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SIMULTANEOUS DETERMINATION OF C_2–C_{22} NON-ESTERIFIED FATTY ACIDS AND OTHER METABOLICALLY RELEVANT CARBOXYLIC ACIDS IN BIOLOGICAL MATERIAL BY GAS CHROMATOGRAPHY OF THEIR BENZYL ESTERS

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SUMMARY

A method for the simultaneous determination of non-esterified short-, medium- and long-chain fatty acids and other types of metabolically relevant carboxylic acids such as hydroxy, keto, aromatic and dicarboxylic acids in biological material by capillary gas chromatography of benzyl ester derivatives is described. Sample preparation avoiding incomplete isolation of carboxylic acids consisted of deproteinization and extraction with ethanol, fixation of carboxylic acids as carboxylates, removal of interfering compounds such as neutral lipids by hexane extraction and amino acids, acyl carnitines and other cations by cation-exchange chromatography, derivatization of keto groups of ketocarboxylic acids into O-methyl oximes and benzyl ester formation by reaction of the potassium carboxylates with benzyl bromide via crown ether catalysis. The sample preparation conditions were investigated, showing the usefulness of this method for quantitative determinations. Chromatograms obtained from human serum, human urine and rat heart ventricle and concentrations of carboxylic acids in these specimens are presented.

INTRODUCTION

For the determination of carboxylic acids in biological material, in addition to methods for particular classes of acids, such as ketocarboxylic acids and long- or short-chain fatty acids, methods that allow the simultaneous determination of a wide range of different carboxylic acid classes (carboxylic acid profiling) have become important. Such methods have been successfully employed in clinical medicine for the diagnosis and study of inherited metabolic disorders resulting in abnormal profiles of carboxylic acids (organic acidurias) [1–4].

Carboxylic acid profiling is most often carried out on urine samples (see ref-

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erences in refs. 1–4) and only a few papers have reported acid profiles of serum [5–10] and tissues [11–14].

Carboxylic acids are commonly isolated from urine either by extraction with organic solvents [15–17], such as diethyl ether or ethyl acetate, or by anion-exchange chromatography on DEAE-Sephadex [18,19]. Solvent extraction is fast but results in poor recoveries of polar acids [20], and the more time-consuming anion-exchange procedure results in losses of the more volatile acids and is not suitable for acids with higher pK_a values such as long-chain fatty acids [20,21].

Neither solvent extraction nor anion-exchange chromatography allows the isolation of volatile short-chain fatty acids. Hitherto, short-chain fatty acid analysis has required special isolation methods such as steam distillation [22,23], vacuum distillation [24], partition chromatography [25] or solvent extraction [26–28]. In addition, methyl or trimethylsilyl ester derivatives, mainly used for gas chromatographic separations, are not suitable for short-chain fatty acids owing to their high volatility. Either after isolation [22–28] or by direct injection of biological material [29–31], short-chain fatty acids are usually chromatographed underivatized using special stationary phases, although the use of derivatives such as *n*-butyl [32], *tert*-butyldimethylsilyl [33] or benzyl [34] esters is recommended. Because serum and tissues contain large amounts of protein and lipids, sample preparation procedures for the analysis of urinary organic acids are not easily adaptable.

Recently, we described a method for the preparation and capillary gas chromatographic separation of benzyl ester derivatives of C_2–C_{20} fatty acids and a large number of other metabolically important carboxylic acids [35]. Benzyl esters were prepared by reaction of the potassium carboxylates with benzyl bromide via crown ether catalysis.

Combining this derivatization and separation method with a newly developed sample preparation procedure, we have developed a method for the determination of C_2–C_{22} non-esterified fatty acids together with other metabolically relevant acids such as keto, hydroxy, aromatic and dicarboxylic acids in protein- and lipid-rich biological material (serum and heart tissue); the method is also suitable for urine.

EXPERIMENTAL

Chemicals

Analytical-reagent grade acetonitrile, *n*-hexane, benzene and ethanol, boron-trifluoride–methanol, glutaric acid and 18-crown-6 were obtained from Merck (Darmstadt, F.R.G.). Sodium 3-hydroxybutyrate, phenylacetic acid, sodium 2-ketovalerate, stearoyl cholesterol, trimyristoyl glycerol, dipalmitoyl phosphatidylcholine, dilauroyl phosphatidylethanolamine, acetyl- and palmitoyl-D,L-carnitine chloride, acetyl coenzyme A (sodium salt), palmitoyl coenzyme A and methoxylamine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Cholesterol was purchased from Boehringer (Mannheim, F.R.G.) and *n*-dodecane, heptadecanoic acid and 2-methylbutyric acid from Fluka (Buchs, Switzerland). Dowex AG 50W-X8 (analytical-reagent grade) and Amberlyst 15 were

purchased from Serva (Heidelberg, F.R.G.), Bond Elut SCX (500-mg columns) from Analytichem International (Harbor City, CA, U.S.A.) and *n*-hexacosane from Applied Science Labs. (State College, PA, U.S.A.).

Benzyl bromide was distilled in vacuo before use. Amberlyst 15 was washed thoroughly with water, methanol and acetone.

Gas chromatograph

A Model 428 gas chromatograph (Packard, Downers Grove, IL, U.S.A.) equipped with a flame ionization detector, a CP-Sil 5CB fused-silica capillary column (50 m × 0.22 mm I.D.) (Chrompack, Middelburg, The Netherlands) and an integrator (Merck/Hitachi Chromato-Integrator D-2000; Merck, Darmstadt, F.R.G.) was used. The temperatures used were: injection port 300°C, detector 330°C, and oven 100–300°C at 3°C/min. Injections were made at a splitting ratio of 1:100. Helium was used as the carrier gas at a linear gas velocity of 25 cm/s.

Biological material

Blood was obtained by venepuncture from a healthy young man after an overnight fast. After centrifugation (1000 *g*) the serum was removed and stored at −20°C until analysis. Urine was collected for 24 h from a healthy 12-year-old boy and stored at −20°C until analysis. The creatinine content was 84 mg per 100 ml.

Rat heart tissue was obtained from Wistar rats fed with a standard diet and water ad libitum. Animals (15—20 weeks old, 270–340 g body weight) were anaesthetized with pentobarbital (5 mg per 100 g body weight i.p.). After tracheotomy and medial thoracotomy, the beating hearts were freeze-stopped with stainless-steel clamps pre-cooled in liquid nitrogen. The atria were removed and the ventricles were stored in liquid nitrogen until analysis.

Preparation of serum samples

To 0.5 ml of serum were added 2 ml of ethanol and the mixture was shaken for 2 min using a vortex mixer for precipitation of proteins and extraction of carboxylic acids. After centrifugation (1800 *g*) the precipitate was washed with 0.5 ml of 80% ethanol. The combined ethanolic solutions were made alkaline to a phenolphthalein end-point with potassium hydroxide solution and extracted three times with 3-ml portions of *n*-hexane. The aqueous ethanolic phase was passed through a cation-exchange column in the H^+ form (either Dowex AG 50W-X8 or Amberlyst 15 packed 5 cm high in a Pasteur pipette, or a Bond Elut SCX solid-phase extraction column with 500 mg of sorbent) and the column was washed with 1.5 ml of 80% ethanol. The eluate was mixed with 100 µl of a solution of methoxylamine hydrochloride (40 mg/ml in ethanol), neutralized to a pH of about 7.0 with potassium hydroxide solution and heated for 30 min at 60°C. After cooling, the pH of the solution was adjusted to a phenolphthalein end-point (exactly still colourless) with potassium hydroxide solution and extracted twice with 3-ml portions of *n*-hexane. The ethanolic phase was then evaporated to dryness in a gentle stream of nitrogen at 30°C (during evaporation the pH of the solution was readjusted with 0.01 *M* hydrochloric acid when the solution became red) and

the residue was dried azeotropically by twice adding a few drops of benzene. Immediately afterwards, 200 μl of a solution of benzyl bromide (200 m*M*) and 18-crown-6 (20 m*M*) in acetonitrile were added and the mixture was ultrasonicated for 5 min. The solvent was evaporated to less than 20 μl in a gentle stream of nitrogen at room temperature and the mixture was heated for 60 min at 90°C in a nitrogen atmosphere. After cooling, 1.5 ml of water were added and the benzyl esters were extracted three times with 3-ml portions of *n*-hexane. The combined hexane extracts were dried over anhydrous sodium sulphate and the solvent was evaporated to about 100 μl in a gentle stream of nitrogen at 30°C. After addition of 50 nmol each of *n*-dodecane and *n*-hexacosane (1 m*M* in *n*-hexane) as internal standards, a few microlitres were injected into the gas chromatograph. The whole preparation was carried out using Pyrex glass culture tubes (13 mm O.D.) with Bakelite screw caps with Teflon liners.

Preparation of urine samples

A 1-ml volume of urine was mixed with 4 ml of ethanol. Further preparation was as described for serum samples, but only Bond Elut SCX was used as the cation-exchange column.

Preparation of rat heart tissue samples

Residual blood and epicardial fat of the tissue were scraped off at liquid nitrogen temperature. A 0.4–0.7-g amount of ventricle was homogenized in a mortar under liquid nitrogen. To the powdered tissue a six-fold (v/w) amount of ethanol was added and the suspension was allowed to warm up to about 0°C and transferred into a Pyrex tube. The mortar was rinsed twice with each 0.75 ml of 80% ethanol and the combined ethanolic suspensions were ultrasonicated for 2 min with cooling in an ice-bath. After centrifugation (1800 *g*), the precipitate was washed with 1 ml of 80% ethanol. Further preparation was as described for serum samples, but only Bond Elut SCX was used as the cation-exchange column.

Determination of carboxylic acids

Carboxylic acids in serum, urine and rat heart ventricle were determined from the capillary gas chromatograms of their benzyl esters by the internal standard method using *n*-dodecane and *n*-hexacosane as internal standards. *n*-Dodecane was used for benzyl esters with retention times up to benzyl benzoate and *n*-hexacosane for benzyl esters with longer retention times. The use of *n*-hexacosane as the internal standard for the determination of high-boiling benzyl esters reduced problems arising from discrimination due to the use of an injector splitter, so that the reproducibility of the relative peak areas of benzyl esters was better than 5%. Relative response factors for quantitation were obtained by derivatization of carboxylic acid standards as described recently [35]. The amounts of dicarboxylic acids determined were corrected according to their recovery of 75%.

Determination of lipids, acyl-CoA and acyl carnitines

In studies of sample preparation, trimyristoyl glycerol, dipalmitoyl phosphatidylcholine, dilauroyl phosphatidylethanolamine, stearoyl cholesterol, palmi-

toyl-CoA and palmitoyl carnitine were determined by capillary gas chromatography as the methyl esters of their acyl moieties. The transesterification method with boron trifluoride–methanol according to Morrison and Smith [36] was used. The gas chromatographic conditions were the same as for benzyl ester separations. Methyl heptadecanoate was used as an internal standard.

Cholesterol was measured by capillary gas chromatography without derivatization as described for benzyl ester separations with a temperature programme from 280 to 320 °C at 2 °C/min. Two minor peaks appeared in the chromatograms before cholesterol with areas less than 1% of that of cholesterol. Possibly it belonged to cholestadienes produced by dehydration of the sterol.

Acetyl-CoA and acetyl carnitine were determined after hydrolysis with 10% potassium hydroxide solution for 1 h at 100 °C, neutralization with perchloric acid and removal of the precipitated potassium perchlorate. The acetic acid was benzylated as described [35] and determined by capillary gas chromatography.

Thin-layer chromatography of lipids

Thin-layer chromatographic separations were performed on silica gel 60 HPTLC plates (Merck). The mobile phases were light petroleum (b.p. 40–60 °C)–diethyl ether–acetic acid (60:30:1, v/v) for neutral lipid separations and dichloromethane–methanol–acetic acid–water (60:30:8:3, v/v) for phospholipid separations. The lipids were detected with iodine vapour and phospholipids additionally with molybdenum blue reagent.

RESULTS

In the ethanolic extract of a serum sample the various neutral and phospholipids were present in markedly smaller amounts than in a conventional Bligh and Dyer [37] lipid extract because of their limited solubility in 80% ethanol. More than 99.9% of each neutral lipid was found in the hexane phase and more than 99% of the fatty acid remained in the ethanolic phase when 500 nmol each of stearoyl cholesterol, cholesterol and trimyristoyl glycerol and 200 nmol of palmitic acid in 80% ethanol were extracted with hexane under the conditions for serum sample preparation.

After removal of the neutral lipids by the hexane extraction, phospholipids were still part of the ethanolic solution and would therefore pass through the cation-exchange column. We determined the portion of the two major phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), that remained on the cation-exchange column. Amounts of 0.25 and 5 μmol of dipalmitoyl-PC and 0.085 and 1.7 μmol of dilauroyl-PE were added to 2.5 ml of 80% ethanol and passed through Bond Elut SCX columns; 80.7% and 93.5% of the PC and 33.4% and 48.4% of the PE remained on the cation-exchange column.

Acyl carnitines and acyl-CoA are further acyl-containing substances that on hydrolysis may lead to erroneous results for the non-esterified fatty acid content in biological material. Acetyl carnitine was removed completely by the cation-exchange step and palmitoyl carnitine and palmitoyl-CoA were removed to ex-

tents of 98.2% and 29.9%, respectively, when 1 μmol each of acetyl and palmitoyl carnitine and 150 nmol of palmitoyl-CoA, each in 80% ethanol, were passed through Bond Elut SCX columns under the conditions for serum sample preparation. Hence considerable amounts of phospholipids and acyl-CoA but only very small amounts of long-chain acyl carnitines may remain in the sample solution after the cation-exchange step. Therefore, we determined the degree of hydrolysis of the two main phospholipids, PC and PE, and acyl-CoA and acyl carnitine during the complete sample preparation procedure. Amounts of 1 μmol of dipalmitoyl-PC, 500 nmol of dilauroyl-PE, 150 nmol of palmitoyl-CoA, 500 nmol of acetyl-CoA and 500 nmol of palmitoyl carnitine were added to a mixture of propionic, lactic, pyruvic, succinic and stearic acids (200 nmol of each) in 2.5 ml of 80% ethanol and the mixture was treated as described for serum samples under Experimental. In neither of the substances examined could any benzyl ester of their corresponding acyl moieties be detected. Therefore, no hydrolysis of the

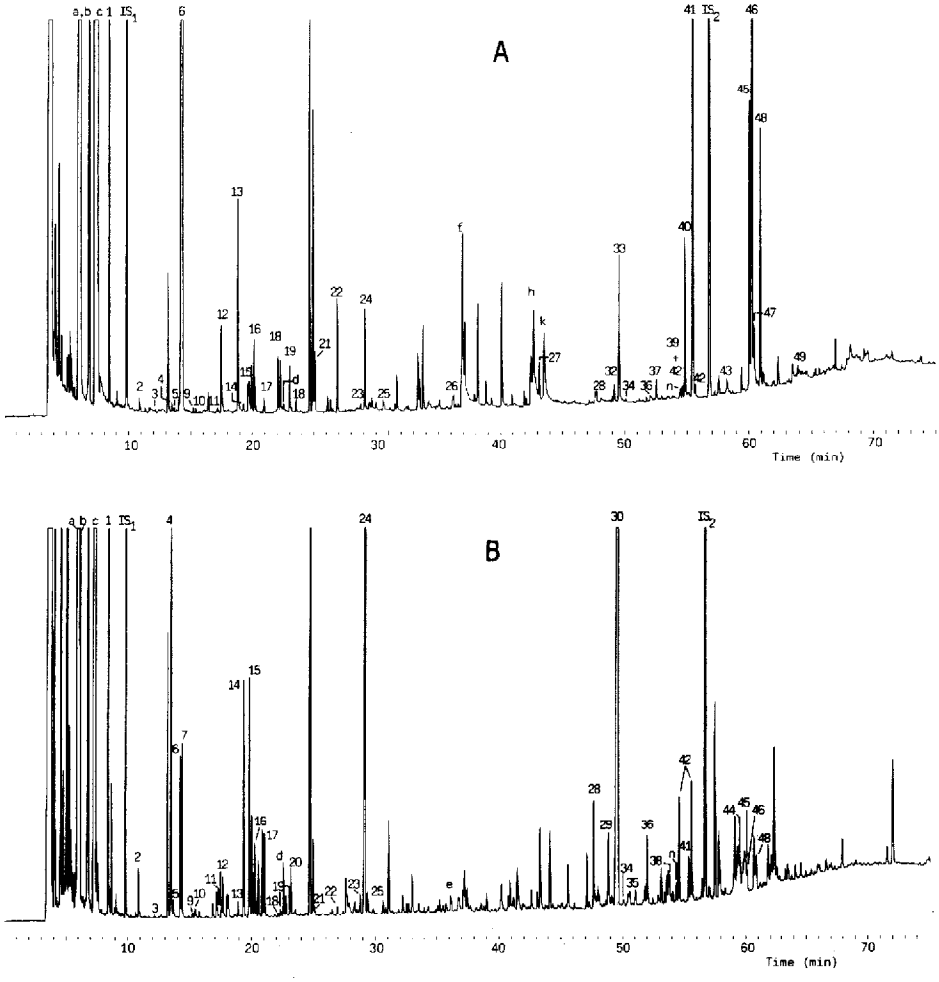

Fig. 1.

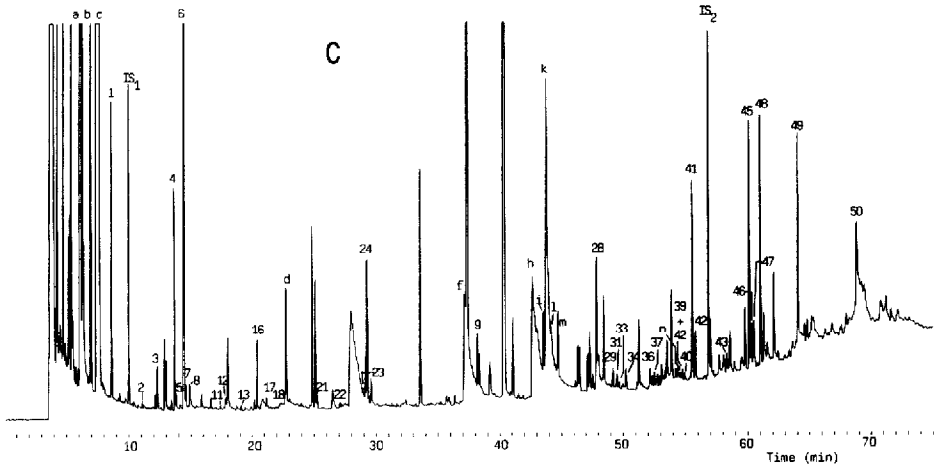

Fig. 1. Gas chromatograms obtained from (A) human serum, (B) human urine and (C) rat heart ventricle. Peaks with numbers are carboxylic acid benzyl esters; numbers correspond to those in Table II. IS_1 = internal standard *n*-dodecane; IS_2 = internal standard *n*-hexacosane. Further peaks were identified as follows: a = benzyl chloride; b = benzyl alcohol; c = benzyl bromide; d = diethyl phthalate; e = dibutyl phthalate; f = palmitic acid; g = ethyl palmitate; h = linoleic and oleic acids; i = ethyl linoleate; k = stearic acid; l = ethyl oleate; m = ethyl stearate; n = bis(2-ethylhexyl) phthalate.

TABLE I

RECOVERY OF CARBOXYLIC ACIDS ADDED TO SERUM

Carboxylic acid	Recovery* (%)
2-Methylbutyric acid	99.3 ± 6.9 (15)
Heptadecanoic acid	94.5 ± 5.0 (13)
3-Hydroxybutyric acid	97.7 ± 7.5 (15)
2-Ketovaleric acid	88.1 ± 7.4 (15)
Glutaric acid	75.4 ± 7.1 (13)
Phenylacetic acid	94.8 ± 5.8 (8)

*Means ± S.D.: number of experiments in parentheses. 100 nmol of carboxylic acid were added to 0.5 ml of serum. Further sample preparation as described under Experimental.

added phospholipids, acyl-CoA and acyl carnitines took place during sample preparation.

After the conversion of keto groups of the ketocarboxylic acids into their O-methyl oximes by reaction with methoxyamine there are two further extractions with hexane, in order to remove long-chain fatty acid ethyl esters produced by transesterification of phospholipids in the aqueous ethanolic solution during O-methyl oxime formation. On reacting 1 μmol of dipalmitoyl-PC in 80% ethanol for 30 min at 60°C at pH 7.0, about 0.25% of the palmitoyl moiety was converted into the ethyl ester. Non-esterified carboxylic acids did not form any ethyl ester under these conditions. Particularly with tissue samples with higher phospholipid contents this transesterification resulted in the formation of several hundred nanomoles of ethyl esters, which could be completely removed by hexane extraction. Small peaks of C_{16} and C_{18} fatty acid ethyl esters could still be detected in the chromatogram obtained from heart tissue (Fig. 1). Owing to the higher phos-

TABLE II

CONCENTRATIONS OF CARBOXYLIC ACIDS IN HUMAN SERUM, HUMAN URINE AND RAT HEART VENTRICLE

No.	Carboxylic acid	Concentration*		
		Serum	Urine	Heart ventricle
1	Acetic acid	86.0	115.2	209.8
2	Propionic acid	2.1	9.2	7.4
3	Isobutyric acid	0.3	0.4	9.3
5	Butyric acid	0.6	2.2	3.4
9	2-Methylbutyric acid	0.8	1.1	0
10	Isovaleric acid	0.6	1.4	0
11	Valeric acid	0.4	1.7	2.0
17	Caproic acid	2.0	13.0	4.7
23	Caprylic acid	0.8	2.8	3.4
26	Capric acid	3.7	0	1.4
27	Lauric acid	4.7	0	N.d.**
32	Tetradecenoic acid	1.8	0	0
33	Myristic acid	16.3	0	4.0
37	Pentadecanoic acid	2.1	0	2.1
39	*cis*-7-Hexadecenoic acid	1.3	0	1.8
40	Palmitoleic acid	15.9	0	1.4
41	Palmitic acid	74.6	3.0	38.5
43	Heptadecanoic acid	1.6	0	2.5
45	Linoleic acid	28.2	1.8	38.3
46	Oleic acid	106.7	1.8	11.1
47	Vaccenic acid	3.6	0	13.4
48	Stearic acid	26.2	2.8	38.6
49	Arachidonic acid	0.9	0	42.8
50	Eicosahexaenoic acid	0	0	31.2
4	Glycolic acid	3.6	260.4	187.5
6	Lactic acid	674.4	32.2	380.8
7	2-Hydroxyisobutyric acid	0	32.7	2.5
8	3-Hydroxypropionic acid	0	0	9.1
12	2-Hydroxybutyric acid	19.1	2.9	19.6
13	3-Hydroxybutyric acid	41.6	3.5	11.6
14	3-Hydroxyisovaleric acid***	1.3	41.3	0
15	2-Hydroxyisovaleric acid	3.7	40.9	0
16	Pyruvic acid	17.3	20.2	80.0
18	2-Ketoisovaleric acid	10.8	0.9	2.5
19	Acetoacetic acid	21.5	3.1	0
20	2-Hydroxy-3-methylvaleric acid***	0	7.9	0
21	2-Keto-3-methylvaleric acid	8.4	1.1	3.7
22	2-Ketoisocaproic acid	15.3	2.0	7.2
24	Benzoic acid	10.8	165.8	14.8
25	Phenylacetic acid	2.0	1.7	0
28	Succinic acid	3.2	19.5	30.6
29	Methylsuccinic acid	0	15.7	0
30	Hippuric acid	0	274.6	0
31	Fumaric acid	0	0	18.1
34	Malic acid	0.8	2.4	41.7
36	Oxaloacetic acid	5.0	25.4	3.7
38	Adipic acid	0	6.3	0
42	2-Ketoglutaric acid	2.9	50.6	79.2
44	Suberic acid	0	8.3	0

*Values expressed as nmol/ml for serum, nmol/mg creatinine for urine and nmol/g wet mass for heart ventricle.

**N.d. = not determinable.

***Tentative identification.

pholipid content in tissue they were produced by ethanolysis during subsequent evaporation.

After the addition of several carboxylic acids to serum samples, the recoveries of different types of acids through sample preparation were determined. As shown in Table I, the recoveries were good for short-chain fatty acids (99.3% for 2-methylbutyric acid), long-chain fatty acids (94.5% for heptadecanoic acid), hydroxycarboxylic acids (97.7% for 3-hydroxybutyric acid), ketocarboxylic acids (88.1% for 2-ketovaleric acid), dicarboxylic acids (75.4% for glutaric acid) and aromatic acids (94.8% for phenylacetic acid). The recoveries were of the same order of magnitude when as little as 20 nmol or as much as 1000 nmol of these acids were added to serum, and also other representatives of these carboxylic acid classes gave similar recoveries (data not presented). Only the behaviour of oxaloacetic acid during sample preparation was less satisfactory. Oxaloacetic acid is a more labile compound with a tendency for non-enzymatic decarboxylation, resulting in the formation of pyruvic acid. After addition of oxaloacetic acid to serum samples we observed 30–40% decarboxylation, resulting in the corresponding amount of benzyl pyruvate, and the recovery of oxaloacetic acid was only about 40%.

In Fig. 1, gas chromatograms obtained from human serum, human urine and rat heart ventricle samples are presented. Peak identifications of carboxylic acid benzyl esters by retention times were confirmed by combined capillary gas chromatography–mass spectrometry [35]. The chromatograms consisted mainly of carboxylic acid benzyl esters (numbered peaks) and other benzylated substances, seen from their electron impact mass spectra with m/z 91 mainly being the base peak. These unidentified peaks must have been produced from components of the biological material because they were not observed in reagent blanks, which revealed only the peak directly before α-keto-β-methylvaleric acid (peak 21 in Fig. 1A–C), which was identified as dibenzyl ether. The amount of benzyl acetate in the reagent blanks was less than 2 nmol. Further, some artifacts were present: phthalate esters and long-chain fatty acids in the chromatograms of serum and heart ventricle and long-chain fatty acid ethyl esters only in the chromatogram of heart ventricle. Benzyl chloride and benzyl alcohol resulted from reaction of benzyl bromide with chloride and hydroxyl ions, respectively. The peak with a retention time of about 34 min in Fig. 1C was 18-crown-6, which may be extracted to a small extent by the final hexane extraction. The large peaks with retention times of about 37 and 40 min in Fig. 1C could not be identified, but were observed to this extent only in heart tissue. In Table II the corresponding concentrations of carboxylic acids in human serum and urine and rat heart tissue are shown.

DISCUSSION

Although favourable gas chromatographic and mass spectrometric properties of benzyl ester derivatives of short- and long-chain fatty acids and other metabolically relevant carboxylic acids are known (see the references in ref. 35), a method for the simultaneous determination of these acids in biological material was still lacking, because of the benzylation reagents used so far. Both phenyldi-

azomethane and N, N′-dicyclohexyl-O-benzylisourea require acids in their undissociated form in organic solvents, but carboxylic acids, especially short-chain fatty acids, cannot be isolated by commonly used solvent extraction or anion-exchange chromatography.

In the method described here, we fixed carboxylic acids as non-volatile carboxylates, removed non-polar components by hexane extraction and amino acids and other cations by cation-exchange chromatography and formed the benzyl esters from the solid potassium carboxylates via phase-transfer catalysis as described recently [35]. The benzyl esters of all types of carboxylic acids were non-polar enough to be separated from the polar substances of the biological matrix by hexane extraction. Thus, the finally obtained hexane solution injected into the gas chromatograph contained mainly benzylated compounds, as was seen from GC–MS measurements.

Ethanol ensured the complete extraction of protein-bound carboxylic acids, inactivation of lipases, solubilization of all types of carboxylic acids and immiscibility of 80% ethanol with hexane. Methanol resulted in a 10-fold higher alcoholysis of phospholipids during O-methyl oxime formation than ethanol. Isopropanol caused no alcoholysis, but 80% isopropanol and hexane were not completely immiscible. Hence removal of neutral lipids without losses of carboxylic acids was not possible.

Removal of amino acids was neccessary because benzylation of an amino acid mixture under the conditions described for carboxylic acids caused a series of peaks in the chromatogram that interfered with the determination of several carboxylic acid benzyl esters. The polystyrene ion exchangers Dowex AG 50W-X8 and Amberlyst 15, in addition to Bond Elut SCX (benzenesulphonate-modified silicic acid) removed the amino acids quantitatively. Cation exchange was additionally favourable for removing acyl carnitines and part of the main phospholipids, and in addition inorganic cations were exchanged for potassium ions, which is neccessary for the benzylation reaction using 18-crown-6, with the highest affinity to potassium.

Some acids could not be determined by this method under the conditions described here, such as citric acid, and polyhydroxy acids such as gluconic acid.

Although this sample preparation method is not as fast as conventional solvent extraction, mainly because of the time-consuming evaporation of the ethanolic solution, the carboxylic acid content of a biological specimen can be obtained within 8 h and several samples can be run in parallel.

The sample amounts used were 0.5 ml for serum, 1 ml for urine and about 500 mg for heart ventricle, but may be reduced to 20% of these values without changes to the sample preparation conditions. Benzyl esters are very stable derivatives. Standard solutions of carboxylic acid benzyl esters in hexane were stable for several months when stored at $-20\,^{\circ}C$. The prepared sample solutions could also be stored for several weeks at $-20\,^{\circ}C$ without signs of decomposition.

The underivatized long-chain fatty acids in the chromatogram obtained from rat heart ventricle must have been produced after the benzylation reaction because benzyl bromide was present in large excess. We assume that the acids were

produced by thermal decomposition of acyl-containing substances at an injector temperature of 300 °C, which was neccessary to ensure rapid vaporization of high-boiling benzyl esters. This substance must have become hexane-soluble on benzylation. Because these underivatized fatty acids were absent or present in even smaller amounts in serum, the compound could be a phospholipid present in heart tissue at higher concentration.

In conclusion, the present method may be useful for the determination of carboxylic acids in biological material when information about the concentrations of the complete range of non-esterified fatty acids (C_2–C_{22}) together with other metabolically relevant carboxylic acids is of interest, especially in the study of metabolic disorders, e.g., acyl coenzyme A dehydrogenase deficiencies, Reye's syndrome and other dicarboxylic acidurias or disorders with increased serum short-chain fatty acids, such as propionic and isovaleric acidaemia.

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