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Organic acid profiling by direct treatment of deproteinized plasma with ethyl chloroformate

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Abstract

Carboxylic acids in plasma or serum can be conveniently determined by capillary gas chromatography (GC) following treatment with ethyl chloroformate (ECF). The mixed organic solvent used for plasma deproteinization is also suitable as medium for the subsequent reaction step. Thus, isolation of the compounds of interest is not necessary. Before treatment of the supernatant with ECF, the neutral lipids and amino acids are removed easily by hexane extraction and cation-exchange chromatography. Ketocarboxylic acids do not require a preliminary oximation. Capillary columns with a length of 15 m and a polar silicone phase proved to be ideal for the separation of mixtures of derivatized keto-, hydroxy-, mono- and dicarboxylic acids. The run time is less than 30 min.

1. Introduction

At present detection of organic acids as metabolic products in body fluids, so called "metabolic profiling", can be performed at various medical centers and clinical laboratories [1]. As far as the analysis is concerned, the following three steps must be carried out: isolation, derivatization and separation of the compounds of interest.

There is no doubt that capillary GC in combination with mass spectrometry (GC–MS) is the most efficient tool for separating complex samples and identifying the individual analytes [2–4]. Concomitantly, problems arise in the isolation and derivatization steps. Quantification of organic acids is notoriously inaccurate, largely due to the inconsistent efficiency of both the solvent and sorbent extraction [1–4]. Moreover, the widely used derivatization procedures, silylation [3] or methylation [5], suffer from some disadvantages, *e.g.* the need to use completely dry samples in the former, and the occurrence of undesired side-reactions in the latter. Both procedures require a long reaction time.

Recently, conditions for the esterification of carboxylic acids with alkyl chloroformates have been developed [6]. The reaction proceeds instantaneously following addition of the reagent to an aqueous medium. The successful derivatization of various classes of carboxylic acids, which also occur as acidic metabolites in body fluids, in an aqueous environment prompted the present application since we were interested in the possibility to avoid the isolation step and to treat the plasma supernatant directly. The idea to use the same medium for deproteinization and

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subsequent derivatization seemed to be especially interesting.

The present study highlights the advantages of this novel approach that markedly facilitates the process of blood profile monitoring.

2. Experimental

2.1. Materials

Ethyl chloroformate (ECF), organic solvents of the highest available grade and all other chemicals, including the standards of carboxylic acids (see Table 1), were obtained from Sigma-Aldrich (Prague, Czech Republic). Both absolute or 96% ethanol can be used. Equimolar solutions of the acid standards were prepared in water; a 50 m*M* solution of aqueous bicarbonate was used for the dicarboxylic acids; and acetonitrile–chloroform was used for the long-chain fatty acids) at concentrations of 10 nmol/μl for the acids and the dicarboxylic acids, and 5 nmol/μl for the fatty acids. Internal standard solution was prepared by dissolving malonic acid (20 μmol/ml) in water. The sodium carbonate–bicarbonate solution (approx. 1 *M*) was prepared weekly by dissolving 0.5 g of carbonate and 1 g of bicarbonate in 20 ml water. The anticoagulant solution consisted of 150 m*M* EDTA-Na_2 and 5 m*M* sodium bisulphite in water.

Reaction vials, 2-ml volume, with rounded bottom (made from 10 mm O.D. glass tubes with 10/14 mm ground joints) were silanized with a 10% solution of dichlorodimethylsilane in hexane (30 min at room temperature) and washed with denatured alcohol, water and acetone before use. Silanization was repeated approximately once a month when loss of surface hydrophobicity during sample extraction became apparent.

Cation-exchange columns were made from 1.5-ml tips of push-button pipettes by placing glass wool together with 10–15 mg of dry AG 50W-X8 resin (100–200 mesh) into the conus. The exchanger was reactivated by flushing with 1 *M* HCl (0.5 ml) followed by the same amount of water and acetonitrile.

A plasma pool from blood of 5 volunteers (aged 25–45 years) was prepared by collecting blood samples of 5 ml into chilled tubes containing 0.1 ml of the anticoagulant solution mentioned above. After centrifugation (1000 *g*, 10 min) the pooled plasma was processed immediately or stored at −20°C.

2.2. Apparatus

A GC 14A gas chromatograph (Shimadzu, Austria) with a flame ionization detector and a split/splitless injector connected with a Shimadzu Chromatopac CR1B integrator were used. The injector and detector temperatures were set at 220°C and 240°C, respectively. A fused-silica capillary 15 m × 0.25 mm I.D. of DB-225 type (0.25-μm film thickness of 50% cyanopropylmethyl–50% phenylmethyl silicone; J and W Scientific, Folsom, CA, USA) was used. The temperature was programmed from 60°C (2 min hold) at 8°/min to 220°C (5 min hold). Hydrogen was used as the gas phase at a head pressure of 50 kPa (the make-up gas was nitrogen at the same pressure). The splitting ratio was 1:20, and the attenuation × 32.

2.3. Procedure

See Fig. 1 for scheme of sample work-up. To 0.4 ml of acetonitrile and 0.2 ml of ethanol were added 2 μl of I.S. solution (containing 40 nmol of malonic acid) and 0.2 ml of plasma. The mixture was shaken for 1 min tp precipitate the proteins and extract the carboxylic acids. After centrifugation at 2000 *g* for 10 min, the supernatant (0.6–0.7 ml) was transferred into another tube, alkalinized to pH > 8 by addition of 2 *M* NaOH (2–3 μl) and extracted two times with 0.5 ml of *n*-hexane. The hexane layer was aspirated off and 500 μl of the aqueous phase were pushed slowly through the cation-exchange column till air bubbles occurred at the exchanger bed. To the acidified supernatant (pH < 3) in the silanized glass tube 20 μl ECF and 40 μl pyridine

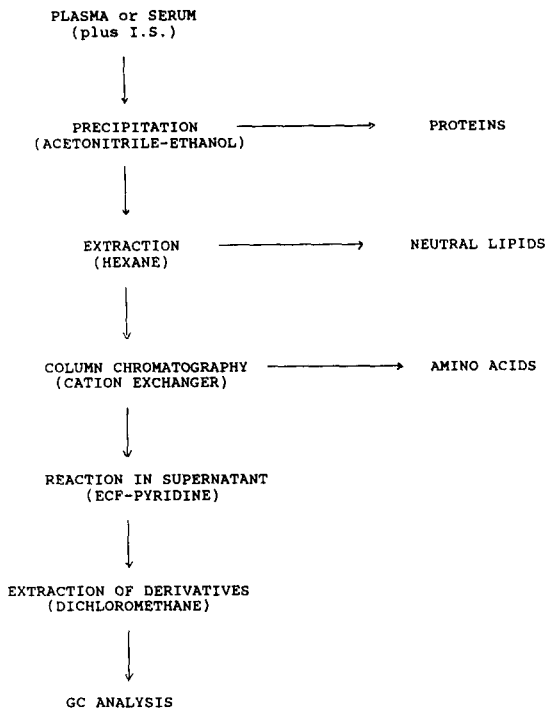

Fig. 1. Scheme of sample work-up.

were added and the mixture was gently shaken for a few seconds while carbon dioxide bubbles are formed (the course of the derivatization and the yield are not influenced by this phenomenon). Following the addition of 0.25 ml of dichloromethane and 0.5 ml of the (bi)carbonate solution, the stoppered tube was shaken for *ca.* 10 s. After standing for 2–3 min to reach phase equilibrium, the upper aqueous layer was aspirated (in the silanized tubes the last water drops can be easily aspirated by pipette-tips when tilting the tube) and the organic phase was dried by addition of few milligrams of sodium sulphate. By blowing nitrogen across the surface of the solvent at room or slightly elevated temperature (36 to 40°C) volume reduction to approximately 60 to 100 μl can be achieved within 2–3 min. A 2–2.5 μl aliquot was then injected onto the column in the split mode (1:20).

The same procedure was carried out for the chromatographic standards by addition of 0.2 ml water instead of plasma.

3. Results

Retention times and response factors of the individual carboxylic acids on the DB-225 column were determined with respect to malonic acid. This compound–unlike some other dicarboxylic acids–can be converted to diethyl ester with a good and reproducible yield. It proved to be a convenient I.S. for plasma profiling since on the specified column it was eluted in a vacant area of the chromatogram and since it was not found in human blood.

The overall reproducibility of the procedure, expressed as percent variation from the mean, was determined from six independent analyses of the standards. As can be seen from Table 1, the C.V.s were remarkably low and close to those found for the derivatization alone. The excellent separation power of the polar silicone phase for this kind of derivatives is documented in Fig. 2.

A normal plasma profile of carboxylic acids is shown in Fig. 3. The chromatogram corresponds to 0.125 ml of plasma, to which I.S. in amount 20 nmol/100 μl plasma was added. Identification of the analytes was based on comparison of the retention times with those of standard solutions. Thus, the structure of the others peaks remains unknown.

The within-day reproducibility of the plasma profiling and the recovery of the acids added to plasma are given in Table 2. Plasma samples were spiked with chromatographic standards in the range of 20 to 100 nmol of the particular standard added to 0.2 ml of plasma.

The average C.V. of the within-day reproducibility was 5%, the C.V. being higher for acids of low abundance (at or below 5 nmol/ml plasma). These minor components are very difficult to detect and are not often included in previous reports [4]. Recovery of the acids from the spiked plasma was surprisingly good; slightly lower values were found for some of the dicarboxylic acids. In contrast, and rather against expectance, the C.V.s for fatty acids were higher than those of the others analytes. Lactic acid, the predominant compound in the profile, is always accompanied (like other hydroxycarboxylic

Table 1
Chromatographic parameters of derivatized standards of carboxylic acids on DB-225 column

Elution order	Carboxylic acid	Abbreviation	t_R (relative)	*RF* (relative)	C.V. (%)
1	Pyruvic	PYR	0.466	0.34	4.4
2	Caproic	6:0	0.517	1.15	7.0
3	2-Ketoisovaleric	KIV	0.645	0.90	2.6
4	3-Hydroxybutyric	3HB	0.730	0.87	6.8
5	2-Ketomethylvaleric	KMV	0.830	1.25	4.1
6	2-Ketoisocaproic	KIC	0.864	1.19	3.2
7	Caprylic	8:0	0.889	1.52	5.9
8	Malonic (I.S.)	M	1.000	1.00	
9	Methylmalonic	MM	1.021	1.03	5.8
10	Benzoic	BA	1.132	0.45	8.3
11	Ethylmalonic	EM	1.146	1.21	7.8
12	2-Hydroxypropionic (lactic)	HP	1.287	0.83	6.0
13	Capric	10:0	1.309	1.89	6.3
14	Hydroxyacetic	HA	1.321	0.56	7.5
15	Phenylacetic	PA	1.395	1.78	2.8
16	2-Hydroxybutyric	HB	1.424	1.07	5.6
17	2-Hydroxyisovaleric	HIV	1.485	0.78	5.0
18	2-Hydroxyisocaproic	HIC	1.648	1.40	4.7
19	Lauric	12:0	1.704	2.24	5.1
20	Adipic	AD	1.718	1.14	7.7
21	Methyladipic	MAD	1.757	1.09	9.2
22	Pimelic	PIM	1.915	1.67	6.4
23	Myristic	14:0	2.064	2.62	6.3
24	Suberic	SUB	2.103	1.82	4.9
25	Palmitic	16:0	2.389	2.98	6.0
26	Palmitooleic	16:1	2.405	2.99	6.9
27	Phenyllactic	PL	2.640	2.14	5.2
28	Stearic	18:0	2.684	3.36	6.5
29	Oleic	18:1	2.706	3.35	5.7
30	Linoleic	18:2	2.766	3.33	5.9
31	Linolenic	18:3	2.829	3.32	6.1
32	Arachic	20:0	2.967	3.70	5.8
33	Arachidonic	20:4	3.067	3.64	6.3
34	Docosahexanoic	22:6	3.546	3.91	5.4

Retention times (t_R) and molar response factors (*RF*) relative to malonic acid (internal standard, I.S.). Reproducibility of derivative formation followed by coefficient of variation (C.V., $n = 6$).

acids) by side-reaction products [7,8]. These are marked by asterisks in Fig. 3 and do not interfere with other compounds under the chosen analytical conditions.

4. Discussion

An organic solvent suitable as an effective deproteinizing agent had to be compatible with the optimum composition of the reaction medium used for the treatment with chloroformates [6]. Although a 1:1 ratio of plasma to organic solvent (acetonitrile–ethanol, 1:1) affords a good yield for most of the analytes and would be desirable with regard to the subsequent volume reduction, precipitation of proteins was proven not to be complete under such conditions. Addition of dichloromethane gave a higher precipitation. Also other authors have used

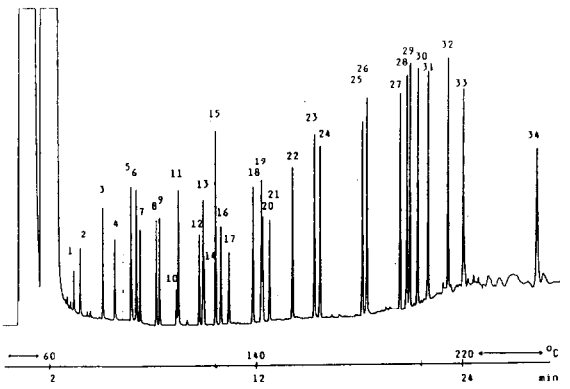

Fig. 2. Chromatographic standards analyzed on the DB-225 column as an equimolar mixture (fatty acids at half molarity). Derivatized amount 20 nmol of each, injected amount 0.5 nmol at 1:20 split ratio. Attenuation: × 32. Peak identities: see Table 1.

organic solvents such as alcohols (methanol, ethanol, isopropanol), acetonitrile or acetone as precipitants [2,3,9–13] even in the ratio 1:1 [9,11], but in none of the previous reports the supernatant was subjected to direct derivatization.

The 3:1 ratio of the present study is closer to the ratio used in a recent report [10] where a 4-fold excess of ethanol over plasma was employed. Furthermore, the authors found that such a mixture could be extracted with hexane without mixing of the phases taking place. Since

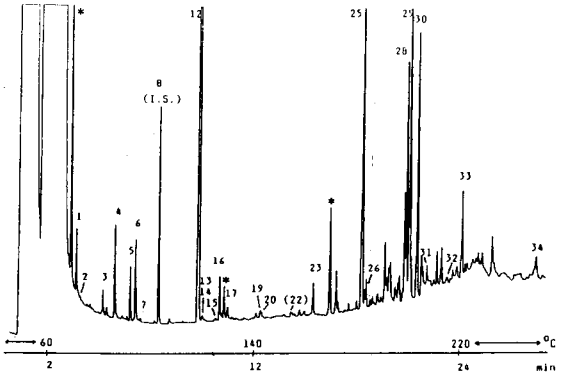

Fig. 3. Organic acid profile corresponding to 0.125 ml of plasma. I.S. added to a concentration of 20 nmol/0.1 ml of plasma. Processed under the same conditions as in Fig. 1. Asterisks (*) indicates side-products of lactic acid. Peak identities: see Table 1.

acetonitrile is also immiscible with hexane, our system was also able to remove the neutral lipids without any negative influence on the derivatization yields.

The chosen ratio with 50% of acetonitrile in the solvent is a compromise, providing optimum yields for both hydroxycarboxylic and dicarboxylic acids. To obtain maximum yields the former require a slightly higher concentration of acetonitrile in the medium [7], whereas the latter require a lower concentration as they tend to form alkoxycarbonyl esters (mixed anhydrides) in its presence [14]. This is also the reason for the failure to detect succinic acid [6] and for the lower response of the higher homologs (diC_6–C_8). The mixed anhydrides decompose when shaken with (bi)carbonate and the corresponding parent acids remain as salts in the aqueous phase. As far as lactic acid is concerned, the addition of ECF prior to pyridine contributes more to the suppression of side-product formation than changing of the acetonitrile content [7].

The highest dependence on the amount of acetonitrile in the medium was observed in the case of keto acids. Any lowering of the 3:1 ratio results in a decline of their response to *ca.* one third at a 1:1 ratio. In contrast, a higher ratio does not result in a higher yield. Determination of acetoacetic acid (3-ketobutyric) was not possible since the compound decomposes in the injector. Acetonitrile is the best reaction medium even for fatty acids [15], and increasing the water content results only in minor decrease of their yield [16]. Any unreacted fatty acids are removed by the (bi)carbonate solution. The same is true for sugars and sugar-related carboxylic acids: they remain "hydrophilic" as the alcoholic hydroxyl groups (except the one adjacent to the carboxyl group) do not react with chloroformates under the described reaction conditions [17].

Exposure of the supernatant to the hexane phase and subsequent cation-exchange chromatography did not alter the reaction yields. Neutralization of the supernatant acidified in the exchange column to pH 1–2 requires *ca.* 4 μl of pyridine. However, this step can be omitted as the added amount of pyridine is more than

Table 2
Within-day reproducibility of plasma organic acid profiling and analytical recovery of organic acids added to plasma

Acid	Plasma		Spiked plasma	
	Concentration (nmol/ml)	C.V. ($n = 6$) (%)	Recovery (%)	C.V. ($n = 6$) (%)
PYR	86.8	4.1	92	6.3
6:0	1.9	14.2	109	10.1
KIV	12.2	4.3	96	8.6
3HB	75.7	3.2	105	7.4
KMV	24.6	4.9	99	8.1
KIC	38.3	3.9	104	6.2
8:0	2.2	13.6	102	10.2
MM	0		100	4.5
BA	0		108	5.9
EM	0		96	8.3
HP	1755.1	7.4	97	8.7
10:0 + HA	3.8	13.0	101	11.2
PA	1.2	9.2	105	9.6
HB	32.5	5.7	93	8.4
HIV	13.4	6.2	104	6.6
HIC	0		100	6.7
12:0	4.3	13.2	98	7.5
AD	1.2	20.7	91	8.1
MAD	0		84	13.7
PIM	1.6	23.5	89	6.4
14:0	17.4	10.8	97	8.8
SUB	0		88	9.4
16:0	112.7	8.2	102	9.7
16:1	14.5	7.9	100	7.3
PL	0		102	5.5
18:0	52.1	5.8	99	8.0
18:1	106.2	5.9	103	7.2
18:2	62.7	4.7	105	8.7
18:3	4.4	11.4	97	9.0
20:0	0		106	10.4
20:4	14.1	5.8	101	7.8
22:6	4.7	12.3	97	7.7

sufficient to give a satisfactory reaction. Even the amount of cation exchanger (10 to 12 mg) was sufficient to remove amino acids and salts completely. The pH of the eluate after passage though the column should always be < 3.

Dichloromethane proved to be a good substitute for the previously used chloroform [6]. Reduction of the volume of the dichloromethane extract to approximately 60–80 μl (the remaining solvent then consists mostly of acetonitrile and pyridine) should be carefully monitored to prevent evaporation to dryness, since in that case loss of the most volatile analytes will occur. In such a condensed medium a progressive decrease of the amount of keto acids in time at room temperature takes place. To prevent this, the sample should be stored refrigerated at -20°C when the analysis is to be done at a later stage.

A pilot study revealed that the values found for the plasma and serum metabolic profiles were comparable. Further studies should be performed in this respect. EDTA as a coagulant does not have an adverse effect on the analysis, as its amount in the sample before derivatization

is less than 1 μmol. Heparinized blood was less suitable as some extraneous peaks, coeluting with some fatty acids, appeared at the end of the chromatogram.

The abundance of the carboxylic acids in control human plasma, as shown in Table 2, is comparable with that reported in some earlier reports using different approaches [4,9,10]. The agreement with literature values is especially valuable for keto acids, which had to be double-derivatized before GC analysis [18] or which were analyzed by HPLC [19,20]. The abundance of the fatty acids in blood fully fits the generally known range.

ECF was preferred over methyl chloroformate (MCF) [6] because it is able to separate pyruvic acid ethyl ester from the solvent peak. The use of a polar silicone phase is perfectly suited to the analysis of the least volatile members of the acid pattern.

A procedure is currently being developed to allow even amino acids to be included in the profile. However, a column with a phase having a higher temperature stability will be required.

5. Conclusion

The novel approach using chloroformates for the instantaneous esterification of carboxylic acids in aqueous media [6] was adapted for the direct treatment of deproteinized plasma supernatants. The within-day reproducibility and the acid recovery from spiked plasma samples demonstrate the usefulness and suitability of this markedly facilitated approach. The procedure uses simpler sample handling and gives results comparable to those of the more tedious methods currently employed. An organic acid blood profile can be obtained within 1 h (including sample pretreatment) with lower costs.

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