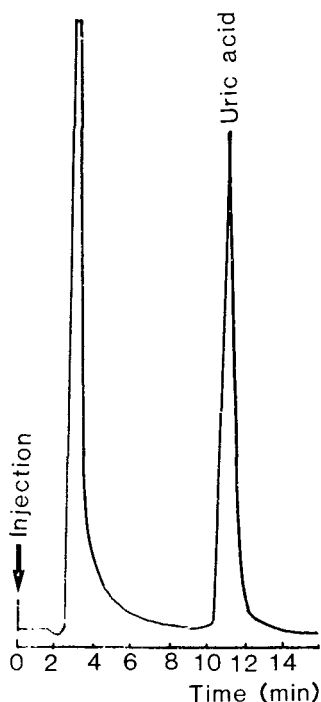
Fig. 1. The typical reversed-phase high-performance liquid chromatogram of uric acid in an artificially prepared sample solution under the present chromatographic conditions. For explanation of the chromatographic conditions, see text.
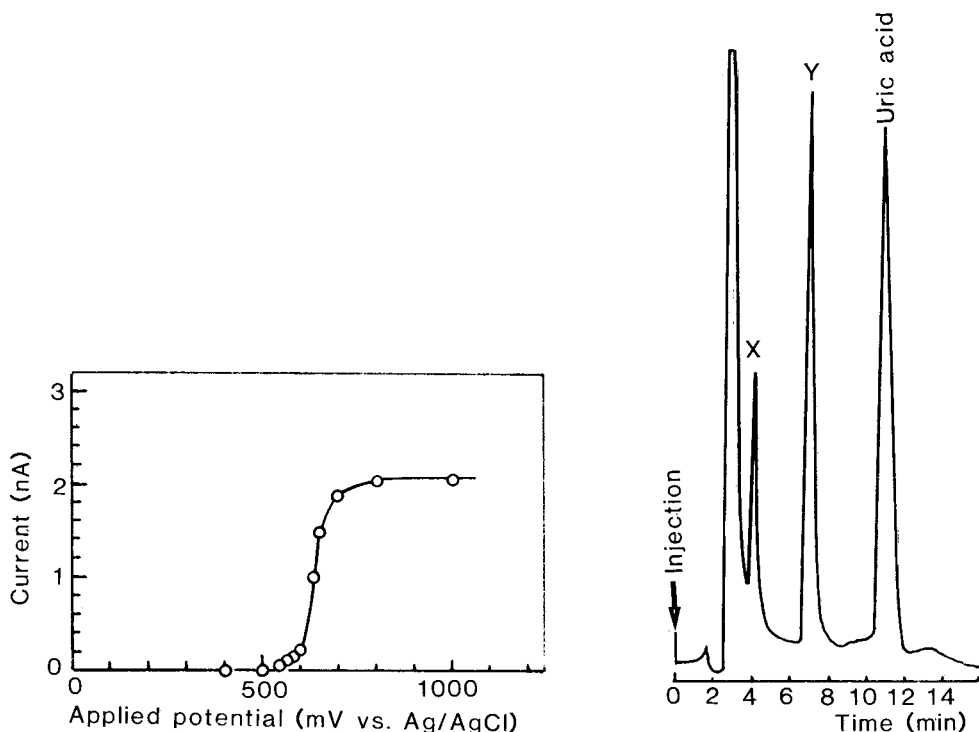
Fig. 2. Hydrodynamic voltammogram for uric acid obtained using the present chromatographic system by repeated injection of 1 ng of uric acid at different electrochemical detector potentials.

Fig. 3. The typical reversed-phase high-performance liquid chromatogram of rat serum after deproteinization under the present chromatographic conditions. For further explanations, see text.

2 illustrates a hydrodynamic voltammogram obtained for UA in the chromatographic system employed in this study. As shown in Fig. 2, the onset potential of UA oxidation is about +550 mV. Thus, the electrochemical detector was set at +800 mV vs. the silver—silver chloride reference electrode.

Fig. 3 shows a typical chromatogram for rat serum after the simple treatment described in the previous section. In the chromatogram, two unidentified peaks (peaks X and Y) are observed. It has been found that the retention time for the peak X component is the same as that for norepinephrine. Peak Y has not yet been identified. When an UA solution and deproteinized rat serum were co-chromatographed, it was found that only the UA peak was enhanced corresponding to the peak height of UA added to the rat serum. The minimum detectable quantity was about 10 pg under the present chromatographic conditions [11]. The content of UA in 1 ml of rat serum was found to be 4.9 ng.

UA standard solutions added to rat serum were analyzed with good precision at concentrations comparable to those in biological samples. The recoveries of UA in different standard solutions added to twenty different rat serum samples prepared on four different days were found to be 98.5 ± 1% under the present experimental conditions.

Excellent precision of retention time for UA was always obtained in routine analysis over a six-day period for 50 different rat serum samples, probably due to the fact that the retention time of UA is not affected by the sample matrix. However, the calibration graphs were obtained before and after the UA determination for the separation study in our laboratory. Both calibration graphs always coincided well with each other. Repeated injections of UA gave an average precision (R.S.D.) of less than 2%.

As described above, we have developed a simple, rapid and sensitive method for the determination of UA by RP-HPLC—ED. Because of its simplicity and applicability to small sample volumes (e.g. 0.1 ml of rat serum), this method is useful in biochemical and basic medical research. The method developed in this study has been used in determining UA in 0.1 ml of human serum, 0.1 ml of human cerebrospinal fluid, and 5 mg of rat brain [12]. As it has been revealed that this method is not subject to interferences encountered in other methods, it should be an attractive alternative to the colorimetric and enzymatic methods now used.

REFERENCES

1   M.E. Balis, Adv. Clin. Chem., 76 (1976) 213—246.
2   Reagent Data Handbook for the Technicon SMR 12/60 System, Technical Publ. No. THO-0160-10, Technicon Instruments Corp., Tarrytown, NY, 1971.
3   P. Fossati, L. Prencipe and G. Berti, Clin. Chem., 88 (1980) 227—231.
4   L.A. Pachla, P.T. Kissinger, L. Yu, F. Watoson, D. Pragay, M.E. Chilcote, L.M. Weiner and B.R. Rennick, Clin. Chem., 25 (1979) 1847—1852.
5   J.A. Miller and E.G. Perkins, Anal. Biochem., 88 (1978) 56—565.
6   E.J. Kiser, G.F. Johnson and D.L. Witte, Clin. Chem., 24 (1978) 536—540.
7   O.C. Ingebresten, J. Borgen and M. Farstad, Clin. Chem., 28 (1982) 496—498.
8   T. Aoki, M. Yoshiura, T. Iwamoto, Y. Ozaki and K. Iriyama, Jikeikai Med. J., 29 (1982) 385—391.
9   K. Iriyama, M. Yoshiura and T. Iwamoto, Jikeikai Med. J., 30 (1983) 35—44.
10 T. Iwamoto, M. Yoshiura and K. Iriyama, Jikeikai Med. J., 30 (1983) 123—128.
11 T. Iwamoto, M. Yoshiura and K. Iriyama, Jikeikai Med. J., 30 (1983) 177—183.
12 M. Yoshiura, T. Iwamoto, K. Iriyama, T. Kanki, Y. Kato, H. Sekino and N. Nakamura, Jikeikai Med. J., 30 (1983) in press.

CHROMBIO. 1846

Note

Quantitation of gangliosides by scanning densitometry of thin-layer chromatography plates*

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Gangliosides are a diversified family of glycolipids characterized by the presence of sialic acid. Although ubiquitous in vertebrate plasma membranes [1], their biological function remains elusive. The human central nervous system is especially rich in gangliosides and their separation by high-performance thin-layer chromatography (HPTLC) in one dimension reveals the presence of at least twelve ganglioside bands [2]. The five major ganglioside species of this tissue were chosen for study in this report (Table I).

The conventional method for determining the relative distribution of individual ganglioside species in tissues employs thin-layer chromatography to separate the ganglioside components of a mixture, resorcinol staining of the sialic acid moiety, and quantitation of the stained ganglioside bands by scanning densitometry [2—7]. The percentage of an individual ganglioside is generally calculated from the ratio of the densitometric detector response due to the ganglioside band relative to the total detector response of all ganglioside bands separated from the mixture. However, this method is valid only if all ganglioside species yield equal densitometric values per mole of lipid-bound sialic acid after chromatographic separation and staining on thin-layer plates.

We report here that gangliosides G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} after migration and resorcinol staining on silica gel thin-layer plates produce different densitometric detector responses per mole of sialic acid. This finding must be taken into account when assessing the content of individual ganglioside species in tissues by scanning densitometry of thin-layer plates.

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*The opinions or assertions contained herein are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense.

TABLE I

CHEMICAL STRUCTURES OF GANGLIOSIDES UNDER STUDY

Abbreviations: gal = galactose; glu = glucose; galNAc = N-acetylgalactosamine; NANA = N-acetylneuraminic acid or sialic acid.

Ganglioside*	
G_{M4}	Ceramide-gal NANA
G_{M1}	Ceramide-glu-gal-galNAc-gal NANA
G_{D1a}	Ceramide-glu-gal-galNAc-gal NANA NANA

G_{D1b}	Ceramide-glu-gal-galNAc-gal
	          NANA
	          NANA

G_{T1b}	Ceramide-glu-gal-galNAc-gal
	          NANA NANA
	          NANA

*Ganglioside nomenclature is that of Svennerholm [11].

EXPERIMENTAL

Materials

Chloroform and methanol were analytical grade. Silica gel 60, extra pure, particle size 0.063—0.200 mm, and HPTLC plates (10 × 10 cm and 10 × 20 cm) precoated with a 0.2-mm layer of silica gel 60 of 5-μm particle size were from E. Merck (Darmstadt, F.R.G.). N-acetylneuraminic acid·$1H_2O$ used as sialic acid standard was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). Resorcinol was from Sigma (St. Louis, MO, U.S.A.).

Ganglioside purification

Gangliosides G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} ·were extracted from adult human central nervous system white matter [8] and purified by the classical procedure of Suzuki [9]. In G_{M4} purification Folch partitioning was omitted. As a final purification step, each ganglioside was chromatographed in a 1.3-m silica gel column using chloroform—methanol—water (60:35:8 or 55:45:10).

Thin-layer chromatography of gangliosides

HPTLC plates were prewashed by migration in chloroform—methanol (2:1) and activated at 140°C for 10 min. Ten samples were applied to each plate in 3 × 1 mm bands with a 1- or 5-μl positive-displacement microsyringe (Hamilton, 7000 series). Bands were placed 10 mm from the bottom of plates and dried under a constant flow of warm air (30—40°C). Two different developing solvents were studied: solvent A, chloroform—methanol—water—1% $CaCl_2$ (55:45:8:2) and solvent B, chloroform—methanol—water—1% $CaCl_2$ (60:35:7:1). Plates were suspended above solvent A for 60 or solvent B for

30 min to insure a saturated atmosphere in the developing tank. The plates were then lowered into the solvent. The height to which the solvent front was allowed to rise above the origin was varied. Following development, plates were heated at 140°C for 10 min to remove traces of solvent prior to spraying with resorcinol—HCl reagent [10]. Sprayed plates were covered with clean glass covers and again heated at 140°C for 10 min to visualize the blue ganglioside bands.

Scanning densitometry

Ganglioside bands were scanned with a Shimadzu Model CS-910 dual-wavelength TLC scanning densitometer (Kyoto, Japan) at 580 nm (sample wavelength) in the transmission mode at 6 mm/min. The slit length was adjusted to be 10% greater than the longest band after development (G_{M4}) and the slit width was 0.2 mm. The peak area of each band was measured after subtraction of the background absorbance at 720 nm (reference wavelength) by means of a Shimadzu data processor (C-R1B). The linearizer was not used.

Ganglioside purity

Ganglioside purity was evaluated by HPTLC of 1000 pmol ganglioside sialic acid in two different solvent systems, solvents A and B. Ganglioside bands were visualized with resorcinol reagent and quantitated by scanning densitometry. Each ganglioside species showed <1% contamination.

Preparation of ganglioside standards

The sialic acid content of ganglioside solutions was determined by a modification of the colorimetric assay of Svennerholm [10] in which the purple color product was solubilized into a single phase with tertiary butanol. A mixed ganglioside standard containing an equimolar sialic acid content of each of the five ganglioside species was prepared in chloroform—methanol (2:1).

Variability in densitometric quantitation of gangliosides

Intra-assay (intra-plate) variability of 5% was obtained when replicate ganglioside bands present on a single HPTLC plate were scanned.

Inter-assay (plate-to-plate) variability of 6% was determined by scanning three HPTLC plates each containing replicate applications of an unknown ganglioside mixture and mixed ganglioside standards (25, 50, 75 and 100 pmol). Amounts of individual ganglioside components within the unknown mixture were determined from densitometric standard curves run on the same plate.

RESULTS AND DISCUSSION

In order to define chromatographic conditions for the effective quantitation of gangliosides by scanning densitometry, TLC of a standard mixture of gangliosides containing equal sialic acid amounts of G_{M4}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} was studied in two popular solvent systems in the range between 10 and 1000 pmol sialic acid for each ganglioside species. When gangliosides
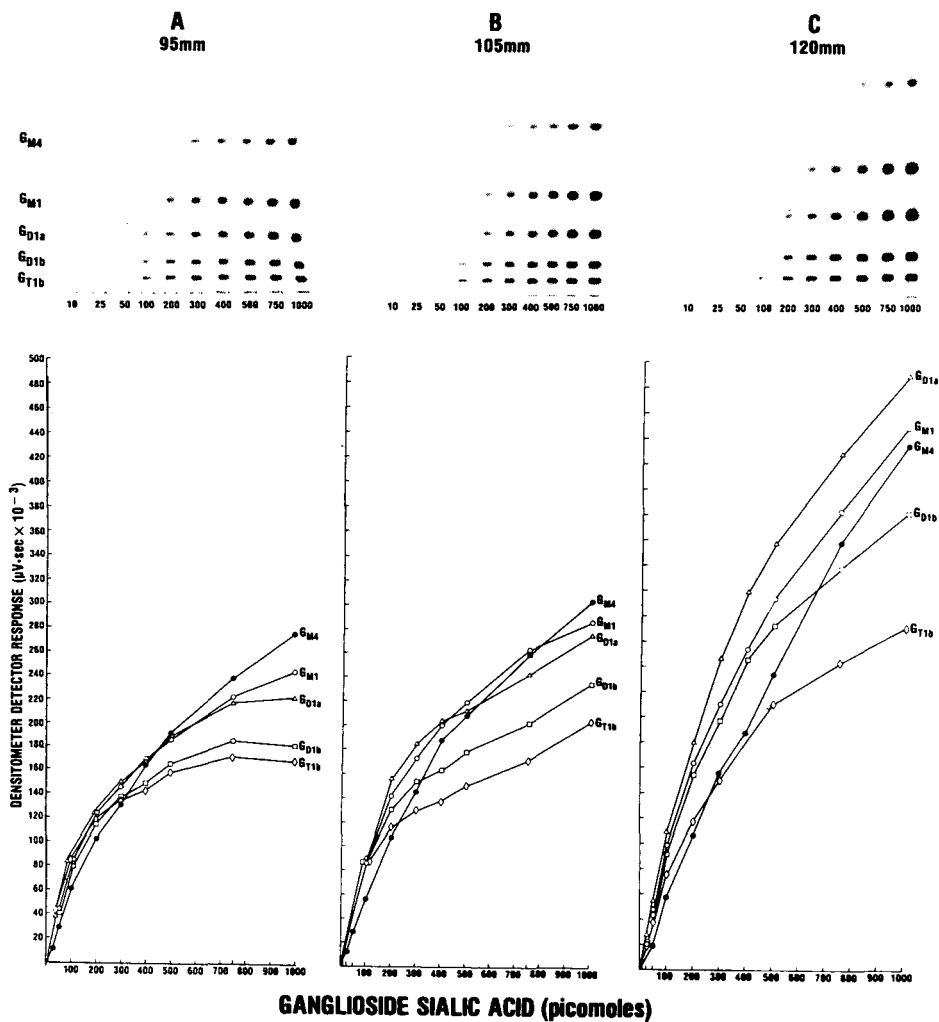
Fig. 1. TLC with corresponding densitometric standard curves of the mixed ganglioside standard in solvent A: chloroform—methanol—water—1% CaCl$_2$ (55:45:8:2). Gangliosides were separated on HPTLC plates and stained with resorcinol reagent. Each lane of the chromatogram contains the indicated pmol sialic acid of each ganglioside species. Each band was scanned at 580 nm in the transmission mode. The densitometer detector response is plotted as a function of amount of ganglioside sialic acid present. Detector response is the peak area due to band absorbance at 580 nm after subtraction of background at 720 nm. Heights of solvent fronts above origin: A, 95 mm; B, 105 mm; C, 120 mm.

164
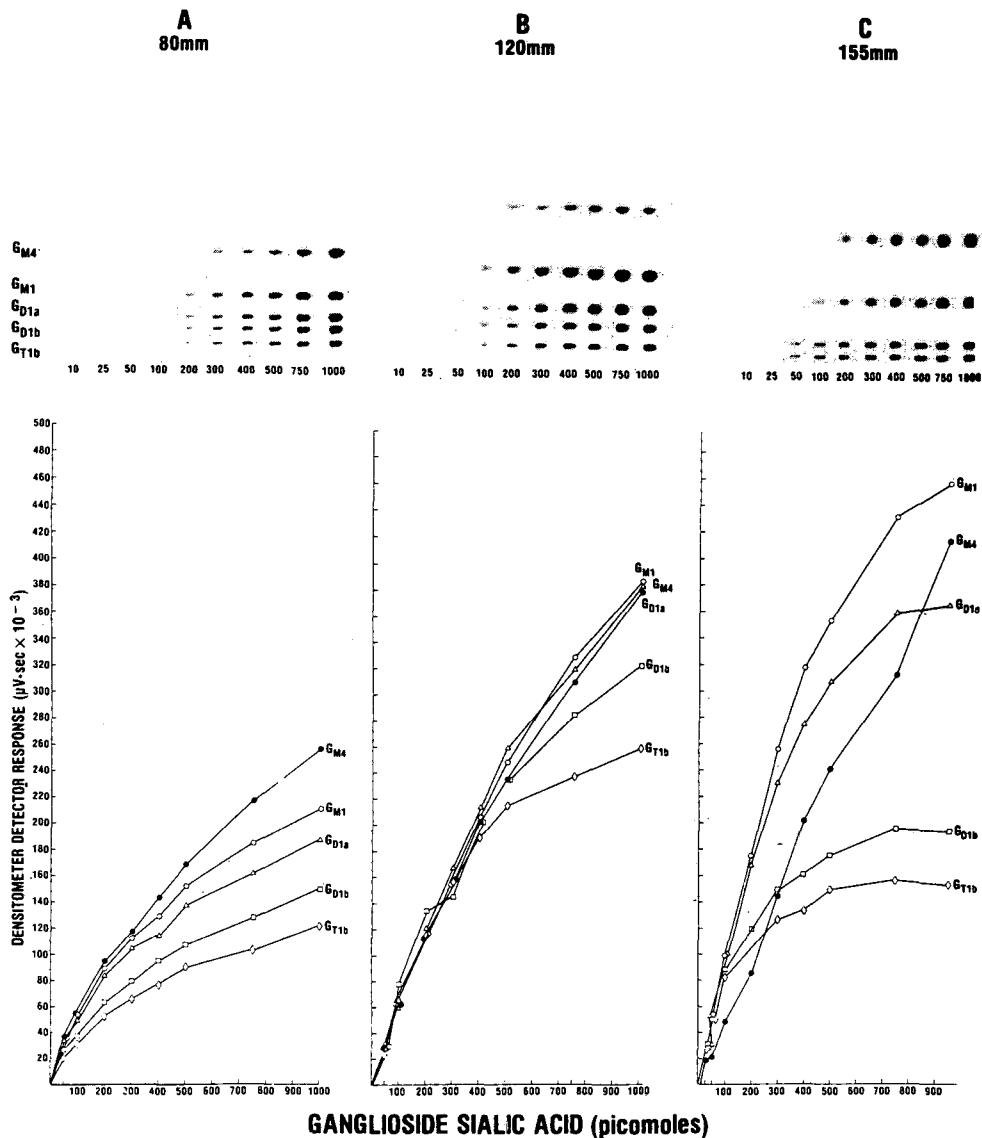
Fig. 2. TLC with corresponding densitometric standard curves of the mixed ganglioside standard in solvent B: chloroform—methanol—water—1% CaCl$_2$ (60:35:7:1). Gangliosides were separated on HPTLC plates and stained with resorcinol reagent. Each lane contains the indicated pmol sialic acid for each ganglioside species. Each band was scanned at 580 nm in the transmission mode. The densitometer detector response is plotted as a function of amount of ganglioside sialic acid present. Detector response is the peak area due to band absorbance at 580 nm after subtraction of background at 720 nm. Heights of solvent fronts above origin: A, 80 mm; B, 120 mm; C, 155 mm.

were chromatographed in solvent A, individual ganglioside species yielded divergent densitometric standard curves (Fig. 1). Standard curves for all gangliosides were essentially non-linear until the solvent front was allowed to reach a height of 105 mm (Fig. 1B). When the front reached 120 mm, all gangliosides had standard curves with an enhanced range of linearity (Fig. 1C). G_{M4} curves were consistently the most linear, while G_{T1b} curves were the least linear. Dense thin ganglioside bands, such as seen with high amounts of G_{T1b} and G_{D1b}, caused the linearity of standard curves to fall off. In contrast, gangliosides of higher R_F which formed larger less dense bands, such as G_{M4} and G_{M1}, had standard curves which were linear throughout a longer range of sialic acid values.

When the ganglioside standard was chromatographed in a less polar system (solvent B), more linear densitometric standard curves were obtained at lower migration heights (Fig. 2A). When the front reached 120 mm above the origin (Fig. 2B), all ganglioside species had overlapping curves which were linear to 500 pmol ganglioside sialic acid. Since the latter chromatographic conditions yielded standard curves with the longest range of linearity, these conditions were chosen when determining the relative distribution of gangliosides within an unknown mixture.

In either solvent system, as little as 10 pmol ganglioside sialic acid could be reproducibly detected, a sensitivity ten-fold greater than that previously reported by Ando et al. [6].

We sought to determine whether differences noted in the densitometric standard curves of individual gangliosides resulted from a differing ability of the gangliosides to form a color product with resorcinol reagent, i.e., had differing molar extinction values. Pure ganglioside solutions were applied to HPTLC plates in 3-mm bands and stained with resorcinol reagent without solvent migration. This resulted in ganglioside bands of nearly identical size and shape, thus negating any influence these factors might have in densitometric measurements. No significant difference in the molar extinction values of gangliosides was detected. This indicated that the individual ganglioside species adsorbed on silica gel were equally reactive with resorcinol reagent. Thus differences in the densitometric standard curves of individual ganglioside species resulted from differences in band size or geometry following migration in solvent. This necessitates the use of standard curves when quantitating individual gangliosides within a mixture.

In summary, when determining the relative distribution of ganglioside species within a mixture by scanning densitometry of thin-layer chromatograms, it is necessary to reference experimental densitometric values to densitometric standard curves of gangliosides present in the mixture. Since the solvent system and distance of solvent front migration can strongly influence band geometry and subsequently densitometric values, unknown mixtures and ganglioside standards must be run on the same plate. TLC conditions which both effectively separate gangliosides and yield linear standard curves are migration in solvent B to a height of 120 mm above the origin.

REFERENCES

1   F.B. Cochran, R.K. Yu and R.W. Ledeen, J. Neurochem., 39 (1982) 773.
2   R.K. Yu, K. Ueno, G.H. Glaser and W.W. Tourtellotte, J. Neurochem., 39 (1982) 464.
3   S. Harth, H. Dreyfus, P.F. Urban and P. Mandel, Anal. Biochem., 86 (1978) 543.
4   H. Rosner, Anal. Biochem., 109 (1980) 437.
5   L.N. Irwin, D.B. Michael and C.C. Irwin, J. Neurochem., 34 (1980) 1527.
6   S. Ando, N.C. Chang and R.K. Yu, Anal. Biochem., 89 (1978) 437.
7   L. Svennerholm and P. Fredman, Biochim. Biophys. Acta, 617 (1980) 97.
8   B.R. Mullin, F.X. DeCandis, A.J. Montanaro and J.D. Reid, Brain Res., 222 (1981) 218.
9   K. Suzuki, Life Sci., 3 (1964) 1227.
10 L. Svennerholm, Biochim. Biophys. Acta, 24 (1957) 604.
11 L. Svennerholm, J. Neurochem., 10 (1963) 613.

CHROMBIO. 1839

Note

Further improvement of the fluorometric assay for hydroxyproline

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The hydroxyprolines (Hyp) constitute a group of amino acids, uncommon in their structure as well as in their metabolism. Their α nitrogen atom is included in a pyrrolidine cycle, a hydroxyl group being attached to either the carbon 3 of the cycle (3-Hyp) or the carbon 4 (4-Hyp). Both natural position isomers are of the *trans* form (*E*) by reference to the carboxyl group located on carbon 2. These amino acids have a special biosynthetic pathway including a preliminary incorporation of proline residues at specific places in polypeptide chains followed by hydroxylation catalyzed by specific enzyme systems. Free proline cannot be hydroxylated, at least in animals.

This biosynthetic pathway is used during the synthesis of proteins belonging to the group of collagens that comprise some twelve different types, all containing a large amount of 4-Hyp. Two types of collagen contain, in addition, a noticeable amount of 3-Hyp: type IV and type V. Also, 4-Hyp is found in several related proteins such as the C_{1q} component of complement, the enzyme acetylcholinesterase, the lung protein alveolyn and also two proteins found in the connective tissue in the immediate vicinity of collagen: elastin and a structural glycoprotein [1]. In addition, some invertebrate proteins seem to contain 4-Hyp synthesized through a different mechanism, whereas many plant species are rich in either 4-Hyp or 3-Hyp.

This distribution explains why 4-Hyp is a marker for all types of collagen while 3-Hyp should be considered as a marker for type IV (basement membrane) and type V (pericellular) collagens. The evaluation of Hyp is of interest in collagen as well as in plant biochemistry.

In several previous papers we described a new technique for the quantitative evaluation of the various isomers of Hyp and also of proline (Pro) [2—4] using the condensation reaction of these pyrrolidine amino acids with the fluorophor 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD—Cl) followed by

thin-layer chromatography on silica gel and fluorometric recording. We also published an application of this method for the evaluation of urinary 3-Hyp [5].

The purpose of this new paper is to introduce a modification of the technique that makes it absolutely specific: prior to the formation of the fluorophor by reaction with NBD—Cl, any remaining primary amine is removed by reaction with o-phthalaldehyde (OPA).

MATERIAL AND METHODS

Reagents

The usual reagents, all of analytical grade, are purchased from Prolabo, Paris. NBD—Cl is obtained from Aldrich, hydrochloric acid and triethylamine from Merck, o-phthalaldehyde from Sigma, Pro, 4-Hyp and 3-methylhistidine from Calbiochem. 3-Hyp is prepared in the laboratory [6]. The standard amino acid mixture is bought from Beckman.

Hydrolysis

Collagen, or protein, or peptide mixture (for instance, originating from urine) is hydrolyzed in 6 M hydrochloric acid at 105°C for 18 h and the hydrolyzate evaporated to dryness.

Ion-exchange chromatography

The fluorometric method is inhibited by inorganic ions, so that two steps of ion-exchange chromatography are necessary. They may be conducted as described in a previous paper [5] or as follows in order to accelerate the operations.

The residues of hydrolyzates are dissolved in 1 ml of 0.20 M citrate buffer, pH 2.2. A 0.20-ml aliquot is layered at the top of a minicolumn of ion-exchange resin suitable for fast elution by centrifugation. This column is made up in the laboratory by drilling a 1-mm diameter hole through the bottom of a conical plastic eppendorf 1.5-ml microtest tube. The hole is covered with a small patch of glass wool, and 0.8 g of M 72 (Beckman) resin, equilibrated with 0.20 M citrate buffer, pH 3.2, is then introduced. The eppendorf microtube is forced into the top of an 8-ml glass centrifuge tube of the same internal diameter and centrifuged at 1000 g in a Heraeus Labofuge III centrifuge equipped with horizontal tubes. The resin is washed four times with 1 ml of the same buffer and centrifuged every time using the same conditions. It takes less than 7 min for the pyrrolidine amino acids to get through the resin and to collect in the bottom space. A set of ten minicolumns may be operated by the same technician within a few minutes. In the case of urine analysis, all pigments and the basic amino acids remain on the column which may be regenerated by 1 ml of 0.2 M sodium hydroxide followed by 3 ml of 0.20 M citrate buffer, pH 3.2.

Then, the effluents of these minicolumns are chromatographed through a 4 × 0.8 cm column of Dowex W-50-X2 in the H⁺ cycle. The pyrrolidine amino acids are bound and, after several washes by distilled water, eluted by 15 ml of 2 M ammonia solution. This solution is evaporated to dryness

under a stream of nitrogen. The residue is carefully dissolved in a minimal volume of 2 *M* ammonia solution and transferred to an eppendorf conical test tube. A second volume of 2 *M* ammonia solution is used for washing the tube and added to the first one. Then the contents of the eppendorf tube are evaporated to dryness. This method permits the final residue to be dissolved in only 20 μl of distilled water.

Derivatization with o-phthalaldehyde (OPA)

The OPA reagent is obtained by dissolving 8 mg of OPA in 1 ml of a 30% (v/v) triethylamine solution. The addition of 0.02% (v/v) mercaptoethanol, proposed by many authors in the evaluation of primary amines by OPA, is not necessary. To the 20 μl of the above amino acid solution are added 20 μl of OPA reagent. The solution is gently shaken and allowed to stand for 5 min at room temperature.

Derivatization with NBD—Cl

The preparation of the NBD—Cl reagent was described in a previous paper [4]. It contains 6 mg of NBD—Cl per ml of ethanol (30 m*M* solution) and must be stored in the dark. Twenty microliters of this reagent are added to the mixture of amino acids and OPA and allowed to react at 65°C for 30 min in the dark.

Thin-layer chromatography

A 5-μl aliquot of the NBD—amino acids solution is spotted on the silica gel thin-layer plate. The system was fully described in a previous paper [4]. A new solvent system was adopted for 3-Hyp separation: chloroform—triethylamine—methanol (80:10:10, v/v). The migration lasts 60 min. The plate is dried at 65°C for 5 min and the fluorescent spots are scanned in a Farrand spectrofluorometer Mark I equipped with a thin-layer plate recording device.

The excitation wavelength is set at 355 nm with an additional violet filter absorbing light over 500 nm and a slit 10 nm wide. The emitted light is read at 525 nm with an additional yellow filter absorbing radiation under 450 nm. The surface areas of recorded peaks are measured and compared with a set of four standards deposited on the same plate. There is a linear relationship between the surface area of the peak and the amount of Hyp deposited on the plate in the range 1—400 pmol [4].

The above proportions have been given for the evaluation of 3-Hyp. When the evaluation of 4-Hyp is required, one has to dilute the residue of hydrolysis in a 1:10 proportion with distilled water, prior to the condensation with OPA. This is particularly so for urine.

RESULTS

Fig. 1 shows a picture of the separations obtained by thin-layer chromatography of the fluorescent NBD derivatives and Fig. 2 is a scan obtained with a sample of urine after OPA and NBD—Cl reactions. It is evident that

some interfering amino acids remain in the vicinity of 3-Hyp or 4-Hyp when OPA is omitted but that the derivatization with OPA eliminates any interfering material.
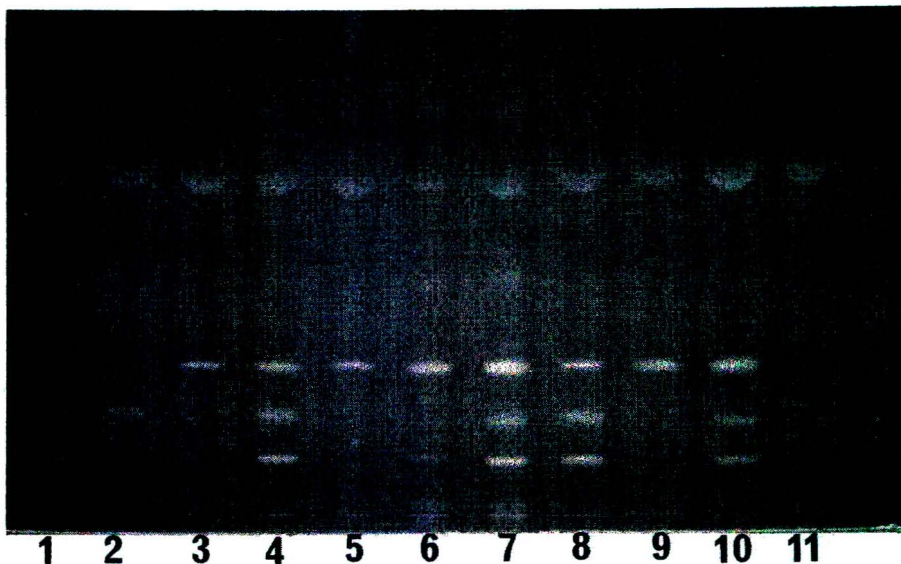
Fig. 1. Thin-layer chromatography of several amino acids after NBD—Cl derivatization. Lanes 1—7: samples directly reacted with NBD—Cl according to the method of ref. 4. Lanes 8—10: samples reacted with o-phthalaldehyde (OPA) prior to NBD—Cl (technique described in this paper). All the samples were deposited in 200-pmol amounts. Lane 1: control trans-4-Hyp. Lane 2: control trans-3-Hyp. Lane 3: control Pro. Lane 4: mixture of 4-Hyp, 3-Hyp and Pro. Lane 5: control cis-4-Hyp (kindly provided by Dr. R. Berg, Piscataway, NJ). Lane 6: a mixture of amino acids (Beckman). Lane 7: the same mixture supplemented with 200 pmol each of trans-4-Hyp, trans-3-Hyp and Pro. Lane 8: the same as lane 4 but previously reacted with OPA. Lane 9: the same as lane 6 but previously reacted with OPA. Lane 10: the same as lane 7 but previously reacted with OPA. Lane 11: reagents.

In order to assess the usefulness of the OPA reaction, we compared the concentrations of 3-Hyp obtained for sixteen samples of urine by three different methods: the ion-exchange technique that we described several years ago [7], the NBD—Cl technique without OPA, and the new improvement. With the fluorometric method performed in the absence of OPA, we found results higher by nearly 30% than with the ion-exchange technique. The results of the OPA + NBD—Cl technique correlated with the colum chromatographic technique ($r = 0.98$, $n = 16$). We found that times of contact of OPA with the amino acid solution ranging from 5 to 30 min did not modify the results.

For the evaluation of efficiency, five samples of urine were supplemented with a known amount of 3-Hyp and the results gave 96.3 ± 3.3% of the amount added. The repeatability of the method was studied fourteen times on the same urine, giving the results 6.0 ± 0.4 μmol/l for 3-Hyp and 82.0 ± 5.7 μmol/l for 4-Hyp.
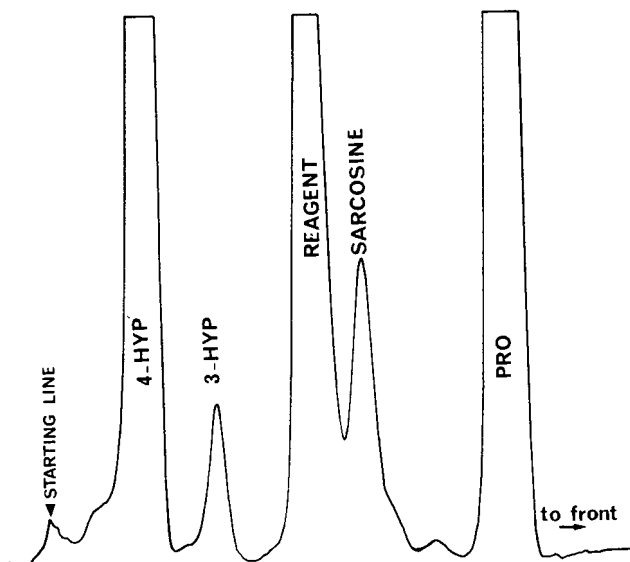
Fig. 2. Scan of thin-layer chromatogram of NBD derivatives of 4-Hyp, 3-Hyp and Pro from a hydrolyzate of urine (100 μl). Silica gel plate. Solvent: chloroform—methanol—triethyl-amine (80:10:10, v/v). Development: 1 h at 20°C. Scanning: spectrofluorometer Farrand Mark I, sensitivity 0.3, speed 150 mm/min. The surface area of the 3-Hyp peak can easily be measured. Surface areas of 4-Hyp and Pro cannot be measured without prior dilution of the sample.

DISCUSSION

The NBD technique for the evaluation of the hydroxyproline isomers is far more sensitive than any of the previously described ion-exchange techniques. It permits the accurate measurement of picomole amounts of these pyrrolidine amino acids. For full efficiency, the reaction must operate in the absence of mineral ions. The fluorescence of primary amino acids is ten to a hundred times lower. Nevertheless, these amino acids may interfere when they occur in very large amounts in the initial medium or when this medium has to be strongly concentrated for improving the sensitivity. In this paper, we described a fast method for completely desalting the solutions or urines and we largely improve the specificity by the use of the OPA reagent. In these conditions, we may apply the technique to larger volumes of initial solution and then increase the sensitivity.

The data obtained for urinary 3-Hyp confirm the reference values that we had previously established by another technique [7]. The values that we obtain for urinary 4-Hyp are lower than those given by the classical reaction with *p*-dimethylaminobenzaldehyde [8, 9]. We suspect that some non-specifically reacting substances such as pyrrole radicals exist in the urine and increase artefactually the results given by this colorimetric technique.

REFERENCES

1 C.H.J. Sear, M.E. Grant and D.S. Jackson, Biochem. J., 194 (1981) 587.
2 A. Szymanowicz, C. Cheron and J.P. Borel, Biochimie, 61 (1979) 425.
3 A. Szymanowicz, G. Poulin, N. Fontaine, J.P. Werquin and J.P. Borel, J. Chromatogr., 190 (1980) 457.
4 A. Bisker, V. Pailler, A. Randoux and J.P. Borel, Anal. Biochem., 122 (1982) 52.
5 G. Bellon, A. Bisker, F.X. Maquart, H. Thoanes and J.P. Borel, J. Chromatogr., 230 (1982) 420.
6 A. Szymanowicz, O. Mercier, A. Randoux and J.P. Borel, Biochimie, 60 (1978) 499.
7 A. Szymanowicz, G. Poulin, A. Randoux and J.P. Borel, Clin. Chim. Acta, 91 (1979) 141.
8 J.P. Borel, J.M. Caranjot and M.F. Jayle, Clin. Chim. Acta, 16 (1967) 409.
9 K.I. Kivirikko, Int. Rev. Connect. Tissue Res., 5 (1970) 93.

CHROMBIO. 1825

Note

Identification of a metabolite of propafenone in human urine by means of high-performance liquid chromatography and gas chromatography—mass spectrometry

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Propafenone, 2'-(3-propylamino-2-hydroxy-propoxy)-3-phenyl-propiophenone, is a new antiarrhythmic drug which has proved effective in recurrent supraventricular and ventricular tachycardias, tachyarrhythmias and ectopic beats [1, 2].

The pharmacokinetic behaviour of the drug in man after intravenous and oral administration has been reported [3]. Propafenone undergoes extensive biotransformation [4] but the metabolism has not been elucidated.

Different methods for the determination of propafenone in plasma have been published [5—7]. It has also been suggested [6] that an unidentified peak eluting near propafenone in plasma extracts could be due to a metabolite. The purpose of the present study is to investigate the nature of this metabolite.

EXPERIMENTAL

Propafenone·HCl was obtained from Knoll (Ludwigshafen, F.R.G.). *n*-Heptane, methylene chloride and absolute ethanol, HPLC grade, were purchased from Merck (Darmstadt, F.R.G.). Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, IL, U.S.A.). All other solvents and chemicals were analytical grade.

High-performance liquid chromatography (HPLC)

A Perkin-Elmer Series 3B liquid chromatograph equipped with a Merck

LiChrosorb® Si 60 5-μm column (25 × 0.46 cm) was used. The mobile phase was dichloromethane—heptane—ethanol—water—ammonium hydroxide (180: 120:200:20:0.2) at a flow-rate of 2.5 ml/min. The effluent was monitored with a Perkin-Elmer LC-75 spectrophotometer at 254 nm.

Gas—liquid chromatography (GLC)

A Perkin-Elmer Sigma 4 gas chromatograph equipped with a ^{63}Ni electron-capture detector was employed. A 180 × 0.2 cm glass column packed with OV-101 3% on Gas-Chrom Q (100—120 mesh) was used. The instrumentation and the conditions were the same as reported previously for gas chromatographic analysis [7].

Gas chromatography—mass spectrometry (GC—MS)

GC—MS analysis was carried out on a Hewlett-Packard HP5792/5970A. A cross-linked fused-silica capillary column (12.5 m × 0.2 mm) coated with OV-1 as stationary phase was used. The carrier gas was helium at a flow-rate of 1.2 ml/min. The oven temperature was programmed from 70°C to 270°C at a rate of 12°C/min. Electron-impact spectra were recorded using 70-eV electron energy and a source temperature of 220°C.

Extraction procedure and derivatization

Plasma and urine samples from the same patient were extracted with benzene after alkalinization as described by Brode et al. [5]. Then 100 ml of urine were extracted using the same procedure to obtain a sufficient quantity of the metabolite for the GLC—electron-capture detection (ECD) and GC—MS analyses. The combined extracts were dried under a stream of nitrogen. The residue was reconstituted with methanol and injected into the liquid chromatograph.

The fractions corresponding to the metabolite were collected in a glass-stoppered tube and dried under a stream of nitrogen at 37°C. The residue was derivatized with TFAA in anhydrous toluene at 37°C for 1 h. Excess anhydride was removed by evaporation. The residue was reconstituted with cyclohexane and divided into two parts: the first was injected into the GLC—ECD system, the second into the GC—MS system.

RESULTS AND DISCUSSION

It has been reported that less than 1% of unchanged propafenone is excreted in urine after its oral administration [4]. These findings suggest that the drug undergoes extensive metabolization and it has not been excluded that one or more metabolites contribute to the antiarrhythmic action of propafenone.

It is well known that the β-blocking agent propranolol, which has common structural features with propafenone, is metabolized to 4-hydroxy-propranolol and that this compound is pharmacologically active. It is therefore important to investigate the structures of the metabolites of propafenone.

Several authors [5, 6] have pointed out that one or two peaks, probably due to biotransformation, can occur in plasma extracts from patients who have taken propafenone orally. Our previous experience using HPLC [5] for

the determination of propafenone confirmed the existence of one peak eluting close to propafenone and with the same retention time in plasma and urine samples extracted by the same analytical procedure (Fig. 1). It is therefore reasonable that it is the same unidentified compound in both plasma and urine.
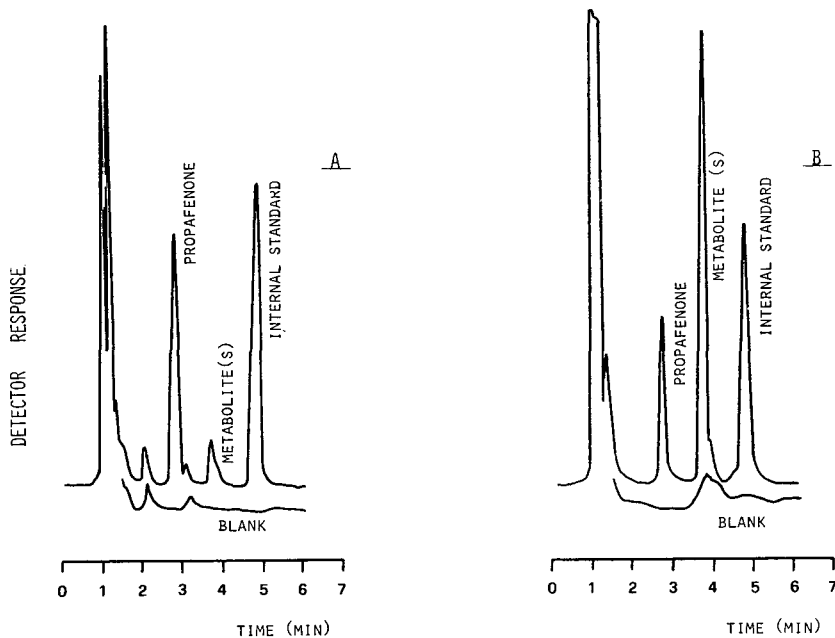
Fig. 1. Representative HPLC chromatograms of plasma (1 ml, A) and urine (2 ml, B) samples after chronic administration of propafenone.

Since the plasma concentration of the unknown compound is unsuitable for identification purposes, the corresponding fraction of urine was collected and analyzed. To obtain some preliminary information on the metabolite, the fraction was dried under a stream of nitrogen, derivatized with TFAA as described above and one portion injected into the GC—ECD system. The chromatogram in Fig. 2 shows two peaks eluting after propafenone: the first at 8.69 min, the second, much smaller than the first, at 10.03 min. These results indicate that the metabolite contains groups reacting with TFAA with a molecular weight probably higher than that of the propafenone derivative.

The second portion was injected into the GC—MS system under the conditions described above. Fig. 3 shows the total ion monitoring and the m/e 308 selective ion monitoring plots of the injected fraction.

Fig. 4 shows the mass spectrum of propafenone—TFA used as a reference for comparison with the spectrum of the unknown metabolite. Propafenone has an alkylaminohydroxypropoxy side-chain in common with most of the β-adrenoceptor antagonists; therefore its spectrum shows many fragment ions characteristic of the spectra of TFA β-blockers. These fragment ions are at m/e 308, 266, and 43 with high relative abundance, and at m/e 194, 168, 152, and 126 with low relative abundance. The attribution of these fragment
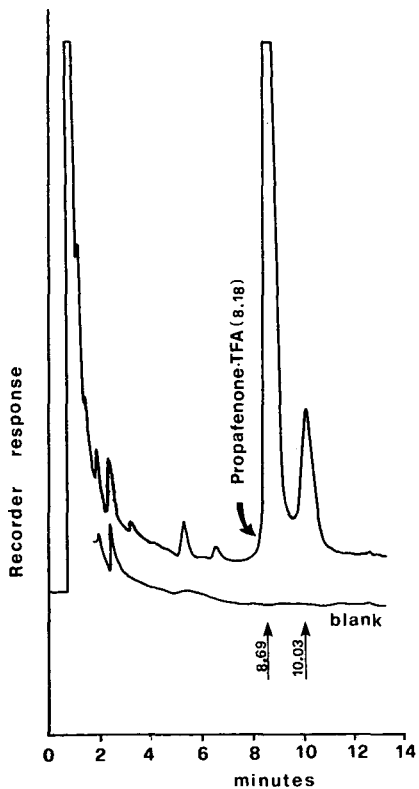
Fig. 2. Representative GLC—ECD chromatogram of the metabolite fraction from urine after derivatization with TFAA.
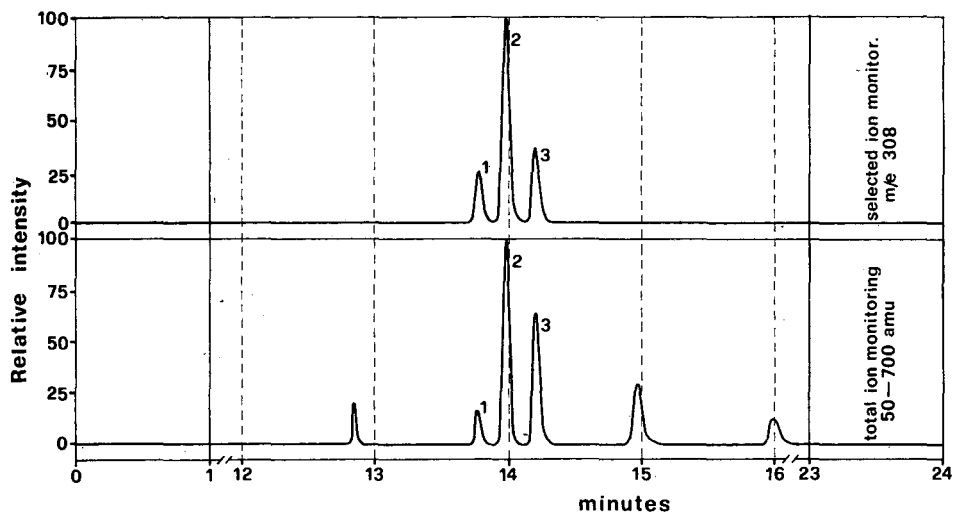
Fig. 3. Total ion monitoring and selective ion monitoring (m/e 308) of the metabolite fraction from urine after derivatization with TFAA. Peaks: 1 = propafenone∘TFA, 2 = hydroxylated propafenone∘TFA and 3 = unidentified compound.

ions and their relative fragmentation mechanism is described in the literature [8]. The propafenone—TFA molecular ion at m/e 533 with low relative abundance is consistent with formation of a di-(trifluoroacetyl) derivative.

The fragment ion at m/e 225 could be reasonably ascribed to the remaining part of the molecule after the loss of the alkylaminohydroxypropoxy side-chain. Its intensity should be relatively weak because, after fragmentation, the charged ion is m/e 308. The fact that the relative abundance of m/e 225 compared with that of m/e 308 is 6.8% supports our hypothesis.
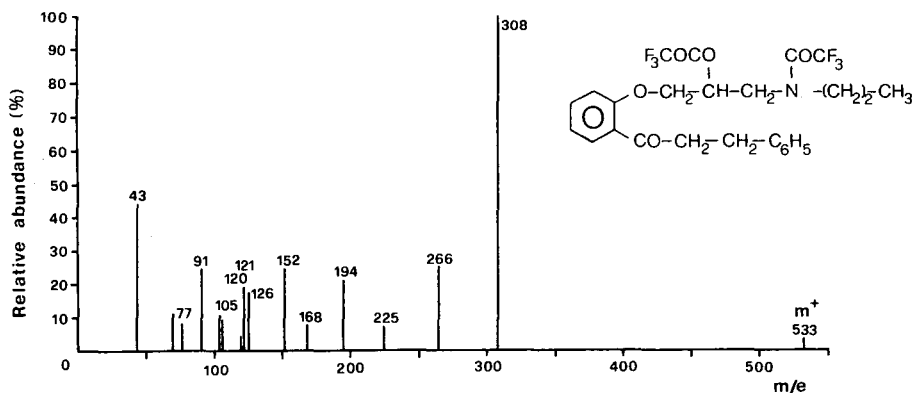
Fig. 4. Mass spectrum of propafenone—TFA and its structural formula.

The fragment ions at m/e 77, 91 and 105 can be explained on the basis of the literature on alkylbenzenic compounds [9]. In analogy with the fragmentation behaviour of alkylbenzenic ethers and phenylalkylketones [9], the fragment ions at m/e 120 and 121 were ascribed to the structures shown below.
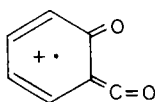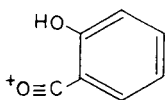
m/e 120 m/e 121

The mass spectra of the three peaks shown in Fig. 3 were recorded. The mass spectrum of the first peak was found to be identical to that of propafenone—TFA. This could be explained by a propafenone contamination during the collection of the HPLC fraction.

The mass spectrum of second peak is shown in Fig. 5. The molecular ion of this compound is at m/e 645, i.e. 112 mass units higher than propafenone—TFA. The same difference was found between the ions at m/e 232 and 233 of this compound and ions at m/e 121 and 120 of propafenone—TFA, and could reasonably be ascribed to a —OCOCF$_3$ group. This hypothesis is consistent with the fact that hydroxylation of the aromatic ring is a metabolic pathway common to these compounds [10]. The presence also of the fragment ions at m/e 77, 91 and 105 in the spectrum of the metabolite indicates that the hydroxylation occurs at the two-fold substituted aromatic ring. Moreover, the

178

possibility of metabolic degradation of the alkylaminohydroxypropoxy side-chain can be excluded because all the characteristic fragment ions are present in the spectrum of the metabolite.
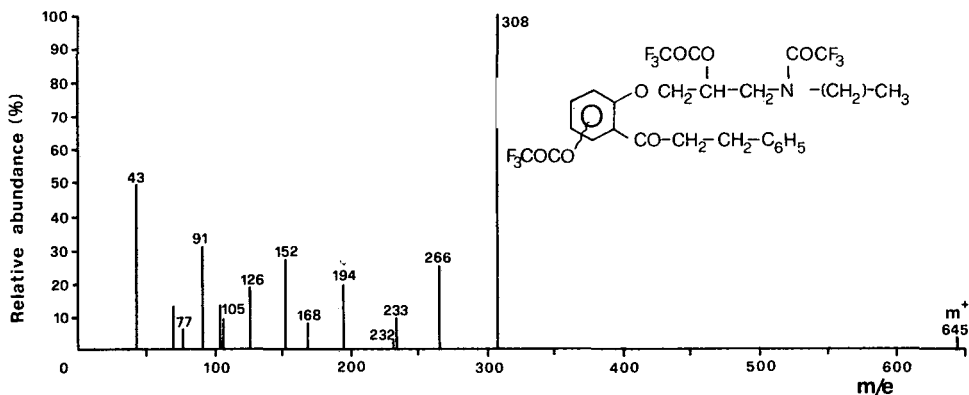
Fig. 5. Mass spectrum of the second peak corresponding to the metabolite.

The mass spectrum of the third peak was very difficult to interpret on the basis of the common metabolic pathways; the structure of this substance thus remains an open problem.

These findings indicate that propafenone undergoes metabolic biotransformation and that the main metabolite is a hydroxylated derivative.

ACKNOWLEDGEMENTS

REFERENCES

1 O.A. Beck, K.D. Kramer, R. Wolf, A. Muller and H. Hochrein, Med. Klin., 70 (1975) 95.
2 H. Hochrein, H.J. Hapke and O. Beck (Editors), Fortschritte in der Pharmakotherapie von Herzrhythmusstörungen. I Internationales Propafenon-Symposium, Gustav Fischer Verlag, Stuttgart, New York, 1977.
3 K. Keller, G. Meyer-Estorf, O.A. Beck and H. Hochrein, Eur. J. Clin. Pharmacol., 13 (1978) 17.
4 B. Stieren, W. Koch and J.A. Schwartz, Pharmacokinetics and bioavailability of propafenone hydrochloride, Knoll Internal Reports, Ludwigshafen.
5 E. Brode, R. Sachse and H.D. Hoffmann, Arzneim.-Forsch., 32 (1982) 1.
6 S.R. Harapat and R.E. Kates, J. Chromatogr., 230 (1982) 448.
7 B. Marchesini, S. Boschi and M.B. Mantovani, J. Chromatogr., 232 (1982) 435.
8 D.A. Garteiz and T. Walle, J. Pharm. Sci., 61 (1972) 1728.
9 D.H. Williams and I. Fleming (Editors), Spectroscopic Methods in Organic Chemistry, McGraw-Hill, Maidenhead, 1966.
10 W.O. Foye (Editor), Principles of Medicinal Chemistry, Lea & Febiger, Philadelphia, PA, 1974.

CHROMBIO. 1842

Note

Gas chromatographic assay of codeine in human plasma utilizing nitrogen-selective detection

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Codeine is a widely prescribed narcotic analgesic and antitussive drug. The determination of codeine in human plasma samples following ingestion of therapeutic doses of codeine requires assays which are both highly sensitive and selective. Various analytical methods [1—10] have been developed for this purpose. They include radioimmunoassay (RIA) (1), gas chromatography—mass spectrometry (GC—MS) (2) and gas chromatography (GC) [3—10]. Among these methods, GC is the technique commonly available in most laboratories. Previously reported GC methods employed either flame ionization detection (FID) [3—7] or electron-capture detection (ECD) [8—10]. These methods require derivatization for improvement of GC properties and/or sensitivity. They are usually time-consuming and difficult to establish.

This paper describes a GC method employing nitrogen-selective detection. No derivatization step is necessary with the present method. The procedure is rapid and sensitive and has been used successfully in analyzing plasma samples from clinical studies.

EXPERIMENTAL

Material

Nanograde-quality methanol and chloroform were purchased from Mallinckrodt (St. Louis, MO. U.S.A.) and glass-distilled grade *n*-butanol from Burdick and Jackson (Muskegon, MI, U.S.A.). All solvents were used without further purification. Glass bottles (15 ml) from Wheaton Company (Millville, NJ, U.S.A.) with polyethylene screw-caps from Poly-Seal Corp. (Baltimore, MD, U.S.A.) were used for the plasma extraction and 12-ml glass-stoppered centrifuge tubes (Arthur H. Thomas, Philadelphia, PA, U.S.A.) were used for the final solvent evaporation. Volumetric glass pipettes were utilized for all solvent transfers. All glassware was soaked in chromic acid for at least 1 h,

rinsed thoroughly with distilled water and heat treated for 3 h at 270°C. Poly-ethylene screw-caps were soaked in n-heptane for at least 1 h and dried at 60°C prior to use. Codeine phosphate and oxycodone hydrochloride (McNeil Pharmaceutical, Spring House, PA, U.S.A.) were used as received from the stock room. Codeine phosphate was of U.S. Pharmacopoeial quality and oxy-codone hydrochloride met all U.S. New Drug Application requirements.

Stock solutions

Stock solutions of codeine phosphate were prepared in methanol in con-centrations of either 10 or 1 μg/ml. A stock solution of oxycodone hydro-chloride, the internal standard, was first prepared in methanol (10 μg/ml), which was further diluted to 3 ng/ml with 10% butanol in chloroform. All concentrations were expressed in terms of the free base. Clinical control plasma obtained from Interstate Blood Bank (Philadelphia, PA, U.S.A.) was used in the preparation of the codeine plasma standards. Plasma standards (volume 10.0 ml) ranging from 5 to 200 ng/ml were prepared by the addition of various amounts of codeine stock solutions (volume 0.05—0.20 ml).

Apparatus

A Hewlett-Packard Model 5710A (Avondale, PA, U.S.A.) gas chromatograph equipped with dual nitrogen—phosphorus-selective detectors was used. The column was a 0.91 m (3 ft.) × 2 mm I.D. silanized glass column packed with 1% OV-17 on Chromosorb W HP (100—120 mesh) from Supelco (Bellefonte, PA, U.S.A.). The column was conditioned at 270°C overnight with a helium flow of 30 ml/min.

Chromatographic conditions were: column oven temperature, 235°C; injection port temperature, 260°C; detector temperature, 300°C. The helium carrier gas flow-rate was 30 ml/min, hydrogen flow-rate was 3 ml/min and air flow-rate was 100 ml/min. The voltage of the detector was set at 18 V. Under these conditions, the retention times for codeine and oxycodone were 2.3 and 3.8 min, respectively.

Assay procedure

A 2-ml volume of plasma containing codeine as standard or unknown, 10.0 ml of internal-standard solution (30 ng of oxycodone in 10% butanol in chloro-form) and 1.0 ml of 1 N sodium hydroxide were added to a 15-ml bottle, and the mixture was shaken on an Eberbach table-top shaker, Eberbach (Ann Arbor, MI, U.S.A.) at 120 oscillations/min for 20 min. After centrifugation at 681 *g*, the supernatant aqueous phase was aspirated and discarded. An 8-ml volume (± 0.5 ml) of the organic layer was transferred to a 15-ml bottle con-taining 5.5 ml of 0.1 N sulphuric acid. The mixture was then shaken for 20 min and centrifuged for 10 min. A 5-ml volume (± 0.5 ml) of the aqueous layer was transferred to a 15-ml bottle containing 8.0 ml of 10% butanol in chloroform and 1.0 ml of 1 N sodium hydroxide. The mixture was shaken for 15 min and centrifuged for 10 min. The aqueous phase was removed and discarded. A 7-ml volume (± 0.5 ml) of the remaining organic phase was pipetted into a 12-ml centrifuge tube, and the solvent was evaporated to dryness under a stream of nitrogen at 40°C. A 1-ml volume of chloroform was added to each

tube to wash down the sides of the tube by vortex action. The chloroform was then evaporated to dryness. The residue was reconstituted in 20 μl of methanol, and 5 μl of the solution were injected into the gas chromatograph.

Clinical study samples

The mean plasma concentration data were taken from the results of a bioavailability study comparing the bioavailability of three drug products all containing codeine and acetaminophen. (The products were found to be bioequivalent.) In this study 24 healthy male adult volunteers were fasted for at least 12 h before and 1 h after administration. Two tablets (each containing 325 mg of acetaminophen and 30 mg of codeine phosphate) and 200 ml of distilled water were administered to each subject.

Blood samples of 15 ml were collected in heparinized vacuum tubes by venipuncture at 0 (pre-dose), 10, 20, 40, 60 and 90 min and 2, 3, 4, 6 and 8 h after administration. The plasma was separated by centrifugation immediately after collection and was stored frozen at $-10°$C in polypropylene snap-cap tubes until analysis.

RESULTS AND DISCUSSION

Codeine is fairly water-soluble and thus a polar extraction solvent such as 10% butanol in chloroform was required. Other solvents, such as ether, ethyl acetate and chloroform, were found to give unsatisfactory extraction efficiencies (<50%) in a three-step extraction procedure. The present procedure has an extraction efficiency of 84 ± 4% (8 determinations) at 50 ng/ml and 81 ± 3% (10 determinations) at 150 ng/ml of codeine in plasma.

Typical chromatograms from plasma samples with and without codeine are
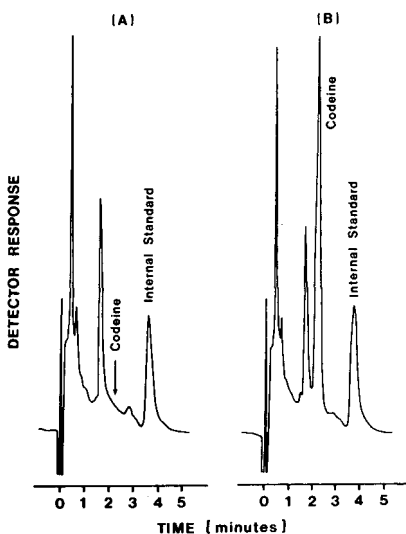
Fig. 1. Typical gas chromatograms from (A) 2 ml of blank plasma containing 30 ng of internal standard (blank without internal standard showed no peak at around 3.8 min and (B) 2 ml of plasma containing 300 ng of codeine and 30 ng of internal standard.

shown in Fig. 1. From these chromatograms, it is clear that there are no peaks in blank plasma samples which interfere with codeine.

The measurable metabolites of codeine in human plasma are morphine, norcodeine and their conjugates [4, 11]. Morphine and the conjugates of morphine and norcodeine are not extracted using this procedure; therefore, they cannot interfere with the assay. Norcodeine is extracted by the procedure, but the concentration of norcodeine in plasma is very low (<10 ng/ml at 1 h) [4] and its retention time is longer than that of codeine. Under the present GC conditions, norcodeine, codeine and oxycodone have retention times of 2.9, 2.3 and 3.8 min, respectively. Drugs that are co-administered with codeine, such as acetaminophen and aspirin, do not interfere in the assay since neither compound is extracted using the present procedure.

The standard curve is linear between 20 and 200 ng/ml. The peak height ratios of replicate samples (expressed as codeine/oxycodone) obtained from the addition of known amounts of codeine to control blank plasma, followed by processing according to the described procedure, are summarized in Table I. A least-squares regression analysis of the data gives a straight-line standard curve with a correlation coefficient of 0.98, a slope of 0.022 and an intercept of -0.06 ± 0.06, which, within experimental error, shows that the line passed through the origin. The assay is reproducible (inter-run) as can be seen in Table I. The coefficients of variation (C.V.) range from 7 to 12% for all selected concentrations.

The concentrations of codeine in plasma following ingestion of a therapeutic dose (60 mg) of codeine are in the range of 5 to 200 ng/ml [11]. At these concentrations, previously reported GC methods using FID or ECD require derivatization for improvement of chromatographic properties and/or sensitivity [3—10]. The nitrogen—phosphorus-selective detector used in the nitrogen mode, however, shows good sensitivity in detecting codeine. The detection limit for codeine was 3 ng injected, and the detector response was linear between 3 and 150 ng. The lowest concentration of codeine in a 2-ml plasma sample that could be detected was 5 ng/ml. However, the lowest level of codeine in plasma (2 ml sample) that could be quantitated both accurately and

TABLE I

MEANS, STANDARD DEVIATIONS AND COEFFICIENTS OF VARIATION OF PEAK HEIGHT RATIOS AT VARIOUS CODEINE CONCENTRATIONS GENERATED OVER A PERIOD OF TWO WEEKS

Codeine plasma concn. (ng/ml)	No. of determinations	Peak height ratios		
		Mean	S.D.	C.V.
20	10	0.33	0.02	0.07
50	18	1.00	0.11	0.12
75	17	1.66	0.18	0.10
125	15	2.69	0.28	0.10
150	19	3.28	0.37	0.11
200	18	4.43	0.39	0.08
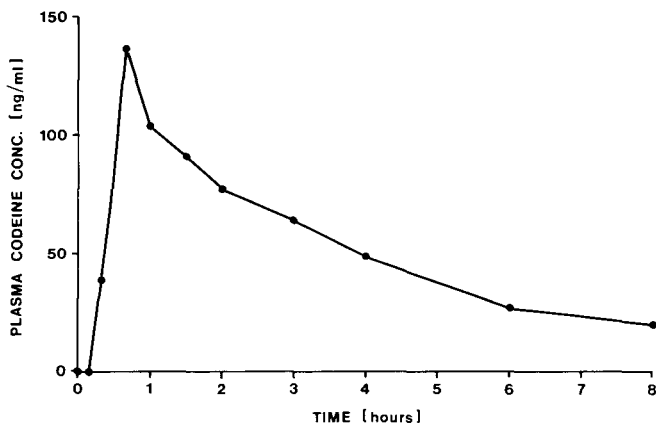
Fig. 2. Plasma-concentration profile of codeine in a human subject following oral administration of 60 mg of codeine phosphate and 650 mg of acetaminophen as two tablets.

TABLE II

MEAN PLASMA CODEINE CONCENTRATIONS FROM A CLINICAL STUDY DETERMINED BY THE PRESENT GC METHOD

Time after drug administration	Mean codeine concn. (ng/ml) from the study (24 subjects)	
	Mean	± S.E.M.
10 min	2.0	1.6
20 min	46.0	8.4
40 min	147.0	12.6
1 h	136.0	10.4
1.5 h	111.0	7.6
2 h	85.0	4.1
3 h	65.0	3.3
4 h	55.0	3.7
6 h	29.0	2.4
8 h	7.0	2.9

precisely (C.V. <15%) was 20 ng/ml. We find that this is adequate sensitivity for clinical samples.

To date, this procedure has been employed successfully in analyzing over 1000 clinical samples for codeine. No interference peaks due to metabolites have ever been observed with the clinical samples. A typical plasma-concentration profile of codeine in man is shown in Fig. 2.

Mean plasma codeine concentrations (± S.E.M.) from a portion of the clinical study as obtained by the present method are shown in Table II. The mean plasma concentration vs. time profile is almost identical to that reported by Findlay et al. [11] for a similar clinical study using the RIA method [1].

The present procedure is rapid since no derivatization step is required. Up to 60 samples can be analyzed routinely in a 8-h working day. Also, the procedure is sensitive enough to have utility in handling clinical plasma samples and a variety of other types of samples, such as urine and animal plasma samples.

REFERENCES

1 J.W.A. Findlay, R.F. Butz and R.M. Welch, Life Sci., 19 (1976) 389.
2 W.O.R. Ebbighausen, J.H. Mowat, P. Vestergaard and N.S. Kline, Adv. Biochem. Psychopharmacol., 7 (1973) 135.
3 E. Schmerzler, W.Yu. M.I. Hewitt and J.J. Greenblatt, J. Pharm. Sci., 55 (1966) 155.
4 M.K. Brunson and J.F. Nash, Clin. Chem., 21 (1975) 1965.
5 G.R. Nakamura and E.L. Way, Anal. Chem., 47 (1975) 775.
6 W.J. Serfontein, D. Botha and L. deVilliers, J. Pharm. Pharmacol., 27 (1975) 937.
7 R.A. Zweidinger, F.M. Weinberg and R.W. Handy, J. Pharm. Sci., 65 (1976) 427.
8 B. Dahlstrom and L. Paalzow, J. Pharm. Pharmacol., 27 (1975) 172.
9 M.J. Kogan and M.A. Chedekel, J. Pharm. Pharmacol., 28 (1976) 261.
10 J.M. Moore, J. Chromatogr., 147 (1978) 327.
11 J.W.A. Findlay, E.C. Jones, R.F. Butz and R.M. Welch, Clin. Pharmacol. Ther., 24 (1978) 60.

CHROMBIO. 1837

Note

Gas chromatographic determination of oxprenolol in human plasma

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Various methods for the assay of oxprenolol in biological fluids are available. A direct evaluation in plasma by thin-layer chromatography and fluorimetric determination has been described [1]. A high-performance liquid chromatographic assay for quantitation in blood, plasma, urine and breast milk has also been reported [2]. Several gas chromatographic (GC) methods have been employed [3—6]. We recently published a GC assay for another β-blocking agent, metoprolol, in plasma, using oxprenolol as internal standard [7]. This method was applied for the determination of oxprenolol in plasma. It differs from the previous GC methods [3—6] in the use of: silanized glassware for the acylation reaction, to avoid adsorption; potassium hydroxide—tripotassium phosphate as buffer instead of sodium hydroxide, to improve the reproducibility of the extraction; heptafluorobutyric anhydride instead of trifluoroacetic anhydride, to improve the stability of the derivatives.

EXPERIMENTAL

Chemicals and reagents

Oxprenolol and the internal standard, propranolol, were supplied by Ciba-Geigy (Basle, Switzerland). Alkaline buffer (pH > 13) was prepared by dissolving 30 g of tripotassium phosphate trihydrate (ref. 5102, E. Merck, Darmstadt, F.R.G.) and 16.8 g of potassium hydroxide (Merck 5033) in distilled water and making the volume up to 100 ml. All reagents and solvents were of analytical grade: diethyl ether (Pestipur quality, S.D.S., Valdonne, France), methylene chloride (Merck 6050) hydrochloric acid (Merck 318), hexane (Pestipur quality, S.D.S.). Pyridine (ref. 82702, Fluka, Buchs, Switzerland) was distilled at 115—116°C over potassium hydroxide pellets and stored over the same reagent. Heptafluorobutyric anhydride was purchased from Ventron (ref. PCR, 1300-3, Ventron, Karlsruhe, F.R.G.); potassium dihydrogen phosphate (Merck 4873) was used as a saturated solution.

Instrumentation

The GC determinations were carried out with a gas chromatograph equipped with an electron-capture detector (Hewlett-Packard, Model 5713 A). The glass column used was 2 m × 3 mm I.D. and packed with 3% SE-30 on 80—100 mesh Gas-Chrom Q. The column temperature was 187°C. The carrier gas (argon—methane, 90:10) flow-rate was 60 ml/min. The column was conditioned as described previously [8]. The glassware was cleaned in a washing machine, and the tubes used for the acylation reaction were then silanized by immersion in a solution containing 1 ml of hexamethyldisilazane, 1 ml of trimethyl-chlorosilane, 1 ml of pyridine and 100 ml of toluene for 15 min. They were subsequently rinsed with methanol and dried at 100°C.

Calibration

One hundred microlitres of suitable oxprenolol hydrochloride aqueous solutions and 100 μl (1692 pmol) of propranolol hydrochloride aqueous solution were added to 1 ml of plasma to obtain calibration samples in the range of concentrations 33.2—3317 nmol oxprenolol per litre of plasma (10—1000 ng/ml). The peak areas were obtained from a Perkin—Elmer Data System (Sigma 10) connected to the chromatograph. The calibration curves were constructed from the ln—ln plot (a linear plot could also be made) of the peak area ratios against the plasma concentrations. Their equations were calculated by the least-squares method. A fresh calibration curve has to be prepared every month and checked once a week by injecting the derivative extracts kept at +4°C.

Extraction procedure

One millilitre of plasma spiked with 100 μl of the internal standard solution (1692 pmol of propranolol hydrochloride), 1 ml of alkaline buffer, and 4 ml of methylene chloride—diethyl ether (1:4) were shaken for 15 min at 300 rpm, then centrifuged for 2 min. The organic phase was shaken with 2 ml of 0.1 *M* hydrochloric acid for 10 min at 300 rpm and centrifuged. The organic phase was discarded and 1 ml of the alkaline buffer and 4 ml of methylene chloride—diethyl ether (1:4) were added and the mixture shaken for 10 min at 300 rpm. After centrifugation, the organic phase was transferred to a silanized tube and evaporated to dryness under a nitrogen stream. Two microlitres of pyridine, 1 ml of hexane and 10 μl of heptafluorobutyric anhydride were successively added. This order of addition — catalyst, solvent, acylation reagent — appeared to result in a more reproducible analytical yield. The mixture was shaken for about 15 sec. After 1 h at room temperature, 1 ml of potassium dihydrogen phosphate saturated solution was added, and the mixture was shaken for 15 min at 300 rpm. Three microlitres of the organic phase were injected into the gas chromatograph.

RESULTS

Plasma interferences

Fig. 1 shows the chromatograms corresponding to an extract of 1 ml of blank plasma and to an extract of the same plasma containing 332 nmol/l oxprenolol (100 ng/ml) and 1692 nmol/l propranolol (500 ng/ml). The metab-
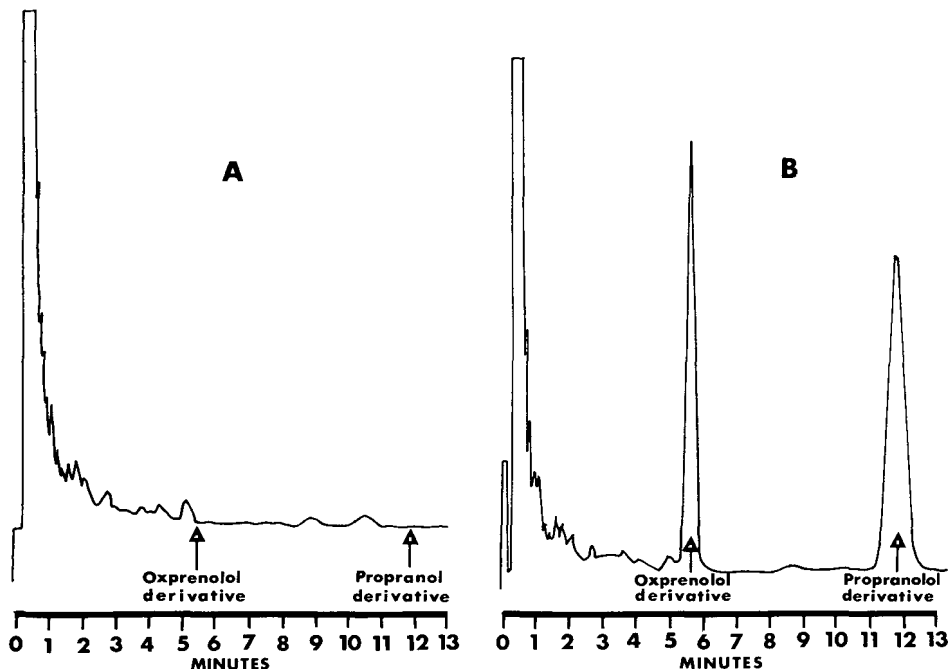
Fig. 1. Chromatograms of: (A) human plasma blank (1 ml plasma); (B) extract of 332 nmol/l oxprenolol and 1692 nmol/l propranolol in plasma.

olism of oxprenolol in man has not yet been fully elucidated, in so far as the presence of metabolites in plasma has not been established [9—15]. Nevertheless, the urinary metabolites found in man and rat [9—15] cannot interfere with the oxprenolol assay as they will not be extracted or derivatized by the described procedure.

Reproducibility and recovery

The results are summarized in Table I. A good reproducibility (coefficient of variation, C.V. = 4.7%) was obtained for the concentration 33.2 nmol/l (10 ng/l), which can be taken as the limit of quantitation. The mean overall recovery was 101.0% (6.5).

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF OXPRENOLOL IN SPIKED PLASMA SAMPLES

Amount added (nmol/l)	Mean amount found (n=6) (nmol/l)	Coefficient of variation (C.V.) (%)	Mean recovery (%)
33.2	33.3	4.7	101
82.9	79.1	2.7	95
332	362	6.4	109
829	818	4.9	99
1658	1680	5.2	101

Mean overall recovery (C.V.) = 101.0% (6.5)

TABLE II

STORAGE STABILITY OF OXPRENOLOL IN HUMAN PLASMA OVER TWELVE
MONTHS AT −20° C

Duration of storage at −20°C (months)	Amount of oxprenolol added to plasma (nmol/l)	
	166	1658
	Amount of oxprenolol found (average of two assays) (nmol/l)	
0	164	1655
1	158	
3	158	1667
5	158	1710
9	176	1615
12	181	1665

Stability of oxprenolol in plasma

Spiked plasma samples were kept at −20°C and analysed regularly. Oxprenolol is stable for up to twelve months under these conditions (Table II).

REFERENCES

1   M. Schäfer and E. Mutschler, J. Chromatogr., 164 (1979) 247.
2   S.E. Tsuei, J. Thomas and R.G. Moore, J. Chromatogr., 181 (1980) 135.
3   D.B. Jack and W. Riess, J. Chromatogr., 88 (1974) 173.
4   P.H. Degen and W. Riess, J. Chromatogr., 121 (1976) 72.
5   D. De Bruyne, H. Kinsun, M.A. Moulin and M.C. Bigot, J. Pharm. Sci., 68 (1979) 511.
6   T. Nambara, S. Akiyama and M. Kurata, Yakugaku Zasshi, 93 (1973) 439.
7   A. Sioufi, F. Leroux and N. Sandrenan, J. Chromatogr., 272 (1983) 103.
8   A. Sioufi and F. Pommier, J. Chromatogr., 183 (1980) 33.
9   W. Riess, T.G. Rajagopalan, P. Imhof, K. Schmid and H. Keberle, Postgrad. Med. J., Nov. Suppl. (1970) 32.
10 D.A. Garteiz, J. Pharmacol. Exp. Ther., 179 (1971) 354.
11 G.A. Leeson, D.A. Garteiz, W.C. Knapp and G.J. Wright, Drug Metab. Dispos., 1 (1973) 565.
12 W. Riess, H. Huerzeler and F. Raschdorf, Xenobiotica, 4 (1974) 365.
13 W.L. Nelson and T.R. Burke, Jr., J. Med. Chem., 22 (1979) 1088.
14 P. Dayer, P. Glasson, A. Gorgia, L. Balant and J. Fabre, Schweiz. Med. Wochenschr., 111 (1981) 1915.
15 F. Goldszer, G.L. Tindell, U.K. Walle and T. Walle, Res. Commun. Chem. Pathol. Pharmacol., 34 (1981) 193.

CHROMBIO. 1829

Note

A simple and sensitive liquid chromatographic method for the determination of 5'-methylthioadenosine in tissues

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Methylthioadenosine (MTA) is formed from S-adenosyl-L-methionine by several pathways, including those leading to biosynthesis of polyamines. This product is a potent inhibitor of enzymes involved in the synthesis of spermidine and spermine. The enzyme responsible for the metabolic degradation of MTA, namely MTA phosphorylase, is a potential chemotherapeutic target enzyme [1], and this enzyme is lacking in some malignant tumors and cells [2—4].

The growing interest in MTA (for review, see refs. 1, 5 and 6) has demanded the development of methods for its measurement in tissues. The concentration of this nucleoside is low in comparison with those of related compounds, and early methods for measurement of MTA suffered from low sensitivity [1]. Recent methods, based on high-performance liquid chromatography (HPLC), allow the determination of MTA in several tissues. Purification of the tissue samples on borate affinity gel [7, 8] or Dowex 50 [9] was required before HPLC.

The present communication describes a liquid chromatographic method for the determination of MTA, involving filtration of neutralized tissue extract through DEAE-Sephadex at pH 7.5. MTA appeared in the effluent while interfering substances were retained on the column. The effluent was lyophilized and subjected to reversed-phase chromatography on a short 3-μm ODS (C_{18}) column eluted at high flow-rate. 2-Chloroadenosine served as a convenient internal standard.

EXPERIMENTAL

Materials

MTA, 2-chloroadenosine, adenine, S-adenosyl-L-homocysteine, S-adenosyl-

L-methionine and adenosine were obtained from Sigma (St. Louis, MO, U.S.A.). ODS Hypersil, 3-μm packing material for HPLC, empty stainless-steel columns (10 $\times$ 0.5 cm) and guard columns (2.5 $\times$ 0.5 cm) were purchased from Shandon (Cheshire, U.K.). Packing material for guard columns, Pelliguard LC 18, 40 μm, was from Supelco (Houston TX, U.S.A.). DEAE-Sephadex A-50 was obtained from Pharmacia (Uppsala, Sweden).

The 3-μm ODS columns were slurry packed using a Shandon column packer. The guard column was dry-packed and subjected to mechanical compression and refilling.

Preparation and purification of tissue extracts

Tissue was homogenized in 0.8 M perchloric acid (1:4, w/v) containing 5 μM 2-chloroadenosine (internal standard). Precipitated protein was removed by centrifugation, using a Beckman type B microfuge. The supernatant was immediately neutralized to pH 7.5 by adding 1.44 M KOH—1.2 M KHCO$_3$. Then 1.5 ml of the solution were applied to DEAE-Sephadex columns (0.6 $\times$ 5 cm) equilibrated with 10 mM Tris—HCl, pH 7.5, and eluted with the same buffer. The first 2 ml were discarded, and the following 5 ml were collected, lyophilized and resuspended in 100 μl of distilled water.

Reversed-phase liquid chromatography

Samples of 5—25 μl were analyzed on a 3-μm ODS Hypersil column (0.5 $\times$ 10 cm) equipped with a guard column (0.5 $\times$ 2.5 cm). The column was eluted isocratically with 8 mM phosphate buffer, pH 6.0, containing 6% acetonitrile. The liquid chromatographic system consisted of a Spectra-Physics SP 8700 solvent delivery system, a Perkin-Elmer ISS 100 autosampler for HPLC, a Beckman ultraviolet (UV) detector, Model 160, and a Hewlett-Packard HP 3390 A integrator. For the determination of UV spectra of MTA in tissue extracts, a variable-wavelength detector from Kratos, Model Spectroflow 773, was used.

RESULTS AND DISCUSSION

MTA and 2-chloroadenosine were not retained on DEAE-Sephadex columns equilibrated with 10 mM Tris—HCl, pH 7.5. More than 95% of MTA and 2-chloroadenosine applied to the column was eluted with this buffer. The recovery of exogenous MTA and 2-chloroadenosine added to the tissue extract was 20—40%, depending on the elution volume collected from the DEAE-Sephadex column. Tissue samples were prepurified by filtration through DEAE-Sephadex columns, followed by lyophilization of the effluent. This step removed UV-absorbing material, which interfered with the determination of MTA by HPLC, and greatly reduced the solvent front of the chromatogram (Fig. 1). The concentration of MTA was increased about 5-fold relative to that in the crude tissue extract.

Some methods for determining MTA include purification of the tissue extract on borate affinity columns prior to HPLC [7—9]. The high cost of this material and the variable quality of some commercial preparations led us to use DEAE-Sephadex to purify the tissue extract. These columns could easily be regenerated by elution with a high salt concentration.
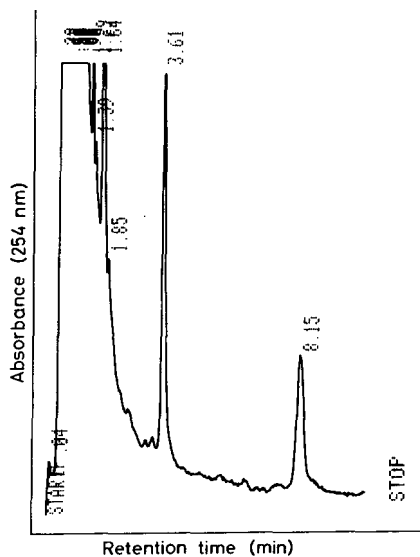
Fig. 1. Chromatographic analysis of rat liver extract. Rat liver was homogenized in perchloric acid containing 2-chloroadenosine, and the neutralized extract was purified on a DEAE-Sephadex column as described in the text. The purified extract was analysed on an ODS Hypersil column eluted isocratically with 6% acetonitrile in 8mM phosphate buffer, pH 6.0, at a flow-rate of 3 ml/min. The retention times of 2-chloroadenosine and MTA were 3.61 and 8.15 min, respectively.
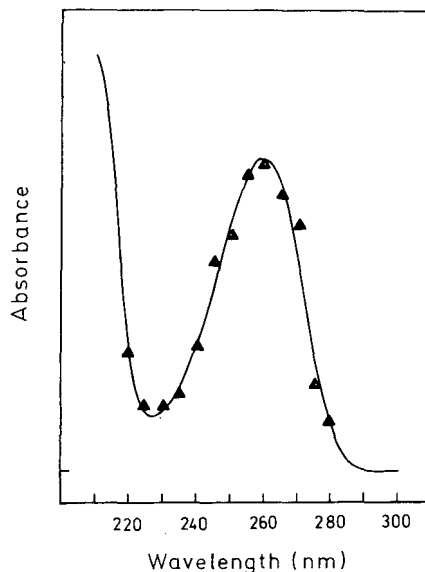
Fig. 2. Ultraviolet absorption spectrum of authentic MTA and the MTA peak in liver extract. The solid line shows the absorption spectrum of MTA dissolved in phosphate buffer, pH 6.0. The absorption spectrum of the MTA peak (▲) was obtained by repetitive analysis of purified rat liver extract. The absorption of the effluent was monitored at the wavelengths indicated.

The lyophilized samples from the DEAE-Sephadex columns were analyzed by reversed-phase liquid chromatography on a short (10 cm) highly efficient (about 9000 theoretical plates) 3-μm ODS column eluted at high flow-rate (3 ml/min). Baseline separation of MTA from interfering substances was obtained, and the internal standard, 2-chloroadenosine, eluted just after the solvent front (Fig. 1). Adenosine, S-adenosylhomocysteine, S-adenosylmethionine, inosine, adenine, and various oxypurines eluted at lower retention times than MTA (data not shown). MTA in the tissue extract was identified by its retention time relative to an authentic standard in various reversed-phase systems (8 mM potassium dihydrogen phosphate pH 6.0 containing 6% acetonitrile or 10 mM potassium dihydrogen phosphate pH 6.0 containing 20—25% methanol), and in a cation-exchange system as described elsewhere [10], by the increase in the MTA peak upon addition of MTA to the tissue extract, and by the UV spectrum of MTA in the tissue extract (Fig. 2). (Fig. 2).

The determination of MTA in tissue extracts was performed by comparison of the area under the peak with that of known MTA standards. The standard curve for MTA was linear in the range 0.2—30 μM, and the amount of MTA was calculated by calibrating the reporting integrator. The variable

TABLE I

THE AMOUNT OF MTA IN VARIOUS TISSUES OF THE RAT

Tissue	MTA (nmol/g wet wt)[*]
Liver	4.24 ± 0.13
Lung	1.31 ± 0.06
Kidney	1.43 ± 0.06
Testis	0.62 ± 0.03
Heart	3.90 ± 0.15
Ventral prostate	2.00 ± 0.07

[*]The values are expressed as the mean ± S.E. of eight parallel determinations.

recovery of MTA during processing of tissue extracts was corrected for by using 2-chloroadenosine as internal standard.

The amount of MTA in various tissues of the laboratory rat was determined with the present method, and the concentrations of MTA obtained (Table I) compared with the tissue levels of MTA reported by others [7, 9]. Using a low-noise, fixed-wavelength detector, equipped with a mercury lamp (254 nm) and a relatively large flow-cell (18 μl), 2 pmol of MTA in tissue extracts could be detected.

REFERENCES

1   H.G. Williams-Ashman, J. Seidenfeld and P. Galetti, Biochem. Pharmacol., 31 (1982) 277.
2   J.I. Toohey, Biochem. Biophys. Res. Commun., 83 (1978) 27.
3   N. Kamatani, W.A. Nelson-Rees and D.A. Carson, Proc. Nat. Acad. Sci. U.S., 78 (1981) 1219.
4   N. Kamatani, A.L. Yu and D.A. Carson, Blood, 60 (1982) 1378.
5   J.I. Toohey, J. Supramol. Struct. Cell. Biochem., 17 (1981) 11.
6   R.E. Parks, Jr., J.D. Stackler, C. Cambor, T.M. Savarese, G.W. Crabtree and S.-H. Chu, in A.C. Sartorelli, J.S. Lazo and J.R. Bertino (Editors), Molecular Actions and Targets for Cancer Chemotherapeutic Agents, Academic Press, New York, 1982, p. 229.
7   J. Seidenfeld, J. Wilson and H.G. Williams-Ashman, Biochem. Biophys. Res. Commun., 95 (1980) 1861.
8   N. Kamatani and D.A. Carson, Cancer Res., 40 (1980) 4178.
9   F. Della Ragione, M. Cartenì-Farina, M. Porcelli, G. Cacciapouti and V. Zappia, J. Chromatogr., 226 (1981) 243.
10 T. Schanche, J.-S. Schanche and P.M. Ueland, FEBS Lett., 137 (1982) 196.

CHROMBIO. 1840

Note

High-performance liquid chromatographic analysis of 5-chloroaminotoluene in rats

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Environmental toxicology, due to uncontrolled use and disposal of chemicals, is becoming a major health management problem. The recent introduction of chlorinated aromatic compounds into open pits and abandoned subterranean oil wells introduces additional health hazards. 5-Chloroaminotoluene (5-CAT) is one of these compounds noted to produce pathological situations in both laboratory animals and man. In 1933 Currie [1] recorded cases of hematuria that occurred from time to time among chemical workers that packaged 5-CAT. Lehmann [2] noted hemorrhagic cystitis and destruction of bladder epithelium in cats exposed to 5-CAT by skin contact. Recently, Folland et al. [3] have reported acute hemorrhagic cystitis in industrial employees who handled the pesticide chlordimeform (CDM); 5-CAT was found in the urine of these workers.

Few analytical procedures have been developed for analysis of 5-CAT in biological media. Kossman et al. [4] reported colorimetric, thin-layer and flame ionization detection gas chromatographic procedures for analysis of 5-CAT content in plant materials. Chemical and enzymatic degradation of CDM yielded the product of 5-CAT which could be analyzed by instrumental methods. The colorimetric and thin-layer chromatographic procedures had detection limits of 1 μg 5-CAT per 50 g of crop material whereas a detection limit of 50 ng 5-CAT per 50 g crop material was obtained by the flame ionization gas chromatographic procedure. Geissbuhler et al. [5] developed an electron capture gas chromatographic procedure for analysis of 5-CAT. This method involved derivatization of 5-CAT via the Sandmeyer iodination reaction giving the derivative electron capturable properties. The extraction procedure was extremely involved and the limit of detection by this method was 50 ng per 50 g of crop material. Folland et al. [3] used a gas chromato-

graphic method with a Coulson detector specific for halogens for analysis of 5-CAT.

As far as the authors can ascertain this paper represents the first high-performance liquid chromatographic (HPLC) method for analysis of 5-CAT. The compound is detectable in rat urine and feces up to three days following single 100-mg/kg intraperitoneal injections. An internal standard of 2-chloro-4-methylaniline (IS) is used for quantitation and the detection limit is 5 ng per ml of urine or gram of feces. The extraction method is rapid requiring approximately 60 min to process samples for HPLC analysis.

EXPERIMENTAL

Materials

The compounds used in this study, 5-chloroaminotoluene and 2-chloro-4-methylaniline (internal standard), were purchased from Aldrich (Milwaukee, WI, U.S.A.). Solvents of methanol and hexane (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Standard solutions of 5-CAT and internal standard were made by adding 20 mg each in 100-ml volumetric flasks and dissolving in methanol—0.05 M KH_2PO_4, pH 4.65 (60:40) (Sigma, St. Louis, MO, U.S.A.). The stock solutions were prepared daily.

Instrumentation

HPLC was accomplished using a Beckman 345T ultraviolet—visible system with a Hewlett-Packard 3390A integrator. Separation was performed on a 15 cm × 4.6 mm I.D. 5-μm ODS Altex column. A precolumn of 6 cm × 4.6 mm I.D. contained 5-μm Spherisorb ODS (Supelco, Bellefonte, PA, U.S.A.). The column temperature was maintained at ambient temperature (approx. 22°C). The mobile phase consisted of methanol—0.05 M KH_2PO_4, pH 4.65 (60:40) at a flow-rate of 1 ml/min.

Sample collection and extraction

Adult male Sprague Dawley rats (Charles River, MA, U.S.A.) were used in all experiments. Three animals were housed in separate metabolic cages during each experiment for a total of five days and fed food and water ad libitum. Each animal was weighed before experimentation and one was given 100 mg/kg normal saline i.p. (control) and the other two were injected with 100 mg/kg 5-CAT i.p. (test rats). The three animals were given only a single injection at the beginning of each five-day trial period at approximately 9 a.m. Each following morning at 9 a.m. the urine and feces content was collected, recorded and frozen at −70°C and new metabolic collection tubes were replaced for another 24-h collection period.

The extraction scheme is presented in Fig. 1. Analysis was performed after all samples had been collected. To screw-topped PTFE-lined 20-ml glass culture tubes were added 500 μl of internal standard and the solution dried by an air stream. Then either 0.50 ml urine or 1.0 g feces (well homogenized in 5 ml water) was added with 1 ml of 10% sodium bicarbonate and 10 ml hexane. The solutions were shaken for 10 min by hand and centrifuged at 500 g for 10 min. The organic layer was transferred to a new tube containing 0.50 ml of

0.50 ml Urine
or
1 g Homogenizend Feces

A.   100 ug Internal Standard
     (2-chloro-4-methyaniline)

B.   1 ml 10% Sodium Bicarbonate

C.   10 ml Hexane

D.   Shake and Centrifuge - 10 minutes each

Aqueous Layer
(Discarded)

Organic Layer

A.   0.5 ml of 0.10 N Sulfuric Acid

B.   Shake and Centrifuge - 10 minutes

Organic Layer
(Aspirated and
Discarded)

Aqueous Layer

20 µl Injections onto
HPLC Column
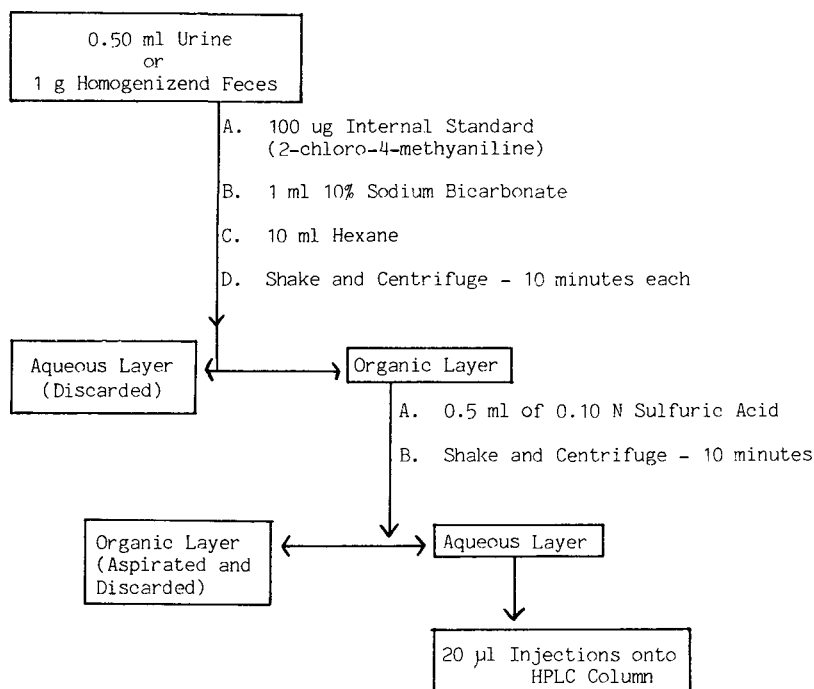
Fig. 1. Extraction scheme for 5-CAT from biological samples.

0.10 N sulfuric acid and shaken for 10 min and centrifuged at 500 g for 10 min. The organic layer was asperated from the tube and 20-μl injections of the aqueous phase were injected onto the column. The recovery of 5-CAT was found to be 75 ± 4% from spiked biological samples.

Standard curves

Standard curves were constructed by addition of 1, 3, 5, 7, 9 and 11 μg of 5-CAT and 500 μl of internal solution to 20 ml PTFE-lined glass culture tubes. These solutions were then dried by a stream of air. To the tubes was added either 0.5 ml of blank rat urine or 1 g of homogenized feces in 5 ml of water. These solutions were then carried through the above reported extraction procedure and 20 μl of each were injected onto the column. A least-squares fit (r = 0.997) of the data was performed, resulting in the equation: $y = 9.520x - 0.083$. This equation was constructed by plotting μmoles 5-CAT/μmol IS versus the ratio of peak area 5-CAT/area IS. After the concentration (in μmol) of 5-CAT was found the value was multiplied by its molecular weight (142 g/mol) and divided by the ml or g of sample. The final values were reported in μg 5-CAT per ml or g of biological sample.

RESULTS AND DISCUSSION

HPLC analysis

In Fig. 2a and b can be seen the analysis of 5-CAT from urine (a) and a blank sample (b), respectively. Fig. 3a and b represents the separation of
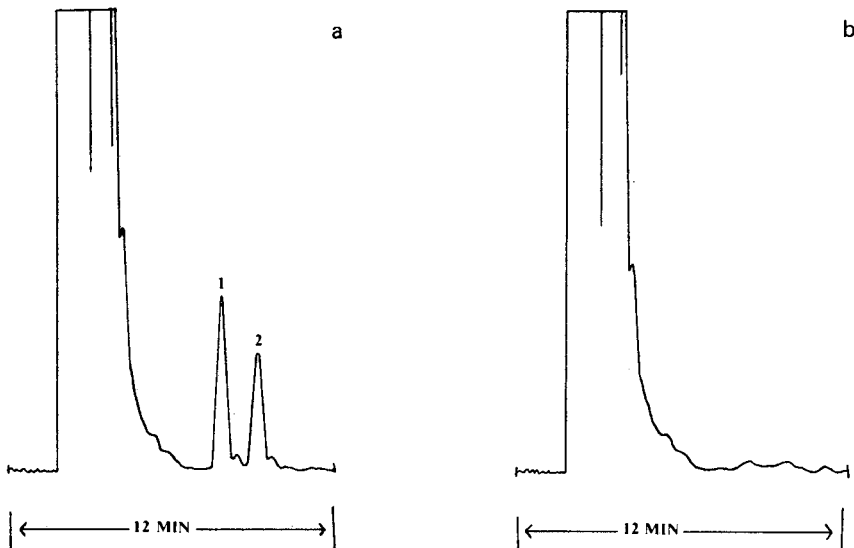
Fig. 2. HPLC analysis of 5-CAT and internal standard from rat urine two days following i.p. injection (a). Analysis of rat urine of a control animal one day following i.p. injection with normal saline (b) without addition of internal standard. Peak 1 is 5-CAT and peak 2 is the internal standard.
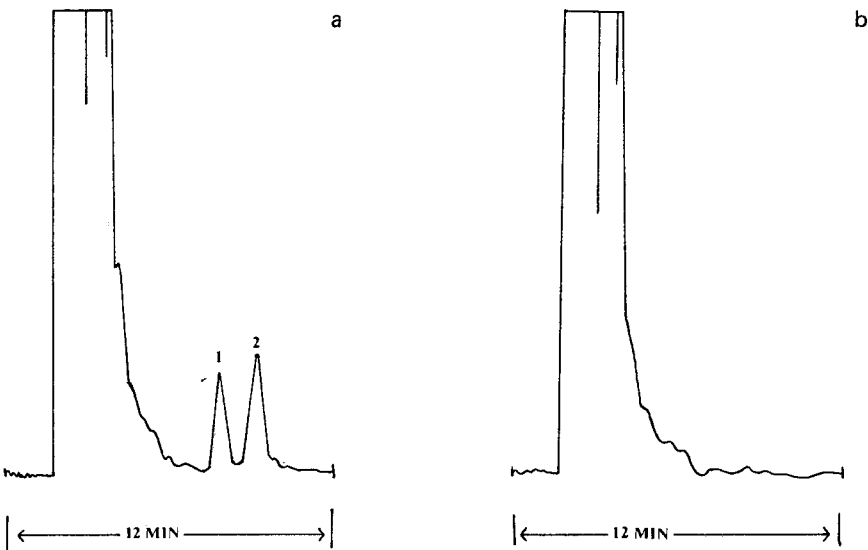
Fig. 3. HPLC analysis of 5-CAT and internal standard from rat feces two days following i.p. injection (a). Analysis of rat feces from a control rat (without addition of internal standard) two days following i.p. injection with normal saline (b). Peak 1 is 5-CAT and peak 2 is the internal standard.

5-CAT and internal standard from feces (sample a) and blank feces (sample b), respectively. The retention times of 5-CAT and IS are 8.20 and 10.53 min, respectively. Positive identification of the samples in question is achieved by peak superimposition (i.e., by injection of 1 to 5 μg of 5-CAT standard with

the previously extracted sample and observing the increase in peak area at its corresponding retention time).

The limit of detection (2:1 signal-to-noise) of this procedure is 5 ng 5-CAT per ml urine or g feces. Repetitive injections of standards gave good reproducibility of retention times (C.V., ± 2.1%) at the 100-ng level. Standard curves were linear in the range of 100 ng to 15 μg 5-CAT and day-to-day reproducibility varied less than 3.0% (C.V.). Standard stock solutions of this compound and its internal standard were stable at least one week when stored at −70°C. Samples extracted from urine or feces gave reproducible results of ±3.5% (C.V.) for two-day duration when stored at −20 or −70°C. However, extracted samples left at room temperature or −4°C had variable losses of up to 25% within 24 h following the extraction and upwards of 50—60% when left at room temperature (approx. 22°C), or −4°C, when analyzed at 48 h. Therefore, it is suggested that all samples should be quantitated immediately within 4—6 h following separation from the biological media unless precautions are taken to store the samples at the lower temperatures as mentioned previously.

Pharmacokinetic study in rats

Three rats were studied during each experiment of five-days duration. One rat was injected i.p. with 100 mg/kg of normal saline and two rats were injected i.p. with 100 mg/kg of 5-CAT as described in the Experimental section. Table I presents the data obtained from this study of the urinary excretion of 5-CAT. This compound was detectable up to three days following injection before its concentration fell below the limits of detection of the analytical methodology. A total of eighteen rats were carried through this experiment and 5-CAT was never detectable on the fourth day of experimentation. Semilog plots of concentration versus time were constructed and the elimination half-life was determined to be 16.5 h.

TABLE I

AVERAGE URINARY EXCRETION DATA OBTAINED FROM RATS IN THIS STUDY

Day	Urine (μg/ml ± S.D.) ($n = 18$)
1	161.3 ± 15.9
2	20.8 ± 5.6
3	4.2 ± 1.9

CONCLUSION

A sensitive and selective analytical technique has been developed for the quantitation of 5-CAT in rats. The emphasis of this work was on the rapid extraction and analysis of low-nanogram amounts of this compound in biological tissue. A structurally similar internal standard was used for quantitation and was well resolved from the compound under investigation and any interfering components. A pharmacokinetic investigation carried out demonstrated

the usefulness of this technique and revealed a 16.5-h half-life in rats following a single i.p. injection. Further studies of the pharmacokinetics of 5-CAT in rats and the possible metabolites are currently under investigation in this laboratory.

REFERENCES

1 A.N. Currie, J. Ind. Hyg., 15 (1933) 205.
2 K.B. Lehmann, Arch. Hyg. Bakteriol., 110 (133) 12.
3 D.S. Folland, R.D. Kimbrough, R.E. Cline, R.C. Swiggart and W. Schaffner, J. Amer. Med. Assoc., 13 (1978) 1052.
4 K. Kossmann, H. Geissbuhler and V.F. Boyd, J. Agric. Food Chem., 19 (1971) 360.
5 H. Geissbuhler, K. Kossmann, I. Baunak and V.F. Boyd, J. Agric. Food Chem., 19 (1971) 365.

CHROMBIO. 1834

Note

Resolution of the enantiomers of propranolol and other beta-adrenergic antagonists by high-performance liquid chromatography

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The important β-adrenergic antagonist propranolol [1-isopropylamino-3-(1-naphthoxy)-2-propanol] is used clinically as the racemic mixture of the R-(+) and S-(−) isomers. The pharmacological properties of the enantiomers, however, are quite different, and the β-adrenergic blocking activity resides in the S-(−) isomer [1,2]. This observation has generated considerable interest in the chromatographic resolution of the enantiomers for analytical and preparative purposes. The most frequently used approach to this problem appears to be derivatization of the drug with a chiral reagent, followed by chromatographic resolution of the resulting diastereomers. Using gas—liquid chromatography (GLC), the propranolol enantiomers have been resolved as the derivatives formed with N-trifluoroacetyl-S-prolyl chloride (TPC) [3] or with S-(−)-1-phenylethyl isocyanate (PEIC) [4]. High-performance liquid chromatography (HPLC) has also been used for the resolution of propranolol, employing TPC [5,6], PEIC [4], *tert.*-butoxycarbonyl-L-alanine anhydride [7], or *tert.*-butoxycarbonyl-L-leucine anhydride [7] as the chiral derivatizing reagent. The latter two amino acid anhydrides have significant disadvantages in that they are not commercially available, and the derivatization procedure is rather elaborate. TPC also suffers from a disadvantage inasmuch as commercial samples contain 6—10% of the R-proline-derived enantiomer arising from partial racemization during the synthesis or storage of the reagent [6,8,9].

Other approaches to the chromatographic resolution of propranolol appear to be limited to an HPLC procedure using ion-pair chromatography with a chiral ion in the mobile-phase [10].

Other β-adrenergic antagonists related to propranolol in chemical structure have been studied less extensively. Alprenolol, oxprenolol, atenolol, pindolol,

4-hydroxypropranolol, pronethalol and nifenalol have been resolved using a complex derivatization scheme based on TPC, followed by capillary GLC [3]. HPLC was applied to the resolution of the enantiomers of alprenolol and metoprolol, using the above-cited amino acid anhydrides [11], or with a chiral ion in the stationary phase [10].

In this communication, we describe a new and convenient HPLC method for the resolution of the enantiomers of propranolol and ten other, related, compounds.

EXPERIMENTAL

Chemicals and reagents

(−)-Alprenolol was purchased from Sigma (St. Louis, MO, U.S.A.). The following compounds were generously donated: (+)-alprenolol (Vega Bio-chemicals, Tucson, AZ, U.S.A.), (±)-propranolol, (−)-propranolol, (±)-pronethalol, and (±)-deacetylpractolol (Ayerst Labs., New York, NY, U.S.A.); (±)-4-hydroxypropranolol (Dr. John Thompson, School of Pharmacy, University of Colorado); (±)-metoprolol (CIBA Pharmaceutical, Summit, NJ, U.S.A.); (±)-pindolol (Mr. Allen Chapman, Pharmacy, University of Colorado Health Sciences Center); (−)-pindolol (Dr. Nancy Zahniser, School of Medicine, University of Colorado); (±)-sotalol and (±)-atenolol (Dr. John Gerber, School of Medicine, University of Colorado); (±)-bupranolol and (−)-bupranolol (Dr. Thomas Dunwiddie, School of Medicine, University of Colorado), and (±)-practolol (Dr. Joe Masserano, School of Medicine, University of Colorado).

The chiral reagents, 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) were purchased from Polysciences (Warrington, PA, U.S.A.), acetonitrile (distilled-in-glass grade) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), ᵃⁿd ammonium phosphate monobasic from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Chromatography

A Waters (Milford, MA, U.S.A.) HPLC system consisting of a Model M-45 and a Model M-6000 solvent delivery system, a Model U6K injector, a Model 440 absorbance detector, and a Model 660 solvent programmer was used. The Model M-45 solvent delivery system delivered acetonitrile, while the Model M-6000 pump delivered a 0.02 M aqueous ammonium phosphate solution. The two liquids were mixed in the M-6000 pump under the control of the solvent programmer. Mixtures of various compositions were used (Table I), and each mixture was delivered to the chromatographic column in the isocratic mode. Separations were carried out on a Beckmann Instruments (Berkeley, CA, U.S.A.) Ultrasphere ODS 5-μm 150 mm × 4.6 mm reversed-phase column. The column effluent was monitored at 254 nm. Chromatograms were recorded using a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3380 A electronic integrator.

Preparation of derivatives

A 1-mg sample of the compound to be derivatized, in the free base form, was

TABLE I

RESOLUTION OF β-ADRENERGIC ANTAGONISTS WITH AITC OR GITC

Chromatographic and derivatization conditions are given in the Experimental section.

Compound	Retention time (min) of diastereomers[*] formed with				Percent acetonitrile in mobile phase[**]		Separation factor[***] (α)	
	GITC		AITC		GITC	AITC	GITC	AITC
Propranolol	8.4 S-(−)	10.5 R-(+)	8.7 R-(+)	9.8 S-(−)	58	56	1.28	1.14
Pindolol	8.6 S-(−)	10.4 R-(+)	6.5 R-(+)	7.0 S-(−)	47	47	1.24	1.09
Alprenolol	6.4 S-(−)	7.7 R-(+)	5.8 R-(+)	6.5 S-(−)	65	65	1.24	1.15
Bupranolol	4.3 (−)	5.0 (+)	4.0 (+)	4.3 (−)	75	75	1.21	1.10
Atenolol	5.3	6.4	4.4	4.7	40	37	1.26	1.09
Pronethalol	6.8	8.7	6.8	8.1	58	56	1.28	1.22
Metoprolol	4.8	5.7	4.8	5.2	58	56	1.24	1.11
4-Hydroxypropranolol	9.2	11.3	6.9	7.5	50	50	1.26	1.10
Practolol	7.9	9.8	5.9	6.5	40	37	1.28	1.12
Deacetylpractolol	7.9	9.8	6.4	7.1	40	37	1.28	1.13
Sotalol	7.3	8.7	5.4	5.9	44	44	1.22	1.11

[*]When known, the identity of the drug enantiomer from which the diastereomer derives is given.
[**]Remainder of mobile phase aqueous ammonium phosphate as described in the Experimental section.
[***]Separation factor, defined in ref. 12.

treated with 200 μl of acetonitrile containing 2 mg of AITC or GITC in a test tube. The tube was tightly capped, swirl-mixed (vortex), and allowed to stand at room temperature for 30 min. Aliquots (5—10 μl) were then injected into the HPLC system.

RESULTS AND DISCUSSION

The chiral reagents GITC and AITC were recently described and applied to the HPLC resolution of enantiomeric amino acids [13,14] and the enantiomers of epinephrine and norepinephrine [15]. The isothiocyanato group reacts rapidly and selectively with primary and secondary amines under mild conditions to form the corresponding thiourea derivatives [13--15]. Since propranolol and related β-adrenergic antagonists are, in general, secondary amines, it appeared worthwhile to examine the applicability of GITC and AITC to the resolution of the enantiomers of these drugs. The results are collected in Table I. The enantiomers of each compound in Table I were well resolved as their GITC derivatives by reversed-phase HPLC; indeed, base-line resolution was achieved in each case, and the chromatographic peaks were remarkably narrow. Representative examples are shown in Fig. 1. The separation factor, α (Table I), remained surprisingly constant within the GITC series considering the wide variation in structure represented by the compounds studied. Individual enantiomers were available for four of the compounds, permitting the identification of the elution order of the diastereomeric derivatives. The diastereomer derived from GITC and the S-(−) enantiomer of propranolol, pindolol or alprenolol elutes before the diastereomer from the corresponding R-(+) enantiomer (Table I). The absolute configuration of bupranolol has not been published, but the levorotatory enantiomer of this drug also provided a GITC derivative with a shorter retention time than that of the diastereomer derived from (+)-bupranolol.
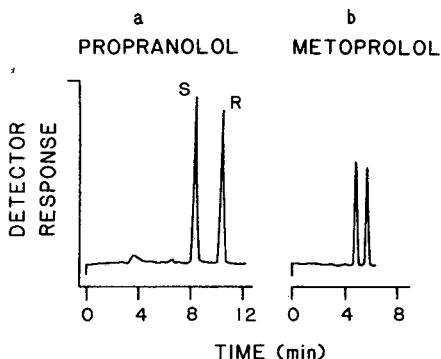
Fig. 1. Resolution of the enantiomers of (a) propranolol and (b) metoprolol as their GITC derivatives. The chromatographic conditions are given in the text and in Table I.

The diastereomers formed from AITC were in general considerably less-well resolved than the corresponding GITC derivatives (Table I). AITC is therefore not recommended for the separation of the enantiomers of the compounds listed in Table I. Interestingly, the order of elution of the diastereomers from AITC was the reverse of the order of elution of the GITC derivatives: the product from the reaction of AITC and the dextrorotatory drug elutes first in each instance (Table I). Such reversal of elution order was previously observed in the chromatography of the AITC and GITC derivatives of amino acids and catecholamines [13—15], and it was suggested that this phenomenon may be related to conformational differences between the two acetylglucosyl residues, especially at the anomeric carbon atom [14].

The procedure has several major advantages over previously described techniques: the derivatization is extremely simple; the chiral reagent is commercially available and is chemically and stereochemically stable; and baseline resolution of the diastereomers is achieved within a remarkably short analysis time. The procedure for the resolution of the enantiomers was not applied to the analysis of these compounds in biological fluids, but it appears that the technique may be adaptable to this purpose, since thiourea derivatives appear to be very sensitive to UV detection [13,14]. Further study will be required to confirm this expectation.

In conclusion, derivatization of propranolol and other β-adrenergic antagonists with GITC is a useful technique for the separation of their enantiomers by HPLC.

ACKNOWLEDGEMENTS

REFERENCES

1 L.T. Potter, J. Pharmacol. Exp. Ther., 155 (1967) 91.
2 A.M. Barret and V.A. Cullum, Brit. J. Pharmacol., 34 (1968) 43.
3 S. Caccia, C. Chiabrando, P. DePonte and R. Fanelli, J. Chromatogr. Sci., 16 (1978) 543.
4 J.A. Thompson, J.L. Holtzman, M. Tsuru, C.L. Lerman and J.L. Holtzman, J. Chromatogr., 238 (1982) 470.
5 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109.
6 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643.
7 J. Hermansson, Acta Pharm. Suec., 19 (1982) 11.
8 G. Manius and R. Tscherne, J. Chromatogr. Sci., 17 (1979) 322.
9 J. Gal, J. Pharm. Sci., 66 (1977) 169.
10 C. Pettersson and G. Schill, J. Chromatogr., 204 (1981) 179.
11 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113.
12 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley, New York, 2nd ed., 1979, p. 840.
13 N. Nimura, H. Ogura and T. Kinoshita, J. Chromatogr., 202 (1980) 375.
14 T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77.
15 N. Nimura, Y. Kasahara and T. Kinoshita, J. Chromatogr., 213 (1981) 327.

CHROMBIO. 1828

Note

Determination of metoprolol in human blood plasma using high-performance liquid chromatography

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Metoprolol is a cardioselective β-adrenoceptor antagonist which has been shown to be effective in the management of hypertension [1,2], angina pectoris [3], and cardiac arrhythmias [4]. Monitoring the concentration of the drug in blood plasma can be helpful in maintaining optimum dosage [5], and is also needed in pharmacokinetic and some pharmacodynamic studies. Published methods for the determination of metoprolol in plasma include procedures based on gas chromatography [6—8], radioisotopes [9,10], gas chromatography—mass spectrometry [11] and high-performance liquid chromatography (HPLC) [12—16]. The HPLC methods are all based on fluorescence detection and appear useful, but some have significant drawbacks, e.g., lack of use of an internal standard [13,16], interference from other drugs [15], unduly long (26 min) retention times [14], long (1 h) extraction time [14], low drug recovery [12,13], or elaborate derivatization schemes [16].

Some time ago we developed a simple HPLC method for the determination of metoprolol in human plasma, and used it in pharmacological studies [17] of the drug. In this communication, we describe the analytical procedure.

EXPERIMENTAL

Chemicals

Metoprolol tartrate was obtained from Ciba Pharmaceutical (Summit, NJ, U.S.A.), and alprenolol tartrate was purchased from Sigma (St. Louis, MO, U.S.A.). Sodium hydroxide, hydrochloric acid, 0.1 N, and sulfuric acid, 0.1 N, all Baker Analyzed reagent-grade, and phosphoric acid (85%, Fisher Scientific, Pittsburgh, PA, U.S.A.) were obtained from routine chemical suppliers.

Methanol, acetonitrile, and *n*-butyl chloride, all distilled-in-glass grade, were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Human blood plasma was provided by the Belle Bonfils Memorial Blood Bank of the University of Colorado Health Sciences Center. Water used was twice distilled.

Standards

Stock solutions of metoprolol tartrate and alprenolol tartrate at 50 μg/ml free-base concentration were prepared in 0.01 *N* hydrochloric acid and were stored at 4—6°C. The working internal standard was prepared by diluting the stock alprenolol solution with 0.01 *N* hydrochloric acid to a concentration of 500 ng/ml. Metoprolol standards in plasma were obtained by appropriate dilutions of the stock solution with plasma to achieve free-base concentrations of 5, 10, 25, 50, 100, 250 and 500 ng/ml.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model U6K injector and Model 6000A pump were used in combination with a Hitachi-Perkin-Elmer (Norwalk, CT, U.S.A.) Model 204-A fluorescence detector. The mobile phase, delivered at 2.0 ml/min, was prepared by mixing 150 ml acetonitrile, 300 ml methanol and 550 ml water, and adjusting the acidity of the mixture to pH 3.0 with phosphoric acid. The chromatographic column, 30 × 3.9 mm, contained octadecylsilane reversed-phase (μBondapak C_{18}, Waters) of 10-μm particle size. The detector was operated at 275 and 300 nm excitation and emission wavelengths, respectively.

Procedure

To a 1.0-ml aliquot of the sample (standard or patient plasma) in a 12-ml conical extraction tube were added 0.5 ml of the working internal standard solution, 0.1 ml of 2 *N* sodium hydroxide and 3.0 ml of *n*-butyl chloride. The mixture was shaken vigorously for 30 sec, followed by centrifugation at 500 *g* for 2 min. The organic (top) layer was then transferred to another conical tube, and 0.1 ml of 0.1 *N* sulfuric acid was added. The mixture was mixed on a vortex-type mixer, followed by centrifugation at 500 *g* for 1 min. Most of the organic (top) layer was removed by aspiration and discarded, and 20 μl of the aqueous layer was injected into the HPLC system.

Calculations

The standard curve was defined by linear-least-squares regression analysis of the data obtained from the spiked plasma samples. The concentration of metoprolol in the unknown samples was determined from the standard curve.

RESULTS

Fig. 1 shows the chromatogram of metoprolol and alprenolol in the HPLC system used. The retention times are: metoprolol, 2.8 min; alprenolol, 6.0 min. The overall recoveries of metoprolol and alprenolol were 74 ± 3% and 74 ± 4%, respectively (*n* = 5). The method was evaluated over the metoprolol concentration range of 5—500 ng/ml, and was found to be linear, with
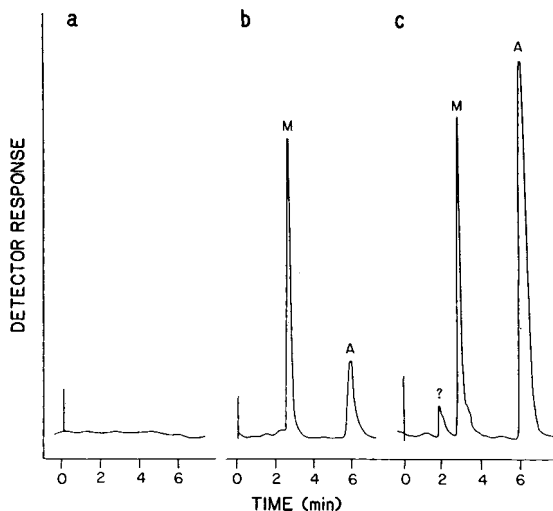
Fig. 1. (a) Chromatogram obtained upon analysis of blank plasma without addition of internal standard; (b) chromatogram of standard metoprolol (M) and alprenolol (A); (c) chromatogram obtained upon analysis of plasma of volunteer receiving metoprolol. Concentration of metoprolol: 79 ng/ml. The unknown peak may be due to α-hydroxy-metoprolol, see Discussion.

metoprolol:alprenolol peak height = 0.010 metoprolol concentration − 0.001 (r = 0.998) being a typical least-squares regression line. The minimum detectable amount (peak height = 3× baseline noise) was 2 ng/ml. The analysis of ten plasma samples containing metoprolol at 100 ng/ml concentration yielded an intra-assay (within-day) mean value of 99 ng/ml and a coefficient of variation (C.V.) of 4.1%. The corresponding values for inter-assay (day-to-day) were 98 ng/ml and a C.V. of 6.7%.

Interference from other drugs was studied including hydralazine, prazosin, furosemide, lidocaine, digoxin, quinidine, procainamide, N-acetylprocainamide, propranolol, salicylic acid, acetaminophen and diazepam. Plasma samples containing high therapeutic concentrations of the potentially interfering drug were analyzed by the procedure for metoprolol and were found to produce no interfering peaks.

DISCUSSION

Alprenolol was selected to serve as internal standard in the determination of metoprolol. The two compounds are very similar in chemical structure (Fig. 2), and the extraction, chromatographic, and fluorescence properties of alprenolol make it highly suitable for the role of internal standard. In addition, alprenolol is readily available from a commercial supplier at low cost (where internal standards are used in previously published HPLC procedures [12,14,15] the compounds are not routinely available from commercial suppliers, but must be obtained as gifts from pharmaceutical companies, a constraint we find somewhat inconvenient). While alprenolol is not available for routine clinical use in the United States, it is marketed in some countries. However, metoprolol
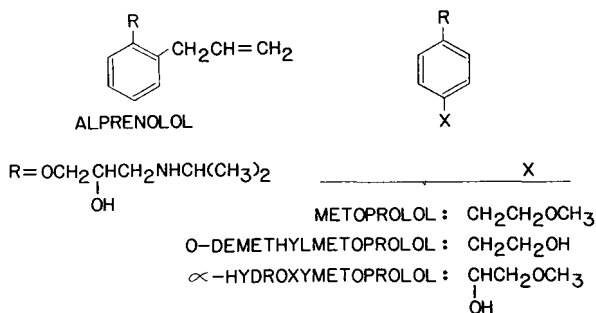
Fig. 2. Chemical structures of the compounds studied.

and alprenolol are not normally administered together, and therefore the two drugs are generally not present simultaneously in patients. Nonetheless, it should be noted that this procedure cannot be used if alprenolol (other than that added as internal standard) is present in the sample (the presence of alprenolol in a sample can be readily determined by analyzing it without addition of internal standard).

Among the previously published HPLC procedures for metoprolol only one used monochromators for both excitation and emission, set at 280 and 300 nm, respectively [13]. In our HPLC system, slightly greater sensitivity was achieved by using 275 instead of 280 nm for excitation.

Among the metabolites of metoprolol [18] only the basic compounds α-hydroxymetoprolol (Fig. 2) and O-demethylmetoprolol (Fig. 2) would be extracted from alkalinized plasma. O-demethylmetoprolol is rapidly further oxidized in vivo and negligible amounts are found in plasma [19]. α-Hydroxymetoprolol has been detected in human plasma at concentrations reaching ca. 50% of those of metoprolol [19,20]. We did not evaluate the chromatographic behavior of this metabolite, but in view of its chemical structure (Fig. 2) it is expected to have a shorter t_R value than that of metoprolol in reversed-phase HPLC, as has been found by others [15]. Indeed, a peak with a t_R of 1.7 min appeared in the chromatogram upon analysis of plasma samples from volunteers receiving metoprolol (Fig. 1). The peak was not present in blank plasma-derived chromatograms, and may well be due to α-hydroxymetoprolol.

The procedure described is selective, simple, rapid, and displays good accuracy and precision. Furthermore, the same HPLC column and mobile phase can be used (with different fluorescence monochromator settings) for the determination of the concentration of propranolol [21] and salicylate [22] in plasma.

ACKNOWLEDGEMENT

REFERENCES

1 B.E. Karlberg, O. Nilsson, K. Tolagen, E. Nitelius and V. Waern, Clin. Pharmacol. Ther., 25 (1979) 399.
2 M. Kubik, M. Kendall, A. Ebbut and V. John, Clin. Pharmacol. Ther., 25 (1979) 25.
3 M.H. Frick and O. Luurila, Ann. Clin. Res., 8 (1976) 385.
4 H.W. Wasir, R.K. Mahapatra, M.L. Bhatia, S.B. Roy and R. Sannerstedt, Brit. Heart J., 39 (1977) 834.
5 G. Johnsson and C.G. Regårdh, Clin. Pharmacokin., 1 (1976) 233.
6 M. Ervik, Acta Pharmacol. Toxicol., 36 (Suppl V) (1975) 136.
7 P.H. Degen and W. Riess, J. Chromatogr., 121 (1976) 72.
8 C.D. Kinney, J. Chromatogr., 225 (1981) 213.
9 R.C. Browning, V.A. John, R.J. Till and W. Theobald, Brit. J. Clin. Pharmacol., 12 (1981) 600.
10 K.U. Seiler, K.J. Schuster, G.J. Meyer, W. Niedermayer and O. Wasserman, Clin. Pharmacokin., 5 (1980) 192.
11 M. Ervik, K.J. Hoffman and K. Kylberg-Hanssen, Biomed. Mass Spectrom., 8 (1981) 322.
12 M.A. Lefebvre, J. Girault and J.B. Fourtillan, J. Liquid Chromatogr., 4 (1981) 483.
13 H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223.
14 D.B. Pautler and W.J. Jusko, J. Chromatogr., 228 (1982) 215.
15 M.T. Rosseel, F.M. Belpaire, I. Bekaert and M.G. Bogaert, J. Pharm. Sci., 71 (1982) 114.
16 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113.
17 J. Sklar, G.D. Johnston, P. Overlie, J.G. Gerber, H.L. Brammell, J. Gal and A.S. Nies, Circulation, 65 (1982) 894.
18 K.O. Borg, E. Carlsson, K.-J. Hoffmann, T.-E. Jonsson, H. Thorin and B. Wallin, Acta Pharmacol. Toxicol., 36 (Suppl V) (1975) 125.
19 K.-J. Hoffman, C.-G. Regårdh, M. Aurell, M. Ervik and L. Jordo, Clin. Pharmacokin., 5 (1980) 181.
20 L. Jordo, P.O. Attman, M. Aurell, L. Johansson, G. Johnsson and C.-G. Regårdh, Clin. Pharmacokin., 5 (1980) 169.
21 R.D. Aarons, A.S. Nies, J. Gal, L.R. Hegstrand and P.B. Molinoff, J. Clin. Invest., 65 (1980) 949.
22 J. Gal, unpublished results.

CHROMBIO. 1841

Note

Quantitative determination of almitrine in plasma by high-performance liquid chromatography

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Almitrine (I) is a new peripheral chemoreceptor [1, 2] agonist for treatment of hypoxia and/or hypercapnia which improves ventilation perfusion inequality [3—5]. Almitrine is currently under phase II investigation in the U.S.A. for use in chronic obstructive lung disease in studies sponsored by the Amaric Corporation, Mentor, OH, U.S.A. This drug also has potential usefulness in other conditions in which oxygen delivery is impaired.

Studies of the pharmacokinetics of almitrine require a separation system with high selectivity and high sensitivity because of the minor chemical differences between almitrine and those of endogenous compounds. This paper presents a method for the separation, detection and quantitation of almitrine by reversed-phase high-performance liquid chromatography (HPLC). The

method is comparable in sensitivity and reproducibility to the gas—liquid chromatography (GLC) method of Baune et al. [6] with nitrogen—phosphorus detection.

EXPERIMENTAL

Chemicals

Almitrine bis mesylate, 2,4-(2-propenylamino)-6-[N_4-(di-p-fluorophenyl-methyl)-1-piperazinyl]-1,3,5-triazine bis mesylate, and S-2082 (internal standard, the dimethylated derivative of almitrine bis mesylate) (Fig. 1) were supplied by Technologie Servier, Orleans (France). HPLC grade acetonitrile, 2-propanol and cyclohexane were obtained from Fisher Scientific. All other chemicals were analytical reagent grade obtained from Fisher Scientific.

Almitrine bis mesylate

2082 (Internal standard)
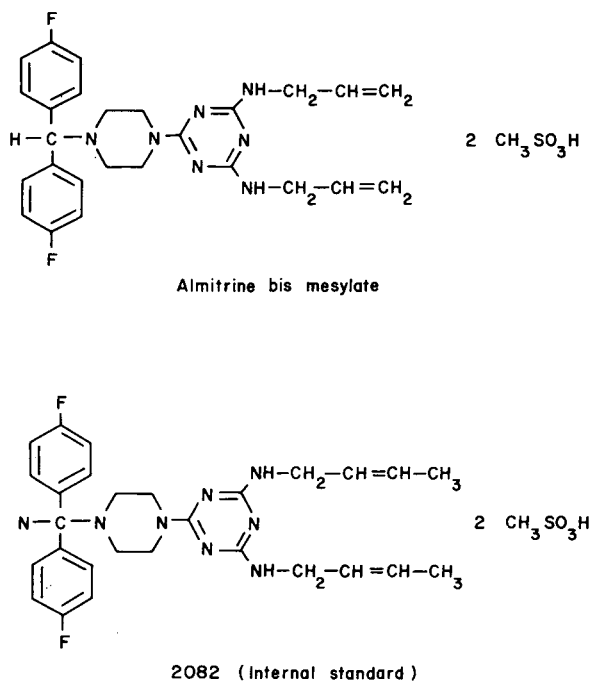
Fig. 1. Structures of almitrine bis mesylate and S-2082 bis mesylate (internal standard).

Standard solutions

Standard solutions of almitrine and S-2082 were prepared as their bis mesylate salts (MW 670 and 698 respectively) in acetonitrile at a concentration of 10 μg/ml. As necessary the almitrine solution was diluted with acetonitrile to produce solutions of desired concentration. Spiked-plasma samples, containing 7.1 to 1000 ng/ml almitrine base (MW 476), were prepared in duplicate by adding 20 to 140 μl of the appropriate solution to 1 ml of heparinized human plasma. Internal standard, 145 ng/ml S-2082 base, was added to each duplicate spiked-plasma sample of the standard curve. The resulting solution was mixed thoroughly on a Vortex mixer.

A Waters Assoc. (Milford, MA, U.S.A.) 6000A pump was used in conjunction with a Bio-Rad Model 1305 variable-wavelength UV detector operated at 225 nm and an IBM Instrument 9540, chromatography data integrator, attenuated to 1 mV full scale at 0.20 cm/min.

Injections were made with a Waters Assoc. WISP 710B with a 200-μl sample loop. The separation was performed on a Waters Assoc. radial compression module 10-μm C_{18} column.

Mobile phase

Acetonitrile—2-propanol—0.006 M K_2HPO_4 buffer pH 7.8 (66.7:8.3:25) was used as the mobile phase at a flow-rate of 1.6 ml/min (pressure drop 35 atm) with development being carried out at room temperature (21—24°C). The mobile phase was filtered through a 0.2-μm Nylon-66 membrane filter and degassed under·vacuum immediately before use.

Limited volume inserts (LVI) for the injector as well as other reusable glassware were cleaned by immersion over night in 95% ethanol. The LVI's were rinsed with mobile phase three times and dried in a 75°C oven. Note: care must be exercised in cleaning glassware as even slight contamination will distort the chromatogram.

Assay procedure

Blood samples (6 ml) were collected in heparinized glass tubes. Plasma was obtained by centrifugation. A 20-μl volume of internal standard (containing 145 ng/ml S-2082 base) was added to the 1-ml plasma samples and mixed on a Vortex mixer. One ml of acetonitrile was added to each of the duplicate samples. The samples were mixed on a Vortex mixer for 15 sec, allowed to stand at room temperature for 25—30 min and centrifuged at 4100 g for 10 min at 4°C. The resulting supernatant was decanted into ethanol-washed 12 × 75 mm disposable glass tubes containing 20 μl of 5 M NaOH. The protein precipitate was discarded. The alkalinized supernatant was then extracted twice with 1 ml of cyclohexane by mixing vigorously on a Vortex for 30 sec. The layers were separated by centrifugation for 10 min at 4100 g at 7°C. The organic layers were transferred by pasteur pipette after each centrifugation to an evaporation tube. The combined organic layers were subsequently evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was redissolved in 100 μl of mobile phase solvent and transferred to an LVI. A sample of 35 μl was injected into the liquid chromatograph.

Calibration graphs

Almitrine concentrations were calculated with the aid of a calibration graph. Spiked plasma samples were processed as described in the assay procedure. Peak heights were calculated and plotted against known plasma concentrations. The slopes of the calibration curves and correlation coefficients were calculated by using a linear regression analysis program on an Apple II Plus computer.

Recovery

Recoveries of almitrine at different concentrations were determined by comparison of the extracted spiked plasma (R_1) to direct injection of almitrine in mobile phase solvent (R_2). Recovery (%) = R_1/R_2 × 100.

Comparison of HPLC vs. GLC

Results obtained in our laboratory using the HPLC technique were compared with results from Bromet's laboratory using the GC nitrogen—phosphorus detection technique. Blood samples obtained after a 200-mg oral dose of almitrine, were split after centrifugation. A portion of the sample was retained for analysis by HPLC and the rest was sent to Bromet's laboratory for analysis by GC with nitrogen—phosphorus detection. When the comparison was made only the topmost organic layer from the extraction was used in the HPLC analysis.

RESULTS

Standard curves were linear over a concentration range of 7.1 to 1000 ng/ml base. The correlation coefficients of the calibration graphs were found to be 0.995 ± 0.003 ($n = 18$). Recoveries from plasma were calculated for almitrine in the range of 7.1 to 1000 ng/ml. Recoveries were consistent over this range with a mean value of $98 \pm 3\%$. In our preliminary precipitation—extraction procedure only the top layer of the three-layer extraction solution was removed with recoveries of $62 \pm 3\%$ from 7.1 to 500 ng/ml.

The lower limit of quantitation in plasma is 1 ng/ml using 1 ml of plasma. However, interferences from endogenous components in plasma have a great influence on the signal-to-noise ratio, which ultimately determine the detection
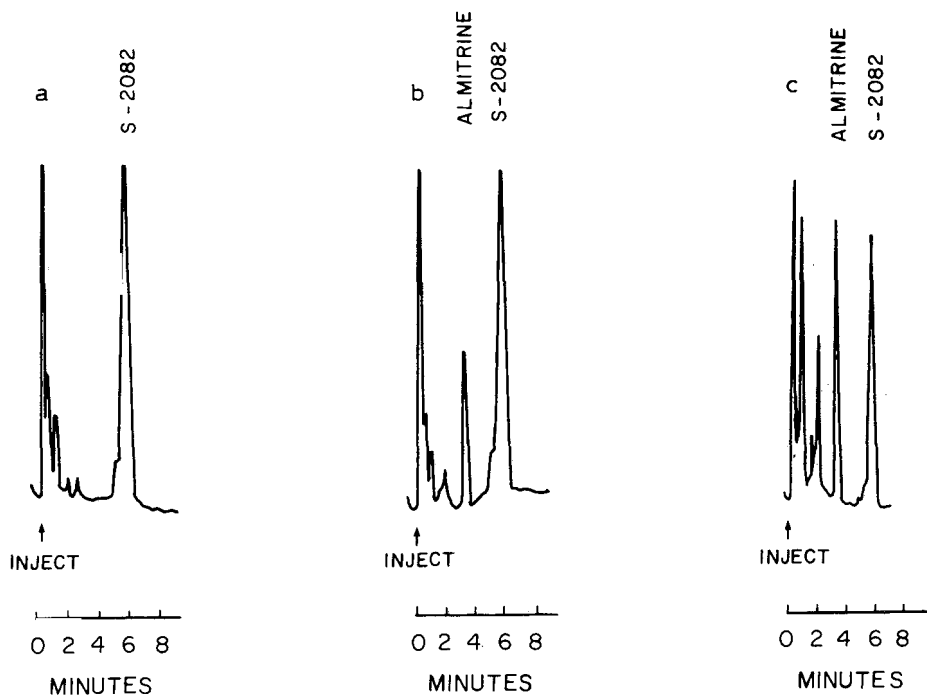

Fig. 2. Chromatograms of (a) spiked plasma containing 7.1 ng/ml almitrine base and 25 ng/ml S-2082 base, (b) spiked plasma containing 25 ng/ml S-2082 base, and (C) subject plasma sample 8 h post 200 mg oral dose of almitrine bis mesylate.
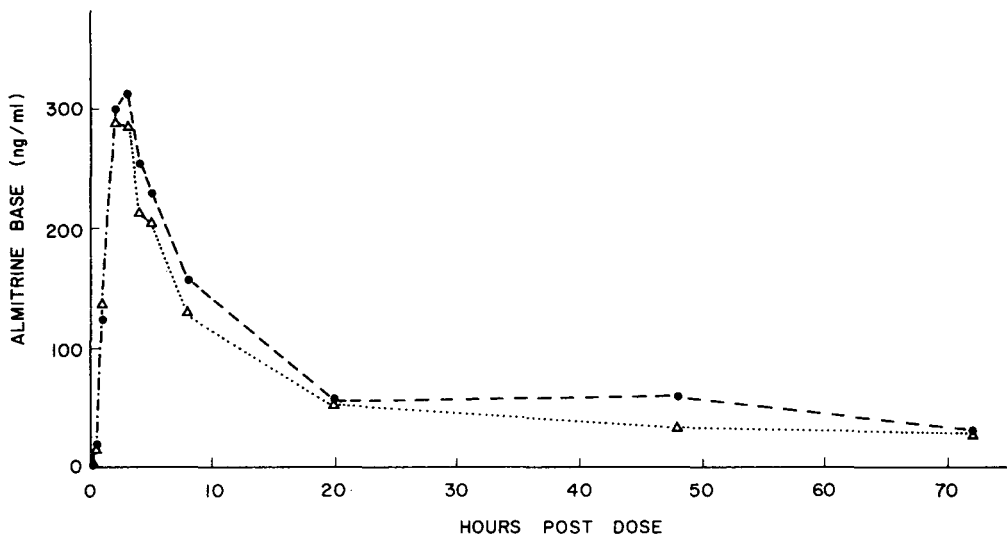
Fig. 3. Method comparison: determination of almitrine (base) by HPLC UV (●) and GC nitrogen—phosphorus (△) for the same human subject after oral administration of 200 mg of almitrine.

limit. Fig. 2a illustrates a chromatogram obtained from the analysis of a spiked plasma sample containing 25 ng/ml S-2082. The chromatogram in Fig. 2b contains both 7.1 ng/ml almitrine and 25 ng/ml S-2082. Fig. 2c illustrates a chromatogram from a subject sample obtained 8 h after oral administration of almitrine bis mesylate. In single-dose experiments in volunteers, almitrine appeared rapidly in the circulation after oral administration. Peak plasma levels were generally reached 3 h after intake and were 173—607 ng/ml for the 200-mg dose. The plasma half life from these data assuming a two-compartment model was approximately 40 h. Even though our preliminary extraction procedure was not quantitative, recoveries were consistent. Comparison of the results from each laboratory suggests that the values are in good agreement and that the methodologies are comparable. Fig. 3 shows a comparison of a plasma concentration—time curve obtained by the HPLC and GC methods after single-dose 200 mg oral administration.

DISCUSSION

Reversed-phase chromatography was chosen as the separation mode because it offers excellent column stability. This is particularly important for routine analysis. Various solid stationary phases were investigated. We chose a chemically modified silica gel as the adsorbent because of high capacity factors (K') that could be obtained and which preserved the separation between almitrine and the internal standard.

The use of the solvent system as described effected a good separation among endogenous plasma components, almitrine and the internal standard with relatively low viscosity. The separation could be optimized by varying the mobile-phase composition, especially by changing the 2-propanol concentra-

tion to maximize the capacity factor and still achieve separation from endogenous components.

Several different isolation procedures were developed for separating almitrine from the other constituents in plasma. Almitrine is tightly bound to plasma proteins but direct precipitation of protein using acetonitrile (1:1) yielded good recovery of almitrine in the microgram-per-milliliter range. With precipitation alone, however, sensitivity of the method was not sufficient to follow the concentrations of almitrine contained in plasma after low to moderate dosage regimens. An extraction procedure appeared to be necessary but we wished to avoid some of the difficulties in the method of Baune et al. [6] (e.g., the internal standard was not added until the final extraction step and large volumes of the extracting solvent were used). We devised a combined precipitation—extraction procedure to minimize the volume of solvents, time of sample preparation for routine analysis, and still obtain quantitative recovery.

Acetonitrile precipitation was used to separate almitrine and the internal standard from plasma protein. After centrifugation the supernatant was alkalinized to enhance the extraction of the internal standard. An extraction was then performed by extracting the aqueous mixture twice with 1 ml of cyclohexane. After the first extraction and centrifugation there was a three-layer system. After assaying each of the layers, it was determined that the top two layers contained almitrine and S-2082. These layers were removed and were transferred to an evaporation tube. After the second extraction and centrifugation there were only two layers. The upper layer from the second extraction was also transferred to the evaporation tube. Combination of the two upper layers from the first extraction and the one upper layer from the second extraction results in recoveries of 98 ± 3%. In order to be able to determine concentrations at the lower nanogram-per-milliliter level, the combined organic layers must be evaporated and the residue redissolved in a minimum amount of mobile-phase solvent. The residue was readily redissolved in 100 μl of mobile phase by shaking the tube on a Vortex mixer for a few seconds.

The method is at present being used to quantitate levels in plasma after oral administration of almitrine to volunteers as well as patients with chronic obstructive pulmonary disease (COPD). We have completed over 1000 samples with the preliminary extraction procedure and 200 with the procedure reported in this communication. We feel that this method offers a highly sensitive and specific alternative method for the routine analysis of almitrine in plasma.

REFERENCES

1 M. Laubie and F. Diot, J. Pharmacol. (Paris), 3 (1972) 363.
2 M. Laubie and H. Schmitt, Europ. J. Pharmacol., 61 (1980) 125.

3   D. Rigaud, F. Dubois, J. Godart and C. Paramelle, Amer. Rev. Resp. Dis., 123 (1981)
    112.
4   D. Rigaud, F. Dubois, J. Boutet, C. Brambilla, A. Verain and B. Paramelle, Rev. Fr.
    Mal. Respir., 8 (1980) 605.
5   D. Rigaud, F. Dubois, J. Godart, J. Boutet and B. Paramelle, Lyon Med., 245 (1981)
    491.
6   A. Baune, N. Bromet, S. Courte and C. Voisin, J. Chromatogr., 223 (1981) 219.

CHROMBIO. 1816

Note

Determination of alcuronium dichloride in plasma by high-performance liquid chromatography without solvent extraction

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A number of analytical procedures have been used for the determination of the neuromuscular blocking agent alcuronium dichloride (diallyl-nortoxiferine, Alloferin) and include bioassay [1], assay of radioactively labelled drug [2] and a spectrofluorimetric technique involving extraction with rose-bengal [3].

A high-performance liquid chromatographic (HPLC) method has recently been reported [4] for the assay of plasma samples of alcuronium dichloride. The method uses ion-pair extraction of the picrate salt to isolate the drug followed by chromatographic separation and ultraviolet detection, *d*-tubocurarine chloride being used as an internal standard. A major disadvantage of this method is that it involves a time-consuming extraction step of 5 h and an evaporation process. Each plasma determination also requires 1 ml of plasma which necessitates taking quite a large total volume of blood from patients if one wishes to do detailed pharmacokinetic studies.

A new selective, rapid and sensitive method for the determination of alcuronium dichloride in 250 μl of plasma is the subject of this report.

EXPERIMENTAL

Standards and reagents

Acetonitrile and methanol were HPLC grade (Waters Assoc., Milford, MA, U.S.A.) and potassium dihydrogen orthophosphate was AR grade (Ajax Chemicals, Sydney, Australia). Alcuronium dichloride was a gift from Roche Products (Sydney, Australia) and a stock solution of 0.2 mg in 1 ml was prepared in distilled water and stored at 4°C until required.

Instrumentation

Reversed-phase HPLC was performed using a Waters Model M45 solvent delivery system, a Rheodyne Model 7125 40-μl injector and a Brownlee RP-8 column (25 cm × 4.6 mm I.D., 10 μm particle size). A Rheodyne column inlet filter (Model 7302) was placed between the injection valve and column. An Omniscribe recorder (Houston Instruments) was used in conjunction with a LC-4A amperometric detector (BioAnalytical Systems) and an LC-17 oxidative flow-cell. The LC-17 flow-cell is composed of a TL-5 cube with a glassy carbon working electrode and a silver/silver chloride reference electrode. Injections were made with a 100-μl SGE syringe.

Assay procedure

To 250 μl of plasma were added 500 μl of acetonitrile. This solution was vortexed for 1 min, then centrifuged at approximately 2000 g for 5 min. An aliquot (40 μl) of the clear supernatant was injected onto the column. The mobile phase consisted of acetonitrile—0.001 M potassium dihydrogen orthophosphate—methanol (10:22:68). The chromatogram was run at ambient temperature with a flow-rate of 1.5 ml/min. The electrochemical detector was operated with an applied voltage of 1.0 V and the recorder at a setting of 10 mV and a chart speed of 2.5 mm/min. The procedure was standardised by analysing drug-free plasma samples spiked with known quantities of alcuronium dichloride solution. Peak heights were used to establish calibration curves.

RESULTS AND DISCUSSION

This assay procedure produces a good, almost noise-free baseline after the initial plasma peaks except for one endogenous plasma peak with a retention time of 16 min (Fig. 1B), the alcuronium peak having a retention time of 12 min. (Fig. 1A).

It has been reported [5] that direct injection of plasma samples onto a reversed-phase column leads to an increase in back pressure of the system due to proteinaceous materials being deposited onto the head of the column. To avoid this problem we used a column inlet filter between the injector and column to protect the column inlet frit from being blocked. These inline filters are inexpensive, easily replaced and there seems to be no loss of resolution. In our preliminary work we initially diluted plasma with an equal volume of acetonitrile but found that the filter needed to be changed regularly. Now that we dilute the plasma with a two-fold volume of acetonitrile we have had no problem with increasing back pressure and the operating pressure
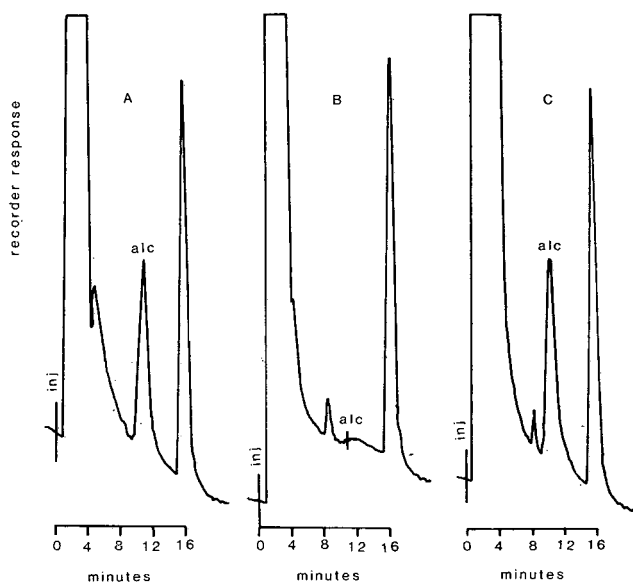
Fig. 1. Chromatograms of (A) plasma spiked with alcuronium dichloride (alc) at a concentration of 1 μg/ml; (B) patient plasma collected at zero time, and (C) patient plasma collected at 10 min after drug administration, concentration approximately 1.1 μg/ml.

remains at approximately 7 MPa. The filters are changed occasionally when the operating pressure rises above 10 MPa.

The analytical method reported here is selective and linear calibration curves were derived over the range 0.1—6.0 μg/ml of alcuronium dichloride giving a correlation coefficient greater than 0.995. There is no extraction or evaporation step and an internal standard is not needed. The sensitivity for this method is at least equal to the previously described procedure (0.1 μg/ml) and with appropriate modification even lower concentrations of alcuronium could be measured. After deproteinating the plasma samples with acetonitrile the supernatant was found to contain 100% of the added alcuronium dichloride. The method is rapid and a single plasma sample could be assayed within 20 min of receipt. Although we routinely use 250 μl of plasma as little as 100 μl of plasma could be used if diluted with 200 μl of acetonitrile. This would be particularly important if one wanted to study the pharmacokinetics of alcuronium in patients where it is difficult to take large quantities of blood.

A 7-days analytical study was undertaken to determine the coefficient of variation of the procedure using a pooled-plasma specimen containing

TABLE I

DRUGS TESTED FOR INTERFERENCE IN PLASMA

Caffeine	Codeine	Diazepam
Fentanyl	Indomethacin	Methadone
Metoprolol	Morphine	Nitrazepam
Oxycodone	Paracetamol	Pethidine
Phenoperidine	Pholcodine	Thiopentone

alcuronium dichloride at 1.0 μg/ml and was found to be 4.7%. The intra-day coefficients of variation were 2.3%, 3.0% and 4.2% at 2.0 μg/ml, 1.0 μg/ml and 0.6 μg/ml, respectively ($n=8$).

Under the above analytical conditions no interference has been observed by a number of drugs used routinely during anaesthesia. Drugs that have been tested for interference are listed in Table I.

REFERENCES

1 J. Thomas, C.R. Climie and L.E. Mather, Brit. J. Anaesth., 41 (1969) 297.
2 J. Raaflaub and P. Frey, Arzneim.-Forsch., 22 (1972) 73.
3 J. Walker, C.A. Shanks and E.J. Triggs, Eur. J. Clin. Pharmacol., 17 (1980) 449.
4 J.E. Parkin, J. Chromatogr., 225 (1981) 240.
5 L.C. Franconi, G.L. Hawk, B.J. Sandmann and W.G. Haney, Anal. Chem., 48 (1976) 372.

CHROMBIO. 1821

Note

High-performance liquid chromatographic determination of aprindine and its active desethyl metabolite in plasma

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Aprindine [AP, N-phenyl-N-(3-diethylaminopropyl)-2-indanylamine] (Fig. 1), an effective anti-arrhythmic agent, has been used successfully to treat both ventricular and supraventricular arrhythmias in Europe and the United States [1,2] and is currently undergoing review for approval for use in Japan.

Measurement of AP in plasma is important because of its narrow therapeutic index. We have reported the rapid and sensitive gas chromatographic—mass spectrometric (GC—MS) determination of AP in plasma [3]. This method, however, has some disadvantages for general use because it requires an experienced technician for the operation of the expensive instrument. Furthermore, since one of the major metabolites, desethylaprindine (DEAP) (Fig. 1), is reported as active as AP in antagonizing ouabain-induced arrhythmias in dogs [4], a specific assay for both compounds is needed for the appropriate drug therapy.

The present paper reports the development of a simple, accurate and sensitive high-performance liquid chromatographic (HPLC) assay for quantitating AP and DEAP in plasma.
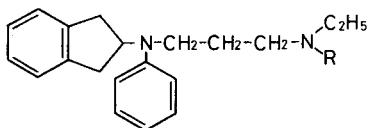
R : C_2H_5 Aprindine (AP)
R : H   Desethylaprindine (DEAP)

Fig. 1. Structures of aprindine (AP) and desethylaprindine (DEAP).

EXPERIMENTAL

Standards and reagents

AP, DEAP and other metabolites were supplied by A. Christiaens (Brussels, Belgium) and amitriptyline hydrochloride was purchased from Kodama Chemicals (Tokyo, Japan). HPLC grade acetonitrile and methanol were from Junsei Chemicals (Tokyo, Japan). All other reagents were of analytical grade and obtained from usual commercial sources. Standard solutions of AP, DEAP and amitriptyline hydrochloride were prepared in distilled water and stored at 4°C throughout the experiments. The hydrochloride salts of AP and DEAP were used and the concentrations were calculated as the salt.

Instrumentation

HPLC was performed on a Shimadzu Model LC-3A liquid chromatograph equipped with a variable-wavelength UV detector (Model SPD-2A). A 30 cm × 4 mm I.D. stainless-steel column packed with 10-μm μBondapak C_{18} particles (Waters Assoc., Milford, MA, U.S.A.) was used and the mobile phase was acetonitrile—0.1 M KH_2PO_4 (45:55, v/v, pH 3.0). The flow-rate was 2.0 ml/min with a pressure of about 10 MPa, and the detector was set at 259 nm because AP and DEAP gave intense absorbance at around 259 nm. The column temperature was ambient.

Procedure

Extraction was carried out basically according to the previously reported procedure [3] except for the use of 500 ng of amitriptyline hydrochloride as internal standard. After evaporation of the final extract, the residue was dissolved in 100 μl of mobile phase, and a 10-μl sample was injected into the chromatograph.

Quantitation

Spiked plasma extracts were chromatographed, and standard curves were constructed by plotting the ratios of AP and DEAP to internal standard peak areas versus the concentrations of the drug. Calibration standards were chromatographed each day when the unknown samples were analyzed. The concentrations of unknown samples were determined by comparison of the peak area ratios to the standard curves obtained that day.

Recovery and precision

Analytical recoveries of AP and DEAP were determined by comparing the peak areas obtained from spiked plasma standards with those obtained when the internal standard was added to the plasma and known amounts of AP were added to the aqueous phase prior to the last extraction with diethyl ether. The precision of the method was estimated by analyzing spiked plasma standards which had been prepared on different days.

Application of the method

Plasma samples obtained from a patient receiving AP [20 mg t.i.d. (ter in die)] were analyzed by the present method. Plasma samples from patients who

received AP orally at different doses were also analyzed by the present method and compared with the results obtained by the GC-MS method [3].

RESULTS AND DISCUSSION

Under the described chromatographic conditions, the retention times of AP, DEAP and the internal standard were 4.40, 2.93 and 3.65 min, respectively (Fig. 2), and no overlap with authentic metabolites which were identified in human urine [5] was observed (Table I). The standard curves of AP and DEAP were linear over the plasma concentration range 50—1000 ng/ml (r^2 = 0.999 and 0.998 for AP and DEAP, respectively). Recoveries of AP and DEAP from spiked plasma samples in triplicate at two different concentrations (200 and 600 ng) were 95—97% and 88—89%, respectively.

The precision and accuracy of the method are shown in Table II The within-day and between-day coefficients of variation were <5% and the sensitivity limit of the assay was 20 ng/ml for AP and 50 ng/ml for DEAP, in the case of a plasma volume of 1 ml. However, this sensitivity could be increased by using a larger volume of sample.
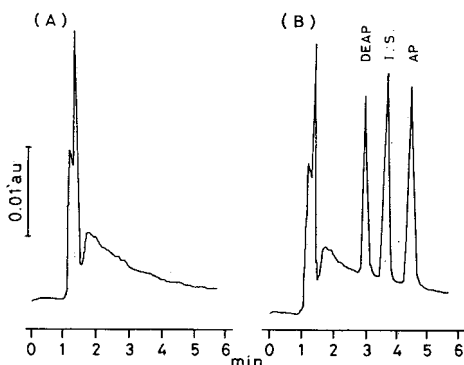
Fig. 2. Chromatograms of (A) a blank plasma extract, (B) an extract of plasma added with aprindine (AP), desethylaprindine (DEAP) (300 ng each) and internal standard (I.S., 500 ng).

TABLE I

RETENTION TIMES OF APRINDINE (AP), ITS METABOLITES AND OTHER ANTI-ARRHYTHMIC AGENTS

Compound	Retention time (min)
Aprindine (AP)	4.40
Desethylaprindine (DEAP)	2.93
Desindanylaprindine	1.82
Desphenylaprindine	1.31
p-Hydroxyaprindine	1.80
Mexiletine	1.70
Quinidine	1.82
Lidocaine	1.87
Disopyramide	2.10

TABLE II

PRECISION AND ACCURACY OF THE METHOD $(n = 9)$

Concentration (ng/ml)	Coefficient of variation (%)			
	Within-day		Between-day	
	AP	DEAP	AP	DEAP
50	3.43	4.74	3.14	4.34
100	4.00	4.65	3.77	4.16
200	3.42	3.63	2.77	4.17
400	3.51	3.41	3.21	3.41
600	2.11	2.58	2.06	2.50
800	2.11	3.19	1.88	3.07
1000	1.49	2.80	1.40	2.69

Since AP may be given concurrently with other antiarrhythmic drugs, several were examined to determine whether they would influence the assay. No interference was observed with mexiletine, quinidine, lidocaine and disopyramide (Table I).

The developed method was applied to the analysis of plasma samples from a patient taking AP chronically for the management of premature ventricular contractions (Fig. 3). After multiple oral administration of AP (20 mg t.i.d.) a steady-state plasma AP level was achieved as late as one week with a trough level of 0.4—0.5 μg/ml. During the early period, the DEAP level was not above the threshold sensitivity of the assay. A steady-state plasma level of DEAP was obtained in less than six days with a trough level of 0.08 μg/ml.

The described HPLC procedure was compared to the earlier GC—MS procedure [3]. There was a good agreement between the values obtained by the two methods (Fig. 4). A correlation coefficient (r^2) of 0.995 and a slope value of 0.951 were obtained for the samples from the patients, analyzed by the two methods.

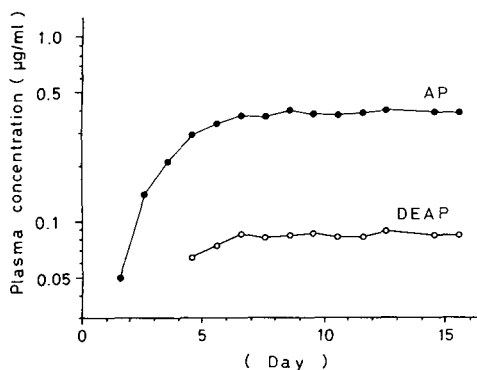The increased sensitivity of the GC—MS procedure [3], which measures

Fig. 3. Time course of plasma aprindine (AP) and desethylaprindine (DEAP) concentrations in a patient after multiple oral doses of aprindine (20 mg t.i.d.). Each value shows the plasma concentration just before the second dose of the day.
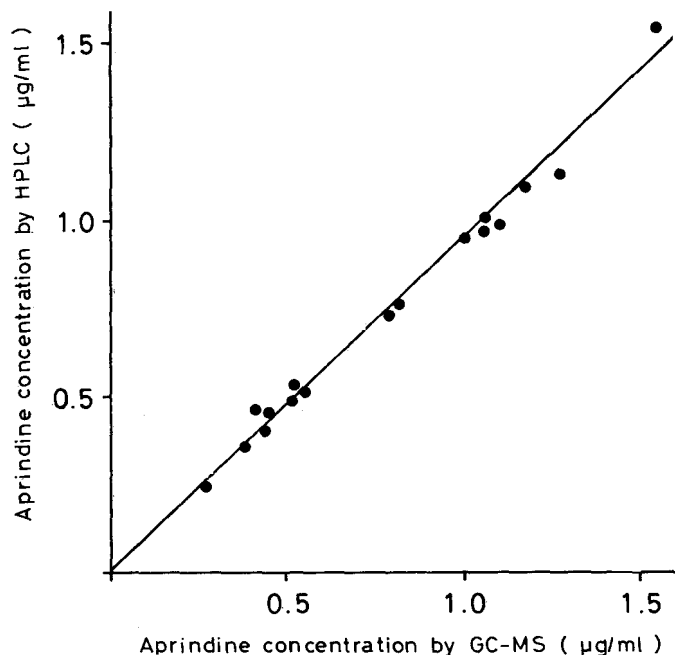
Fig. 4. Plot of plasma aprindrine concentration in patients by GC—MS method [3] versus plasma concentration of aprindine measured by the described HPLC method (n = 17, r^2 = 0.995, and slope = 0.951).

concentrations less than 5 ng/ml of plasma, is offset by the ease of the present method, which makes use of technology readily available in most laboratories and is satisfactory for routine clinical analysis. This method could be applicable to urine analysis because no interference substances were encountered in urine samples.

REFERENCES

1 P. Danilo, Jr., Amer. Heart J., 97 (1979) 119.
2 D.P. Zeipes, V. Elharrar, R.F. Gilmour, J.J. Heger and E.N. Prystowsky, Amer. Heart J., 100 (1980) 1055.
3 T. Ito, H. Namekawa and T. Kobari, J. Chromatogr., 274 (1983) 341.
4 P.J. Murphy, Acta Cardiol. (Brux) Suppl., 18 (1974) 131.
5 L. Dodion, J.M. Suray, M. Deblecker and A. Georges, Therapie, 29 (1974) 221.

225

CHROMBIO. 1838

Note

Determination of a novel antitussive agent 2′,4′-dimethyl-6′-methoxy-3-(2-methylpiperidyl)-propionaldehyde in plasma by high-performance liquid chromatography

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(First received May 3rd, 1983; revised manuscript received June 27th, 1983)

Opium alkaloids are the most effective antitussive compounds available. They have, however, several well-established side-effects and a risk of abuse as well [1]. Therefore a need for a new drug is evident.

A novel compound, 2′,4′-dimethyl-6′-methoxy-3-(2-methylpiperidyl)-propionaldehyde (OR K-242 hydrochloride) (Fig. 1, II), which is chemically related to lidocaine, was found in several types of animal experiments to be an effective inhibitor of the cough [2]. To establish the drug levels in plasma for the use in experimental and clinical pharmacokinetics a simple high-performance liquid chromatographic (HPLC) assay was developed.
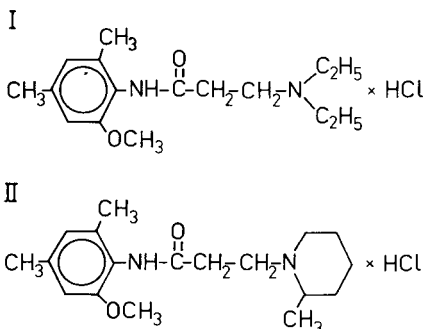

Fig. 1. Structures of the internal standard (I) and OR K-242 (II).

EXPERIMENTAL

Reagents

OR K-242 hydrochloride and the internal standard OR K-269 hydrochloride were obtained from Orion Pharmaceutical (synthesized by Ms. A. Pippuri

and Dr. E. Honkanen, Research Center, Espoo, Finland). Chemical structures are shown in Fig. 1. Acetonitrile, HPLC grade S, was purchased from Rathburn Chemicals (Walkerburn, U.K.) and dichloromethane, Uvasol grade from E. Merck (Darmstadt, F.R.G.). All inorganic reagents were of analytical grade and purchased from commercial sources.

Sample preparation

Plasma samples (0.2—0.5 ml) were spiked with 125 ng of the internal standard. Plasma was alkalinized with 0.1 ml of 1.0 *M* sodium hydroxide in stoppered tubes and extracted with 6 ml of dichloromethane by shaking 10 min with a universal laboratory shaker. After centrifugation (5 min at 1100 *g*) 5 ml of the organic layer were evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.1 ml of the mobile phase and 20 μl were injected into the HPLC column.

Chromatography

The modular liquid chromatographic system consisted of a Waters Model 6000 A pump, a Waters Intelligent Sample Processor (WISP) Model 710 B (Waters Assoc., Millford, MA, U.S.A.), a 250 × 4.5 mm 5-μm Spherisorbnitrile column (Phase Separations, Queensferry, U.K.). The eluted components were detected by ultraviolet (UV) absorption at 214 nm with a Kratos Model 773 variable-wavelength detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). The elution was carried out isocratically at ambient temperature using 30% acetonitrile in 15 m*M* sodium phosphate, pH 4.0, at a flow-rate of 1.5 ml/min.
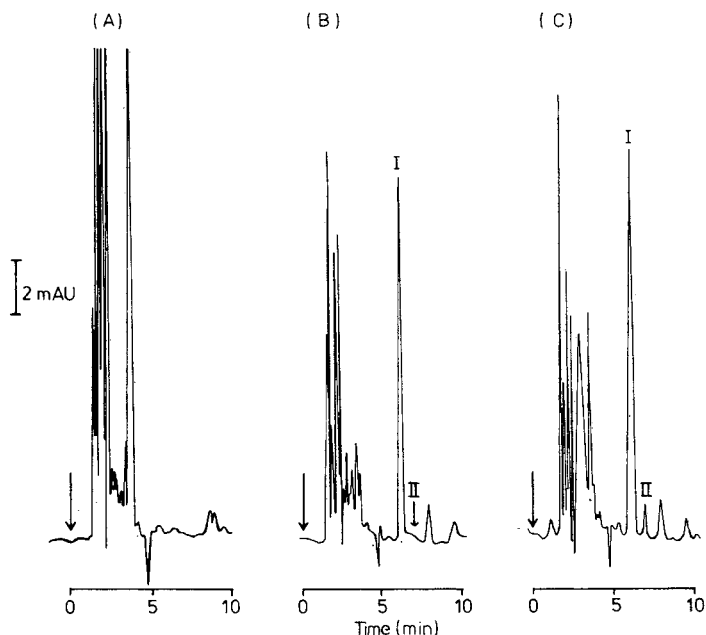

Fig. 2. Representative chromatograms of OR K-242 in plasma. (A) Blank plasma, (B) plasma spiked with 125 ng of internal standard, and (C) plasma containing 15 ng/ml OR K-242 and 125 ng/ml internal standard. Peaks: I = internal standard, II = OR K-242.

RESULTS AND DISCUSSION

Fig. 2 shows representative chromatograms of rabbit blank plasma (Fig. 2A), plasma spiked with 125 ng of the internal standard (Fig. 2B), and plasma 2 h after an intravenous dose of 20 mg/kg OR K-242 (Fig. 2C). The described extraction procedure and the low detection wavelength produced a couple of extra peaks in the chromatogram. These peaks were found to be due to impurities in the dichloromethane. Since they did not interfere with the compounds of interest, no further purification of the extraction medium was carried out. There was no interference with the common antitussive agent codeine, which eluted at 5.6 min (retention times for internal standard and OR K-242 were 6.2 and 7.0 min, respectively).

A plot of peak height ratio versus concentration of OR K-242 is linear over the range 10—1000 ng/ml and is described by the equation $y = 0.005x + 0.015$ ($r = 0.9997$). Detection limit with a signal-to-noise ratio of 3:1 is 2 ng/ml. The recovery of added OR K-242 (12.5 mg/ml and 1000 ng/ml, $n = 6$) from rabbit plasma was 95.5 ± 10.4% and 80.7 ± 4.4%, respectively. The precision of the assay was established by multiple measurements ($n = 10$) of quality control samples (100 ng/ml). The intra-assay mean ± S.D. was 95.5 ± 5.2 ng/ml, C.V. = 5.5%, and the inter-assay mean ± S.D. was 92.5 ± 6.8 ng/ml with C.V. = 7.3%.

The described HPLC assay with UV detection at 214 nm provides a fast, simple and sensitive method to determine plasma levels of the novel antitussive agent OR K-242. It has been successfully used to determine OR K-242 concentrations in plasma of different animal species necessary for experimental pharmacokinetic analysis.

REFERENCES

1 J.H. Jaffe and W.R. Martin, in A. Goodman Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, pp. 494—543.
2 P. Männistö, P. Karttunen, S. Lahovaama, E. Nissinen, J.E. Davies, D.R. Algate and M.W. Baines, Arzneim.-Forsch., submitted of publication.

CHROMBIO. 1831

Book Review

Reversed-phase high-performance liquid chromatography — Theory, practice and biomedical applications, by A.M. Krstulović and P.R. Brown, Wiley-Interscience, New York, 1982, XIII + 296 pp., price £ 27.00, ISBN 0-471-05369-4.

This monograph is, according to the subjects discussed, focused on the problems of reversed-phase high-performance liquid chromatography (HPLC), which today represents the most common approach in liquid column chromatography. The biomedical applications themselves appear to be secondary and are dealt within about 25% of the whole text.

For didactic reasons it was inevitable for the authors to include chapters of a general character in liquid chromatography. The book starts with chapters on the basic theory and nomenclature of chromatography, instrumentation (including detectors), sorbents, and a chapter on column performance. Another part of the book is constituted of chapters that, in spite of their general validity to the reversed-phase technique, have a flavour of biomedical applications. Among these, the chapters on separation mechanisms in reversed-phase systems, ion associated techniques, the strategy and selection of conditions for HPLC, quantitative analysis, and, finally, further peak characterization can be listed. The last part of the book summarizes some selected examples of biochemical/biomedical applications.

The monograph of Krstulović and Brown represents one of the most complete and exhaustive reviews that have been written to this moment about reversed-phase techniques. This justifies the existence of this book and its publication is certainly to be welcomed. There are a number of chapters which are very useful and written in a new, non-traditional way: the chapter on separation mechanism, the chapter on strategy in selection of chromatographic conditions and on peak characterization can serve as representative examples. The approach in introducing biomedical applications lays behind the high standard set by the authors in the general part of the book. Here the authors emphasize in part the areas in which they themselves are recognized experts (nucleic acid components, biogenic amines), while in other areas they remain on the surface of the problem.

This part of the book suffers from considerable disproportionality. Generalization of the whole topic of biomedicine will probably be missed by the readers as well as the introduction of some special techniques like sample preparation, selection of conditions according to the purpose of the analysis, and some others. Naturally, some of this information can be found in the

general part of the book. In a number of applications the references are out-dated. On p. 255 the authors report that the evolution of the technique is so fast that by the time of publishing the review this must be so. However, this statement is documented somewhat too obviously: of 153 references about drugs there is only a single one from 1979 and two from 1980 — all other are from the year 1978 and older. It has to be noted that between 1979 and 1982 an explosion of papers can be seen in the area of drug monitoring and phar-macokinetic studies.

From the typographical point of view the book is very nicely presented. However, in several places the figures and tables are included within the con-text of other chapters. The number of errors is within the acceptable range (see, for example, Table 5 — polargraphic, p. 120 — size/m).

In conclusion, it can be said that the book will represent an enrichment of the HPLC literature and will be welcomed mainly by those applying re-versed-phase separations in their work, including those in the biomedical field. However, for a special monograph devoted to biomedical applications the book market still remains open.

Prague (Czechoslovakia)   KAREL MACEK

PUBLICATION SCHEDULE FOR 1983

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

MONTH	D 1982	J	F	M	A	M	J	J	A	S	O	N	D
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Chromatographic Reviews					271/1		271/2		271/3				
Biomedical Applications		272/1	272/2	273/1	273/2	274	275/1	275/2	276/1	276/2	277	278/1	278/2

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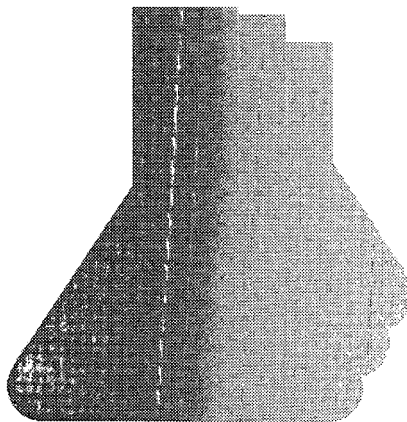
ANALYTICAL METHODS AND PROBLEMS IN BIOTECHNOLOGY

AN INTERNATIONAL SYMPOSIUM

17–19 April, 1984, Leeuwenhorst Congress Center, Noordwijkerhout, The Netherlands

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