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PROFILES OF STRONGLY POLAR AND LESS POLAR ACIDS OBTAINED FROM HUMAN BLOOD, PLASMA AND SERUM BY TWO-STEP ULTRAFILTRATION

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SUMMARY

A two-step ultrafiltration method combined with anion-exchange chromatography is described for the separation of lipophilic and strongly lipophobic acids occurring in human blood, serum and plasma. After treatment with diazomethane, the acid fractions are separated further by gas chromatography. The acids were identified by their mass spectra. Profiles obtained from the strongly acidic fraction of blood samples of healthy individuals are characterized by main peaks corresponding to organic phosphoric acid esters. The peaks are absent in plasma and serum.

INTRODUCTION

Organic acids are obtained from biological fluids either by extraction [1—5] or anion-exchange chromatography [6—11]. Highly polar acids, e.g. amino acids or organic phosphoric acid esters, are lost due to their high water solubility if extraction methods are applied.

These polar acids can be adsorbed on anion-exchange columns. They are recovered by washing the columns with strong acids. This procedure requires the removal of accompanying high-molecular-weight compounds (mainly proteins) which easily clog the columns. The necessary preseparation can be done by precipitation [11]. This procedure is therefore more time-consuming than the extraction procedure and suffers sometimes by the fact that acids are coprecipitated. Therefore a better method for removing the interfering proteins is required.

Recently Issachar and co-workers [6, 7] described the profiling of organic acids obtained from human plasma using ultrafiltration. Although the

reproduced profiles are of excellent quality, only a limited number of compounds could be structurally identified by mass spectrometry. It seemed to us a challenging task to identify the remaining unknown compounds.

Structure elucidation of unknown compounds requires the separation of larger samples than used by Issachar and co-workers [6, 7]. When we repeated the separation technique used by Issachar and co-workers [6, 7], the microporous filter membrane became very clogged from the start of filtration. Almost no filtrate was obtained. Therefore we tried to remove the interfering cell wall components and proteins by ultrafiltration using a filter with rather large pores (pore size 0.45 μm) followed by a second ultrafiltration with a filter having small pores (exclusion volume MW 10,000). With this method no difficulties were encountered in obtaining an ultrafiltrate. The second ultrafiltrate was subjected to adsorption onto an anion-exchange column as described by Issachar and co-workers [6, 7].

To improve the filtration and to avoid the various rather time-consuming separation steps, ultrafiltration and anion-exchange chromatography were combined by using an integrated system. This system could separate acids not only from plasma and serum but also from whole blood, thus avoiding the preparation of plasma or serum.

Highly polar and weakly polar acids could easily be separated. They were identified after derivatization with diazomethane by mass spectrometry. The results of these investigations are reported in this paper.

EXPERIMENTAL

Materials

Freshly drawn human blood was heparinized and stored at −40° C until used. Blood samples of individuals who died by accident or suicide were drawn 1 h to two days after death. Human plasma and serum were obtained from the hospitals of Bayreuth and Marktredwitz.

Two-step ultrafiltration

A schematic diagram of the ultrafiltration system is shown in Fig. 1. The system comprised the following components: stock vessel (200 ml) (a), filter cassette (c) with a polysulphone membrane filter HVLP (pore size 0.45 μm; Millipore, Neu-Isenburg, F.R.G.), filter cassette (d) with a polysulphone

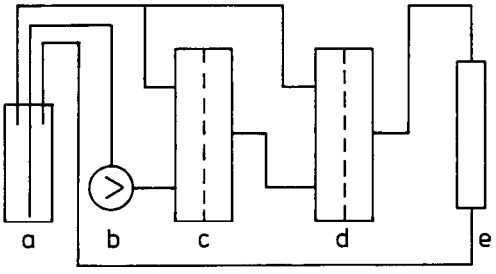


Fig. 1. Schematic diagram of the ultrafiltration system. a = stock vessel, b = toothed wheel pump, c and d = filter cassettes, e = glass column with Dowex 1-X8.

membrane filter PTGC (exclusion volume MW 10,000; Millipore), glass column (e) packed with 500 g of Dowex 1-X8 (mesh size 50—100, counter-ion Cl^-), toothed wheel pump (b) (2600 U/min; Multifix, Schwäbisch-Gmünd, F.R.G.). The components were connected by PTFE tubes (I.D. 0.8 cm).

Filtration conditions were as follows: entrance pressure at the HVLP filter, 3 bars, back-pressure at the HVLP filter, 2 bars; entrance pressure at the PTGC filter, 0.5 bar; back-pressure at the PTGC filter, 0 bar.

Procedure

To prepare the apparatus for filtration it was rinsed with 100 l of distilled water, activated with 10 l of physiological sodium chloride solution [12] and washed again with 50 l of distilled water (time: 30 min).

For filtration, 50 ml of the biological fluid (human blood, serum or plasma) are poured into the stock vessel; then 150 ml of distilled water are added to fill up the stock vessel (a). The stock vessel is connected to the filtration apparatus and to the outlet of the anion-exchange column (see Fig. 1). Then the toothed wheel pump is started and the filtration conditions are adjusted.

After the filtration (about 2 h) the anion-exchange column is disconnected and the remaining system rinsed with 800 ml of distilled water. These washings contain the less polar acids (acid fraction B).

The filtration apparatus can be cleaned by pumping through 20 l of physiological sodium chloride solution followed by 20 l of 0.1 *M* sodium hydroxide solution. After standing for 30 min with 0.1 *M* sodium hydroxide the apparatus is washed with 100 l of distilled water.

When not in use filters were stored in 0.1 *M* sodium azide solution [12].

Isolation of the fraction of polar acids

The anion-exchange column is washed with 1 l of distilled water. Then the adsorbed highly polar acids (fraction A) are eluted by treating the column with 2.5 l of 0.1 *M* methanolic hydrochloric acid. The eluate is evaporated on a water bath (40° C) to dryness. The residue is dissolved in 5 ml of methanol, ethereal diazomethane solution is added until the colour remains yellow and the solution is concentrated to 1 ml; 1 μl of this solution is used for gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis.

Isolation of the fraction of less polar acids

The washings of the filter apparatus containing the less polar acid fraction B are acidified to pH 1 by dropwise addition of concentrated hydrochloric acid. The solution is extracted three times with 600 ml of ethyl acetate each time. The combined ethyl acetate extracts are dried over sodium sulphate. The solvent is evaporated to dryness. The residue is treated as described for fraction A to prepare samples for GC and GC—MS analysis.

Instrumentation for GC—MS

The GC apparatus and conditions were as follows: Carlo-Erba gas chromatograph 4160; hydrogen flow-rate, 2 ml/min (OV-101); helium flow-rate, 3 ml/min (OV-1701); 30-m thin-film glass capillary coated with OV-101 or 50-m

thin-film quartz capillary Durabond OV-1701 (J & W Scientific); injector temperature, 270°C; oven temperature, 80—300°C (OV-101) or 70—270°C (OV-1701); temperature programme, 2°C/min; flame-ionization detector.

For MS measurements a Varian MAT 312 mass spectrometer was used with electron-impact ion source at an electron energy of 70 eV. The mass spectrometer was combined with a Varian 3700 gas chromatograph. The columns and temperature programme were identical to those described above. The instrument was combined with an SS 200 data system, using a PDP 11/34 computer.

High-resolution data were obtained with the same instrument.

^{1}H-NMR measurements were made with a Bruker WM 250 instrument.

Conditions for preparative GC were: Carlo-Erba Fractovap 2400 T; carrier gas, nitrogen; column, 1.5 m × 6 mm I.D., 3% OV-17 on Supelcoport 100—120; injector temperature, 250°C; oven temperature, 100—270°C; temperature programme, 2°C/min; detector temperature, 270°C; flame-ionization detector.

Reference compounds

The reference samples, phosphoenolpyruvic acid monopotassium salt, 2-phosphoglyceric acid sodium salt, 3-phosphoglyceric acid sodium salt and 2,3-diphosphoglyceric acid tris sodium salt, were obtained from Sigma (Munich, F.R.G.). These substances were transformed into the free acids by anion-exchange chromatography (see isolation of polar acids).

The free acids were transformed into their methyl esters by treatment with ethereal diazomethane solution.

Trimethylsilylated derivatives were obtained by evaporating the eluate from the anion-exchange column to dryness, treatment of the residue with an excess of MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) and heating the mixture in a fused tube for 18 h at 60°C. Methyl esters and trimethylsilyl esters were analysed by GC and GC—MS.

^{1}H-NMR and MS data of the compounds isolated from blood samples by preparative gas chromatography

1-Methylpyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.94 (s, 3H, $COOCH_3$), 4.00 (s, 3H, $N—CH_3$), 6.82 (d, J = 2 Hz, N—CH=CH), 7.4 (d, J = 2 Hz, N—CH=CH) ppm. MS: 140 (M^+, 25%), 110 (19), 109 (100), 82 (18).

Pyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.94 (s, 3H, $COOCH_3$), 6.89 (d, J = 2 Hz, 1H, N—CH=CH), 7.65 (d, J = 2 Hz, 1H, N—CH=CH) ppm. MS: 126 (M^+, 80%), 95 (100), 81 (2), 71 (2), 68 (6), 66 (5), 59 (3).

2-Methylpyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.89 (s, 3H, $COOCH_3$), 4.19 (s, 3H, $N—CH_3$), 6.84 (d, J = 2 Hz, 1H, N—CH=CH), 7.47 (d, J = 2 Hz, 1H, N—CH=CH) ppm. MS: 140 (M^+, 25%), 110 (19), 109 (100), 82 (18).

Phosphoenolpyruvic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.86 (d, J = 4 Hz, 6H, $P—OCH_3$), 3.92 (s, 3H, $COOCH_3$), 5.65 (dd, J = 2.2 Hz, J = 2.6 Hz, 1H, H_2C=), 5.98 (dd, J = 2.2 Hz, J = 2.6 Hz, 1H, H_2C=) ppm. MS: 210 (M^+, 18%), 179 (20), 151 (12), 150 (8), 141 (7), 139 (6), 127 (57), 109 (100), 96 (6), 79 (13).

1-Phosphocyclopropane-1-carboxylic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.80 (d, J = 4 Hz, 6H, P—OCH_3), 3.86 (s, 3H, COOCH_3), 1.50 (m, 4H, CH_2—CH_2) ppm. MS: 224 (M^+, 3%), 209 (4), 193 (19), 192 (84), 165 (19), 164 (10), 136 (16), 127 (18), 113 (9), 109 (100), 93 (11), 79 (16), 59 (8), 55 (76).

E,Z-3-Methylphosphoenolpyruvic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 1.90 (dd, J = 7.3 Hz, J = 2.8 Hz, 3H, CH_3—C=C), 2.08 (dd, J = 7.3 Hz, J = 2.8 Hz, 3H, CH_3—C=C), 3.83 (d, J = 4 Hz, 6H, P—OCH_3), 3.93 (s, 3H, COOCH_3), 6.26 (m, 1H, H—C=C), 6.57 (m, 1H, H—C=C) ppm. MS: 224 (M^+, 11%), 193 (15), 192 (84), 165 (6), 164 (8), 136 (19), 127 (25), 113 (8), 109 (82), 93 (11), 79 (15), 59 (5), 55 (100).

RESULTS

Two-step ultrafiltration

The difficulties encountered in the ultrafiltration of volumes larger than a few millilitres of biological liquid are caused by the presence of high-molecular-weight particles, e.g. proteins and also tissue components, clogging the microporous filter. Therefore we tried to remove proteins of high molecular weight first using a macroporous filter (pore size 0.45 μm). After that filtration step we removed proteins with a molecular weight larger than 10,000 in a second step. This stepwise filtration is rather time-consuming. Therefore we combined the two filtration processes and anion-exchange chromatography and enhanced the filtration procedure by applying a toothed wheel pump. An advantage of the method is the possibility of separating large quantities of biological liquids. The apparatus described in the experimental part is able to filter 20 l of human blood within 10 h if used in a continuous manner only by adjustment of the filtration conditions described in the experimental part. The filtration can be applied equally well to serum, plasma and blood.

A disadvantage of the method is the destruction of blood cells in the apparatus, rendering the addition of physiological sodium chloride solution useless. Therefore we used water to dilute the blood samples. This allowed the analysis of blood cell components as well.

The polar acids in ultrafiltrates from patients suffering from uraemia can also be isolated with this method.

The efficiency of the two-step ultrafiltration method using polysulphone membrane filters was checked by filtration of synthetic mixtures of organic acids with different polarities. The amounts of acids retained and passed were determined by preparing methyl derivatives of the acids followed by analysis of the mixture by GC. The results of these measurements are given in Table I.

Monocarboxylic acids with more than seven carbon atoms are not able to pass through the filters, nor are dicarboxylic acids with more than twelve carbon atoms. Dicarboxylic acids up to a carbon number of 12 are partly able to pass through the filters, the percentage decreasing with increasing carbon number. Aromatic acids like benzoic acid may pass through the filters, while the less polar urofuranic acids are mainly retained. Thus separation into a fraction A of highly polar acids and a less polar acid fraction B is achieved.

TABLE I

AMOUNTS OF ACIDS PASSED THROUGH HVLP AND PTGC MEMBRANE FILTERS

Acid	Percentage of acid passed through filters
Heptanoic acid up to undecanoic acid	0
Palmitic acid	0
Stearic acid	0
Oleic acid	0
Tetracosanoic acid	0
Undecenoic acid	0
Succinic acid	71
2-Methylsuccinic acid	67
Glutaric acid	65
3,3-Dimethylglutaric acid	63
Adipic acid	63
3-Methyladipic acid	59
Heptanedioic acid	59
Octanedioic acid	55
Nonanedioic acid	44
Decanedioic acid	27
Dodecanedioic acid	<1
Benzoic acid	100
Phenylacetic acid	100
Phenylpropionic acid	50
Phenylbutanoic acid	23
Propionylurofuranoic acid	65
Valeroylurofuranoic acid	45
Pentylurofuranoic acid	<1
Pentenylurofuranoic acid	2
Propenylurofuranoic acid	7

Separation of fractions A and B

The gas chromatograms of polar and less polar acid fractions of blood, serum and plasma, after derivatisation with diazomethane, are reproduced in Figs. 2—5. The compounds identified in the chromatograms are numbered and listed in Tables II and III. Saturated hydrocarbons were added to determine retention indices [13]. They are not numbered and not listed in the tables. Mass spectral data are given only if they have not previously been published, otherwise the literature is cited.

Since we were not able to identify many of the compounds of the highly polar acid fraction from their mass spectra, 2 l of pig's blood were worked up as described for human blood. The profiles looked rather similar to those of human blood. Peaks 13, 23, 27, 32, 37, 40 and 41 could be collected by preparative GC.

High-resolution mass measurements (peak matching) and the analysis of the ^{1}H-NMR spectra allowed structure determination. A commercial sample of phosphoenolpyruvic acid available as sodium salt was converted to the free acid by anion-exchange chromatography and treated with diazomethane to prepare the methylate. The gas chromatogram of the reaction product showed several peaks. The mass spectra of these peaks were identical with those of compounds

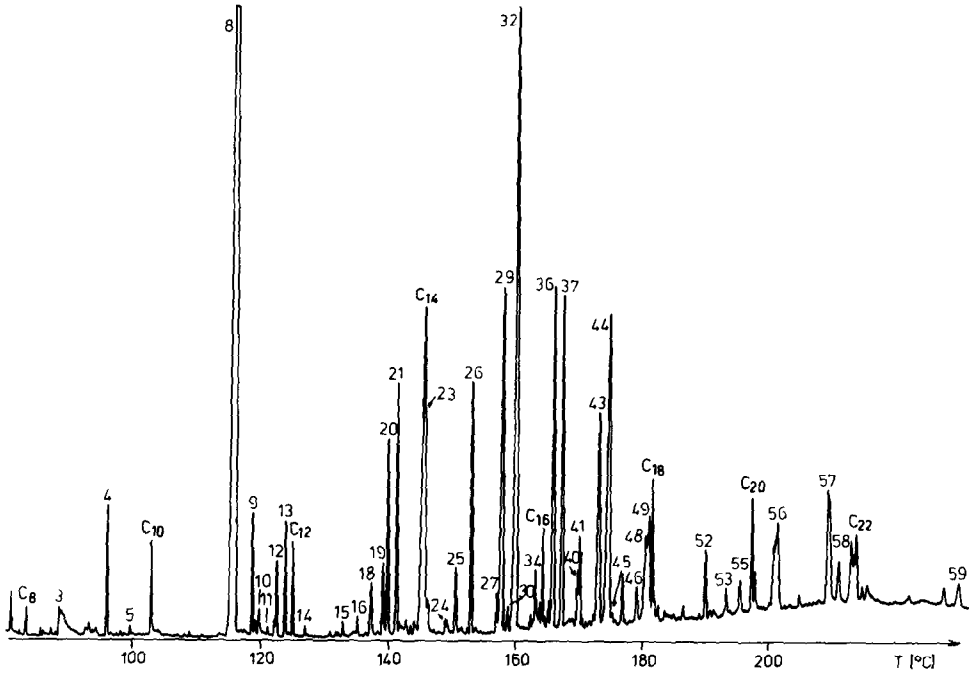


Fig. 2. Gas chromatogram of the strongly polar methylated acids (fraction A) obtained from the blood of a healthy male individual aged 20 years.

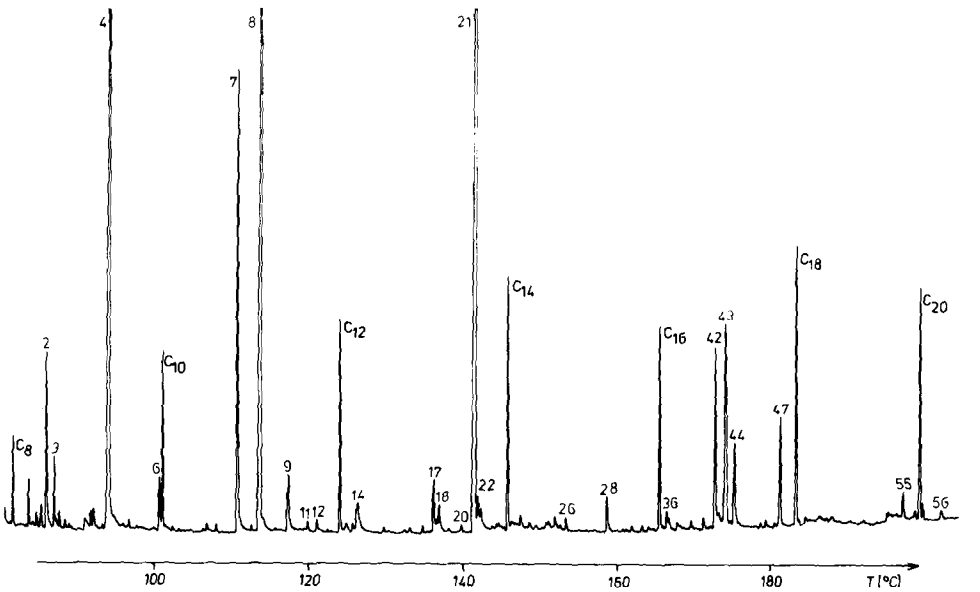


Fig. 3. Gas chromatogram of fraction A obtained from blood plasma.

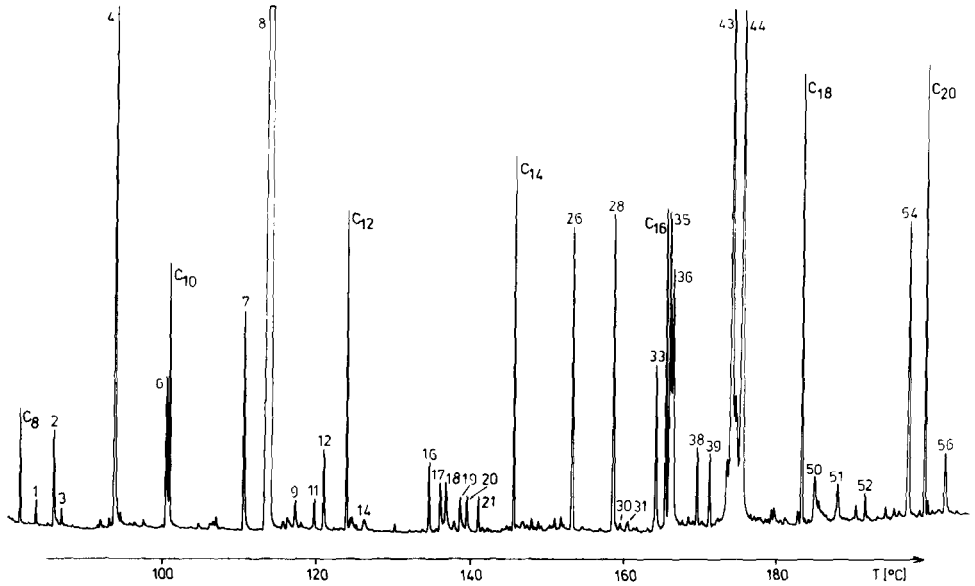


Fig. 4. Gas chromatogram of fraction A obtained from blood serum.

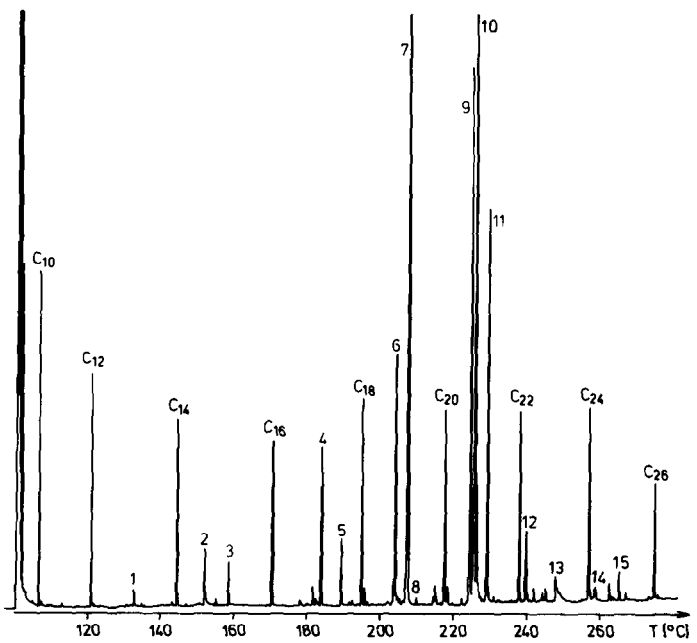


Fig. 5. Gas chromatogram of the less polar methylated acids of blood fraction B of a healthy individual.

13, 23, 27, 32, 37, 40 and 41, isolated by preparative GC. Therefore they were obviously produced by reaction of diazomethane with phosphoenolpyruvic acid in the way shown in Fig. 6 [24–30].

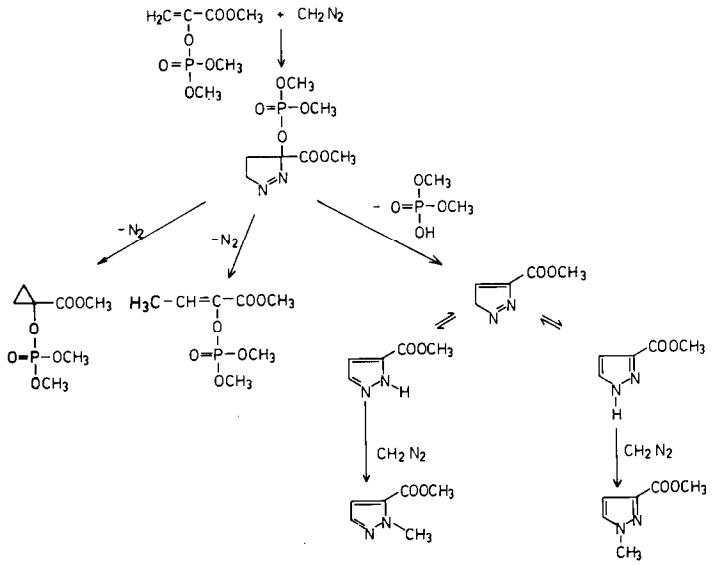
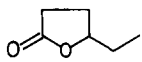
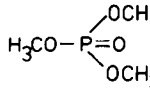


Fig. 6. Reaction of diazomethane with phosphoenolpyruvic acid.

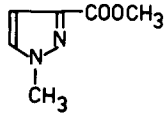
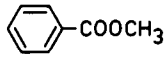
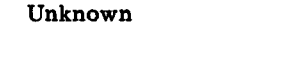
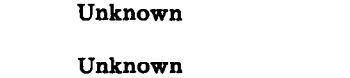
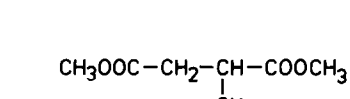
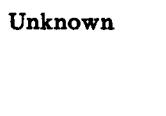
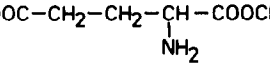
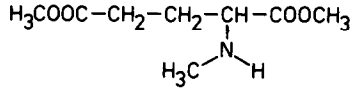
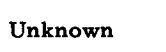
TABLE II

STRONGLY POLAR METHYLATED ACIDS IDENTIFIED IN FIGS. 2—4

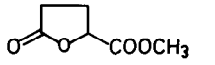
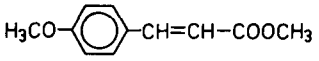
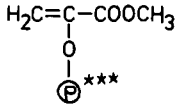
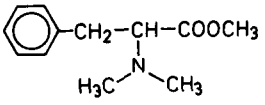
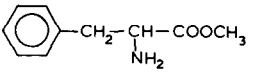
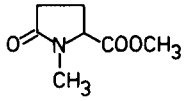
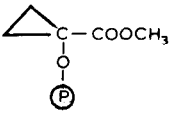
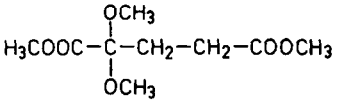
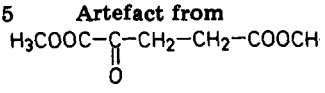
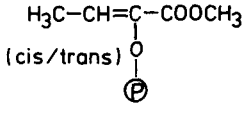
Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
1	835	$H_3C-CH=CH-COOCH_3$	[14]**	
2	845	$H_3C-\underset{\underset{OH}{|}}{CH}-COOCH_3$	[15]	
3	853	$H_3C-\underset{\underset{O}{||}}{C}-COOCH_3$	[16]	
4	927	Unknown	89 (70%), 74 (33), 59 (24) 58 (100), 44 (25)	
5	967	$H_3COOC-COOCH_3$	[15]	
6	996	$H_3C-\underset{\underset{OH}{|}}{CH}-CH_2-COOCH_3$	[17]	
7	1084		[18]	
8	1118		[16]	
9	1144	$H_3COOC-CH=CH-COOCH_3$ (trans)	[15]	
10	1153	Unknown	104 (52%), 89 (14), 75 (100), 72 (8), 71 (4), 59 (7), 55 (6), 47 (2), 45 (38), 43 (42)	
11	1167	$H_3COOC-CH_2-CH_2-COOCH_3$	[16]	
12	1177	Unknown	153 (4%), 139 (6), 127 (100), 110 (22), 109 (44), 103 (2), 96 (18), 95 (20), 79 (8), 66 (4), 65 (4), 47 (4), 43 (5)	

(Continued on p. 26)

TABLE II (continued)

Compound No.*	Retention index	Structure	Key ions (m/z)	Remarks
13	1191		140 (M^+, 25%), 110 (19), 109 (100), 82 (18)	Artefact from compound no. **32**
14	1220		[16]	
15	1269	**Unknown**	139 (2%), 129 (3), 117 (64), 110 (6), 101 (5), 85 (44), 75 (8), 74 (5), 64 (3), 59 (10), 58 (6), 43 (100)	
16	1290	**Unknown**	157 (3%), 98 (100), 70 (5), 59 (3), 55 (3), 42 (8), 41 (17)	
17	1308	**Unknown**	150 (2%), 117 (3), 102 (58), 91 (10), 87 (24), 59 (12), 58 (68), 55 (12), 45 (100), 43 (11)	
18	1321		[16]	
19	1336	**Unknown**	147 (8%), 103 (60), 100 (21), 88 (100), 87 (46), 74 (26), 71 (86), 61 (28), 59 (42), 57 (22), 44 (56), 43 (62)	
20	1347		[19]	
21	1362		[19]	Artefact from compound No. **20**
22	1368	**Unknown**	175 (1%), 161 (5), 129 (7), 116 (12), 102 (84), 88 (6), 74 (42), 58 (4), 45 (22), 44 (21), 43 (18), 42 (100)	
23	1400		126 (M^+, 80%), 110 (2), 95 (100), 81 (2), 71 (2), 68 (6), 66 (5), 59 (3)	Artefact from compound No. **32**
24	1437		[16]	
25	1456	**Unknown**	128 (62%), 113 (12), 100 (10), 85 (54), 69 (8), 59 (12), 57 (32), 43 (100)	
26	1480		[19]	Artefact from compound No. **20**
27	1521		140 (M^+, 25%), 110 (19), 109 (100), 82 (18)	Artefact from compound No. **32**
28	1531		188 (M^+, 2%), 173 (3), 157 (5), 141 (10), 129 (40), 128 (22), 125 (30), 115 (98), 113 (10), 101 (12), 97 (20), 87 (20), 69 (22), 59 (55), 45 (100)	Artefact from 

ABLE II (continued)

mpound).*	Retention index	Structure	Key ions (m/z)	Remarks
)	1533	Unknown	198 (12%), 167 (20), 166 (28), 154 (7), 139 (82), 127 (20), 124 (98), 109 (100), 93 (14), 87 (10), 79 (32), 59 (7), 47 (10), 45 (22), 42 (11)	
)	1540		144 (M^+, 4%), 85 (100), 59 (3), 57 (5), 55 (2), 41 (2)	
	1550		[20]	
!	1553		210 (M^+, 18%), 179 (20), 151 (12), 150 (8), 141 (7), 139 (6), 127 (57), 109 (100), 96 (6), 79 (13)	
	1590	Unknown	134 (36%), 119 (8), 102 (100), 91 (6), 77 (3), 67 (4), 42 (36)	
	1592	Unknown	168 (2%), 140 (10), 112 (11), 111 (17), 97 (40), 84 (39), 83 (66), 70 (68), 69 (76), 57 (64), 56 (80), 55 (99), 43 (100), 41 (70)	
	1605		207 (M^+, 1%), 148 (38), 133 (10), 116 (100), 105 (5), 91 (9), 88 (11), 84 (6), 77 (5), 56 (10), 42 (16)	Artefact from 
	1620		[21]	Artefact from compound No. 44
	1635		224 (M^+, 3%), 209 (4), 193 (19), 192 (85), 165 (19), 164 (10), 136 (16), 127 (18), 113 (9), 109 (100), 93 (11), 79 (16), 59 (8), 55 (76)	Artefact from compound No. 32
	1646		202 (M^+, 1%), 171 (5), 155 (16), 152 (18), 143 (40), 129 (26), 128 (32), 115 (54), 111 (33), 101 (26), 97 (22), 83 (50), 59 (88), 55 (100), 45 (94), 42 (60)	Artefact from 
	1664	Unknown	174 (10%), 163 (20), 160 (12), 131 (60), 128 (100), 127 (59), 115 (12), 105 (20), 103 (22), 91 (96), 77 (22), 65 (26), 59 (6), 55 (12), 51 (21), 42 (82)	
/41	1665/70		224 (M^+, 11%), 193 (15), 192 (84), 165 (6), 164 (8), 136 (19), 127 (25), 113 (8), 109 (82), 93 (11), 79 (15), 59 (5), 55 (100)	Artefact from compound No. 32

(Continued on p. 28)

TABLE II (continued)

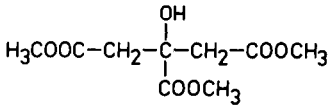
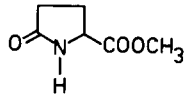
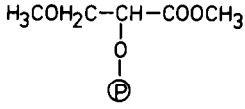
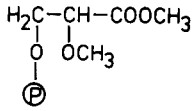
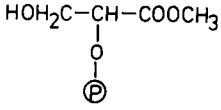
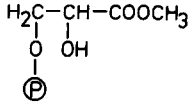
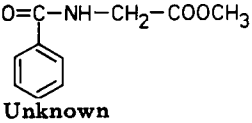
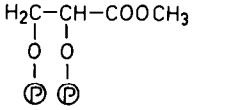
Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
42	1682	Unknown	223 (3%), 187 (10), 174 (10), 166 (24), 164 (80), 138 (6), 136 (23), 128 (11), 106 (23), 102 (10), 74 (5), 63 (8), 59 (7), 45 (100), 42 (50)	
43	1702		[16]	
44	1719		[21]	
45	1730		212 ($M^+ - 30$, 8%), 183 (19), 166 (4), 151 (4), 141 (22), 127 (35), 116 (11), 109 (30), 87 (28), 85 (25), 79 (8), 59 (3), 55 (8), 45 (100)	Artefact from compound No. 48
46	1776		212 ($M^+ - 30$, 4%), 183 (100), 151 (12), 141 (8), 139 (4), 127 (34), 116 (8), 109 (30), 87 (4), 79 (5), 75 (4), 58 (8), 57 (4), 45 (3)	Artefact from compound No. 49
47	1777	Unknown	233 (6), 202 (5), 174 (76), 146 (16), 143 (18), 116 (100), 103 (40), 88 (32), 84 (8), 75 (20), 58 (18), 57 (19), 45 (56), 42 (44)	
48	1791		198 ($M^+ - 30$, 5%), 169 (59), 166 (10), 140 (23), 137 (100), 127 (45), 113 (15), 110 (17), 109 (79), 102 (15), 96 (27), 95 (13), 79 (46)	
49	1779		198 ($M^+ - 30$, 4%), 169 (47), 166 (8), 140 (16), 137 (100), 127 (30), 113 (10), 110 (11), 109 (58), 102 (8), 96 (18), 95 (8), 79 (44)	
50	1818	Unknown	146 (2%), 128 (4), 114 (6), 89 (60), 74 (24), 59 (22), 58 (100), 57 (26), 44 (14), 42 (16)	
51	1857	Unknown	200 (18%), 169 (5), 168 (3), 143 (5), 141 (12), 140 (44), 127 (31), 115 (6), 83 (5), 72 (4), 59 (5), 56 (28), 55 (31), 43 (8), 42 (100)	
52	1908	Unknown	209 (2%), 207 (7), 201 (5), 199 (6), 161 (8), 133 (6), 119 (100), 105 (2), 83 (3), 74 (8), 67 (5), 59 (45), 53 (4), 47 (10), 43 (67)	
53	1951	Unknown	197 (12%), 176 (28), 174 (96), 165 (6), 157 (8), 153 (10), 138 (7), 126 (9), 119 (20), 117 (66), 109 (36), 91 (8), 89 (30), 76 (12), 74 (7), 58 (8), 52 (100)	

TABLE II (continued)

Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
54	1972	Unknown	214 (10%), 183 (17), 182 (47), 154 (10), 141 (24), 140 (62), 127 (21), 114 (4), 98 (24), 69 (12), 56 (22), 55 (15), 42 (100)	
55	1977	Unknown	269 (1%), 149 (100), 122 (6), 95 (4), 85 (4), 75 (5), 68 (6), 53 (3), 42 (9)	
56	2049		[16]	
57	2145	Unknown	260 (2%), 229 (2), 218 (2), 196 (7), 169 (22), 140 (12), 127 (100), 117 (7), 110 (10), 109 (20), 100 (38), 97 (26), 87 (27), 75 (22), 71 (56), 68 (23), 45 (14), 41 (12)	
58	2188	Unknown	248 (6%), 246 (10), 177 (8), 169 (9), 140 (6), 127 (27), 116 (100), 111 (10), 100 (22), 88 (8), 87 (6), 71 (14), 68 (7), 56 (5), 45 (6), 42 (9)	
59	2348		336 (M^+, 1%), 306 (6), 305 (8), 277 (58), 249 (18), 235 (39), 210 (29), 203 (16), 179 (34), 167 (14), 166 (16), 152 (25), 151 (46), 141 (22), 139 (16), 127 (37), 113 (16), 109 (100), 96 (24), 93 (9), 79 (18)	

*Refers to numbered peaks in Figs. 2—4.
**Literature references are quoted in square brackets.
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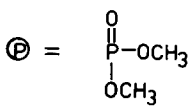
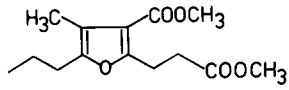
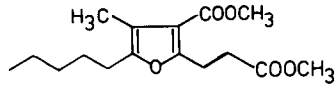
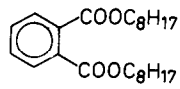


Comparison of the mass spectra and retention indices obtained after diazomethane treatment of other commercially available glycolysis products (see experimental part) allowed the identification of compounds 45, 46, 48, 49 and 59. Phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate and 2,3-diphosphoglycerate are therefore the main compounds found in the acid fraction A of human blood. They are absent in plasma and serum profiles as expected, since they are typical glycolysis products which occur in the erythrocytes.

The composition of the less polar acid fraction B was nearly identical for blood, serum and plasma (see Fig. 5).

TABLE III

LESS POLAR METHYLATED ACIDS IDENTIFIED IN FIG. 5.

Compound No.*	Retention index	Structure	Key ions (*m/z*)
1	1310	$H_3C-(CH_2)_8-COOCH_3$	[22]**
2	1459	Unknown	140 (12%), 125 (6), 112 (13), 111 (26), 98 (27), 97 (48), 84 (45), 83 (80), 82 (34), 70 (82), 69 (88), 68 (34), 57 (72), 56 (90), 55 (100), 45 (7), 43 (97), 41 (67)
3	1510	$H_3C-(CH_2)_{10}-COOCH_3$	[22]
4	1710	$H_3C-(CH_2)_{12}-COOCH_3$	[22]
5	1755		[23]
6	1884	$C_{15}H_{29}-COOCH_3$	[22]
7	1910	$H_3C-(CH_2)_{14}-COOCH_3$	[22]
8	1937		[23]
9	2072	$C_{17}H_{31}-COOCH_3$	[22]
10	2081	$C_{17}H_{33}-COOCH_3$	[22]
11	2110	$H_3C-(CH_2)_{16}-COOCH_3$	[22]
12	2227	$C_{19}H_{31}-COOCH_3$	[22]
13	2308	Unknown	154 (1%), 140 (2), 128 (2), 126 (6), 114 (4), 112 (5), 98 (6), 97 (5), 86 (5), 83 (7), 72 (58), 69 (10), 67 (5), 59 (100), 55 (21), 44 (8), 43 (18), 41 (20)
14	2418	Unknown	239 (8%), 221 (4), 166 (5), 161 (3), 159 (5), 148 (10), 147 (10), 145 (10), 133 (16), 131 (14), 119 (34), 117 (24), 108 (40), 105 (40), 93 (44), 91 (70), 79 (100), 77 (24), 74 (20), 67 (61), 57 (31), 45 (21), 43 (36), 41 (69)
15	2494		[22]

*Refers to the numbered peaks in Fig. 5.

**Literature references are given in square brackets.

DISCUSSION

Clogging of filters is one of the main difficulties encountered in the one-step ultrafiltration of serum and plasma introduced by Issachar and co-workers [6, 7]. This can be circumvented by use of the two-step ultrafiltration described in this paper. Filters and anion-exchange column can be combined into an almost automatical system able to work up serum and plasma on a scale between 5 ml and 20 l, in one day if commercially available filter cassettes are used with the appropriate volumes.

Issachar and co-workers [6, 7] recommended the addition of oleic acid to serum before ultrafiltration to replace carboxylic acids, particularly fatty acids, bound to albumin. Since albumin and consequently also albumin-bound carboxylic acids can not pass through our filters, as well as fatty acids (see Table I), the addition of oleic acid does not improve the yield of less polar acids. To determine if the yield of polar acids could be increased by addition of oleic acid, we added oleic acid to serum and blood in different amounts. The results of the analysis demonstrated no effect at all. Therefore the addition of oleic acid is useless and even causes interference if later the less polar acid fraction is extracted, since in large amounts it renders the determination of low amounts of other less polar acids more difficult. Addition of oleic acid is therefore not advisable.

The two-step ultrafiltration offers the possibility of analysing blood without the time-consuming and costly preparation of plasma or serum. The separation of blood compounds into polar and less polar acid fractions is a major advantage in the analysis of acids occurring in blood, not previously possible.

But the method still suffers from the difficulty of analysing the highly polar acid fraction quantitatively. The fraction obtained requires further separation. This is usually achieved by GC after appropriate derivatization, allowing the quantitative determination of single components. In the case of phosphoric acid derivatives, the necessary derivatization by methylation or trimethylsilylation [31] causes the production of several products, rendering quantitative measurement by summation of the peak heights of the corresponding peaks difficult.

Since the phosphoric acid derivatives do not show ultraviolet absorption their detection is also difficult. A possibility just for analysis of phosphoric acid derivatives may be hydrolysis after separation of the components and determination of phosphoric acid by reaction with molybdate, as shown by Bessman et al. [11]. But this method would exclude the analysis of all other polar components (e.g. amino acids).

In spite of these difficulties at least a semiquantitative analysis can be achieved by glass capillary GC. Since very often great changes in the composition of biological fluids are observed, if the metabolism of the individual is changed this semiquantitative analysis is sufficient to detect such events.

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