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GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF CARBOXYLIC ACIDS IN TISSUES AS THEIR *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

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SUMMARY

Krebs cycle and related acids were isolated from liver, pancreas and stomach of rats by pulverizing frozen tissue samples in liquid nitrogen with an aqueous solution of perchloric acid and methoxylamine hydrochloride. Perchloric acid and basic and neutral compounds were removed by ion-exchange chromatography on DEAE-Sephadex and SP-Sephadex columns. Phosphate was removed without loss of citrate by partition chromatography on Sephadex G-25 in a butanol—water system. Loss of acids during evaporation of water was prevented by keeping the temperature low and by addition of ammonia and tetrabutyl-ammonium bromide. *tert.*-Butyldimethylsilyl derivatives were prepared, purified by chromatography on a Sephadex LH-20 column and separated by gas chromatography on a non-polar capillary column. Recoveries in the procedure were above 45% for all acids except acetoacetate (25%).

The mass spectrometric fragmentation of the methoxime-*tert.*-butyldimethylsilyl derivatives was studied by analysis of the derivatives of deuterated acids. The intense ions at M — 57 present in all spectra appeared to contain all original hydrogen atoms. Thus, the method should be suitable for quantitation by isotope dilution and in metabolic studies with deuterated compounds.

INTRODUCTION

A gas chromatographic—mass spectrometric (GC—MS) method for analysis of Krebs cycle and related acids in tissues is desirable since it allows identification and quantitation by isotope dilution techniques [1]. Such a method could also be used in metabolic studies with stable isotopes [2, 3]. It is then important that no exchange of hydrogen occurs during the work-up procedure and that the ions used in GC—MS contain all the carbon and hydrogen atoms of the original compound. Available methods do not fulfil these criteria [1, 3].

Carbonyl groups must be derivatized in order to avoid partial formation of enol ethers and exchange of hydrogens. O-Methyloximes can be prepared directly in the tissue extract, and this protects against exchange of α-hydrogens [3]. However, preliminary experiments showed that acetoacetic acid had exchanged hydrogens during preparation of the O-methyloxime methyl ester by that method. Furthermore, methyl esters have mass spectra with very few peaks in the high mass region, and the abundant ions contain only a part of the molecule [3].

Mass spectra of *tert.*-butyldimethylsilyl (tBDMS) derivatives [4] contain abundant ions produced by loss of the *tert.*-butyl group, and the esters are stable and easily prepared [5—7]. Inorganic phosphate also forms a derivative [8], and it was found to be necessary to remove phosphate. The separation of citrate and phosphate is difficult [9], but it was achieved by partition chromatography in butanol—water with Sephadex G-25 as support [10].

EXPERIMENTAL

Materials

The organic solvents were of analytical-reagent grade from E. Merck (Darmstadt, F.R.G.) and were dried and distilled before use. Organic acids and salts were also obtained from Merck, except for calcium L-lactate (Kebo, Stockholm, Sweden), citric acid (Riedel-de Haen, Seelze-Hannover, F.R.G.), lithium acetoacetate (Sigma, St. Louis, MO, U.S.A.) and fumaric acid and 3-hydroxybutyric acid (Fluka, Buchs, Switzerland).

[1,5-^{14}C]Citric acid, [2,3-^{14}C]fumaric acid, sodium D-3-hydroxy-[3-^{14}C]-butyrate, sodium [1-^{14}C]pyruvate, L-[U-^{14}C]malic acid, sodium 2-oxo-[5-^{14}C]-glutarate, [2,3-^{14}C]succinic acid, sodium L-[U-^{14}C]lactate and ethyl-[3-^{14}C]-acetoacetate were obtained from the Radiochemical Centre (Amersham, U.K.). The last-named was converted into lithium [3-^{14}C]acetoacetate before use [11]. Deuterium oxide (^{2}H$_2$O) was obtained from Norsk Hydro (Oslo, Norway).

SP-Sephadex (40—120 μm), DEAE-Sephadex A-25 (40—120 μm), Sephadex G-25 (20—80 μm) and Sephadex LH-20 (90—150 μm) were obtained from Pharmacia (Uppsala, Sweden).

Imidazole (Merck) was recrystallized in benzene, and *tert.*-butyldimethylchlorosilane (Janssen, Beerse, Belgium) was distilled immediately before mixing; the solution was stored dry at −18°C. The distillation was performed by heating in a tube at 160°C in an oil-bath. Crystals formed in a water-cooled glass bulb connected to the tube and to a drying tube. Tetrabutylammonium bromide and methoxylamine hydrochloride were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and were recrystallized in toluene and methanol, respectively.

Reference compounds

The O-methyloxime-*tert.*-butyldimethylsilyl (MO-tBDMS) derivatives of [3,3,3-^{2}H$_3$]pyruvic, [2,2,4,4,4-^{2}H$_5$]acetoacetic, [3,3-^{2}H$_2$]oxaloacetic and 2-oxo-[3,3-^{2}H$_2$]glutaric acid were prepared from the corresponding unlabelled acids. Sodium pyruvate, lithium acetoacetate and 2-oxoglutaric acid (1 mg each)

were treated for 18 h at 37°C with 0.5 ml of ^2H_2O and 3 mg of sodium ethylate. Oxaloacetic acid was treated for 3 h at room temperature with 0.5 ml of ^2H_2O and 30 mg of sodium ethylate. The samples were acidified with 5 M sulphuric acid and O-methyloximes were formed by addition of 10 mg of methoxylamine hydrochloride. After 20 min, the O-methyloximes were extracted twice with 2 ml of diethyl ether. The solvent was evaporated to dryness and tBDMS derivatives were prepared [6].

The tBDMS derivatives of [2,3,3,3-$^2H_4$]lactic acid, 3-hydroxy-[2,2,3,4,4,4-$^2H_6$]butyric acid and [2,3,3-$^2H_3$]malic acid were prepared from the corresponding perdeuterated oxoacid (1 mg) by addition of 2 mg of NaB^2H_4 to the reaction mixture described above. After 2 h the samples were acidified with 5 M sulphuric acid, extracted twice with 2 ml of diethyl ether and evaporated to dryness and tBDMS derivatives were prepared [6]. A mixture of sodium pyruvate, lithium acetoacetate, calcium L-lactate, 3-hydroxybutyric acid, succinic acid, fumaric acid, oxaloacetic acid, 2-oxoglutaric acid, L-malic acid and citric acid was treated as described below for freeze-clamped biological samples. The derivatized mixture was used as standard in GC—MS.

Animal experiments

Female Sprague—Dawley rats weighing ca. 200 g were used. The abdomen was opened under light ether anesthesia, and a portion of a liver lobe (ca. 0.1 g), the pancreas (0.5 g) and the stomach (1.2 g) were removed and pulverized in liquid nitrogen.

Isolation and derivatization of carboxylic acids

Frozen samples of liver, pancreas or stomach were pulverized in liquid nitrogen, together with 5 ml of 0.6 M perchloric acid containing 10 mg of methoxylamine hydrochloride. After thorough grinding to a powder, the mixture was allowed to thaw and was centrifuged. The pellet was washed twice with 0.5 ml of 0.6 M perchloric acid and the supernatants were combined.

The combined supernatants were transferred by washing twice with 1 ml of redistilled water to a 2-g column of DEAE-Sephadex A-25 (1 cm I.D.) in the hydroxy form. The column was washed with 10 ml of water and eluted with 0.1 M hydrochloric acid. The collection was started ca. 3 ml before elution of a yellowish band and stopped after the elution of this band and an additional 3 ml. If no band was visible, the volumes were determined for each type of sample with a radioactive organic acid. The DEAE-Sephadex gel is partially destroyed by the low pH in the perchloric acid extract, and is thus not reused.

The eluate was transferred by washing with 2 ml of water to a 1-g column of SP-Sephadex C-25 (1 cm I.D.) in the protonated form. The collection was started immediately and 30 ml of water were used for complete elution.

After addition of 2 ml of 2 M ammonium hydroxide, the solution was concentrated to ca. 0.5 ml in a rotary evaporator at less than 40°C. It was acidified with 0.1 ml of 5 M hydrochloric acid and transferred by washing with 1 ml of water and 2 × 1 ml of water-saturated butanol to a 4-g column of Sephadex G-25 (1 cm I.D.). The column of Sephadex G-25 was packed in butanol-saturated water and equilibrated and eluted with water-saturated butanol. The first 10 ml were discarded and the following eluate was collected until a yellow-

ish band and an additional 3 ml were eluted. If the colour of the band was too weak the phosphate concentration was determined in 3-ml fractions by a spectrophotometric method [12]. The fractions were pooled so that the phosphate content was less than 0.3 μmol.

The solution was concentrated to 2—3 ml in a rotary evaporator at 40°C after addition of 2 ml of 2 M ammonium hydroxide and 5 mg of tetrabutylammonium bromide. The residue was transferred with 2 ml of 2 M ammonium hydroxide and 2 × 5 ml absolute ethanol to a flask that had been silanized with 5% (v/v) dimethyldichlorosilane in toluene. The solvent was removed in a rotary evaporator at 45°C. Complete drying was achieved by inserting a glass bulb filled with 4-Å molecular sieve between the flask and the evaporator.

The tBDMS derivatives were formed in the flask by addition of 0.5 ml of silylating mixture (0.5 M tert.-butyldimethylchlorosilane and 1.25 M imidazole in pyridine). After 1 h at 60°C the content was transferred with 2 × 2 ml hexane to a small tube and washed with 1 ml water. The hexane phase was dried with anhydrous sodium sulphate.

The sample was concentrated to 0.5 ml under a stream of nitrogen at 40°C and percolated through a 2 × 1 cm column of Sephadex LH-20 in hexane—ethylene dichloride (3:1, v/v) [13]. The column was eluted with 5 ml of this solvent. The sample was concentrated to 50—100 μl under a stream of nitrogen at 40°C.

Gas chromatography and gas chromatography—mass spectrometry

A 25 m × 0.32 mm I.D. fused-silica capillary column with a chemically bonded polydimethylsiloxane phase, CP Sil 5 CB (Chrompack, Middelburg, The Netherlands), was used with a falling-needle solid injection system. Nitrogen (17 cm/sec) and helium (11 cm/sec) were used as carrier gases in GC and GC—MS, respectively. The column temperature was 90°C for 10 min and raised to 250°C at 4°C/min in GC and 60°C for 10 min and raised to 250°C at 5°C/min in GC—MS. Mass spectra were recorded at an electron energy of 22.5 eV and an ion source temperature of 290°C in the LKB 9000 or LKB 2091 instrument.

Radioactivity measurements

Radioactivity was measured in duplicate with a liquid scintillation counter using Aqualuma Plus (Lumac, Schaesberg, The Netherlands).

RESULTS AND DISCUSSION

Recovery

The recovery was studied by addition of ca. $5 \cdot 10^4$ cpm (0.03—0.5 μg) each of ^{14}C-labelled pyruvate, acetoacetate, lactate, succinate, fumarate, 3-hydroxybutyrate, 2-oxoglutarate, malate and citrate in the perchloric acid solution used in the work-up of biological samples. The recoveries were between 25% (acetoacetate) and 91% (2-oxoglutarate) after the final chromatography on Sephadex LH-20 (Table I).

Isolation of carboxylic acids

O-Methyloximes of oxoacids were prepared as compounds suitable for GC

TABLE I

RECOVERY OF ACIDS ADDED TO LIVER EXTRACTS

[14]C-Labelled organic acids were added to liver extracts and samples were prepared for GC by the procedure described in the text.

Compound	Percentage recovery (mean ± S.D.)	n
Lactate	78.6 ± 7.1	4
3-Hydroxybutyrate	86.1 ± 15.4	4
Malate	76.0 ± 2.8	4
Citrate	62.9 ± 8.8	6
Fumarate	63.5 ± 11.1	4
Succinate	75.8 ± 1.7	4
Pyruvate	48.2 ± 6.2	6
Acetoacetate	24.7 ± 2.6	4
2-Oxoglutarate	91.4 ± 13.1	4

and to prevent formation of enol tBDMS ethers. The derivative also prevents exchange of the α-hydrogens with the medium as previously demonstrated [3], and this is necessary in studies on the metabolic incorporation of deuterium [3] or when deuterated compounds are used as standards [1].

The perchloric acid used to quench the metabolic processes is usually removed by precipitation with potassium hydroxide. This removal is incomplete and loss of organic acids may occur. Complete removal was found to be essential for formation of tBDMS derivatives and was achieved by ion-exchange chromatography on DEAE-Sephadex. Neutral and basic components were also removed in this chromatography by washing the column after binding of the acids. The organic acids were eluted with 0.1 M hydrochloric acid [14] without any losses. The elution volumes differed with the amount and type of tissue. Both the volumes discarded and collected were 50 ml for liver samples and half-stomach samples, whereas with pancreas samples, 40 ml were discarded and 30 ml collected.

Amphoteric compounds (phospholipids, bile pigments) were removed on a column of SP-Sephadex [3].

For the elimination of phosphate by partition chromatography it was necessary to reduce the volume of the eluate from the SP-Sephadex column. The losses in the rotary evaporator were minimized by the addition of ammonia and by the low temperature of the water-bath. The low temperature was found to be essential for a high recovery of acetoacetate and 3-hydroxybutyrate, which otherwise are degraded to acetone [15].

The presence of large amounts of phosphate in biological extracts constitutes a problem when tBDMS derivatives are prepared, since stable tBDMS esters of phosphate and pyrophosphate are easily formed [8] and have about the same retention time as some carboxylic acid derivatives in GC. Several methods have been described for removal of phosphate, but they cause extensive loss of citrate [9]. Selective extraction from silica gel has been reported to separate citrate from sulphate and phosphate [16, 17]. We were unable to remove phosphate from purified liver extracts without large losses of citrate by this method, even when it was combined with chromatography in the same system. Attempts to use ion-pair extraction [18] were unsuccessful, whereas

extraction of organic acids as adducts with trioctylphosphine oxide [19] could be used but was impractical since it was difficult to release the acids from the adduct.

Separation of phosphate and citrate was achieved by normal-phase partition chromatography in a butanol—water system with Sephadex G-25 as support. It was essential to acidify the sample. It was possible to remove 95% of the phosphate with a 95% recovery of citrate when the method was applied to samples obtained from different tissues (Fig. 1). The samples usually contained a yellowish material that eluted together with citrate and could be used as a marker in the separations.

Complete removal of water was necessary for the preparation of tBDMS derivatives. When only ammonia was added, at least 85% of pyruvate was lost from liver samples. It was observed that the pH decreased during evaporation to about pH 5, indicating loss of ammonium hydroxide. Possibly the undissociated pyruvic acid methoxime was formed and evaporated. To prevent this, a non-volatile counter-ion was added. Tetrabutylammonium bromide was chosen since it can be easily purified and does not interfere with the derivatization when 5 mg are used. Addition of 25 mg or 50 mg of tetrabutylammonium bromide caused a decreased recovery in the derivatization of citrate to 61% and 34%, respectively. With this procedure, the recovery of [14C]-pyruvate methoxime was improved (Table I).

Derivatization and gas chromatographic analysis

The derivatization was performed in silanized flasks in order to increase removal of the acids from the glass wall. Pyridine was chosen as solvent [7] since phosphate and other inorganic anions react less well in this solvent [8]. The reagent was also more stable in pyridine than in dimethylformamide. The lowest recovery in the derivatization was observed for citrate [78 ± 3% (S.D.), $n=6$]. No difference in this recovery was seen when the reaction was carried out for 30, 60 or 90 min. The amount of reagent could not be decreased without decreasing the recovery of citrate when tetrabutylammonium
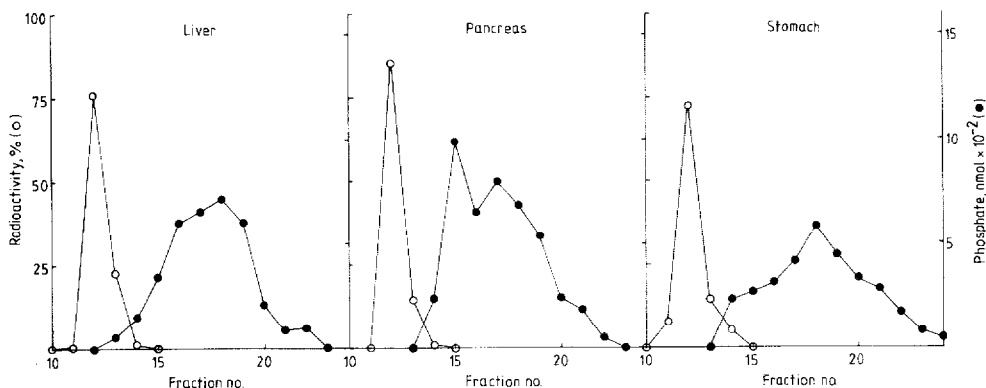

Fig. 1. Elution of [1,5-14C]citrate and phosphate during chromatography of purified rat tissue samples prior to derivatization. A 4-g Sephadex G-25 column was used with butanol-saturated water as stationary phase and water-saturated butanol as mobile phase. The volume of each fraction was 3 ml.
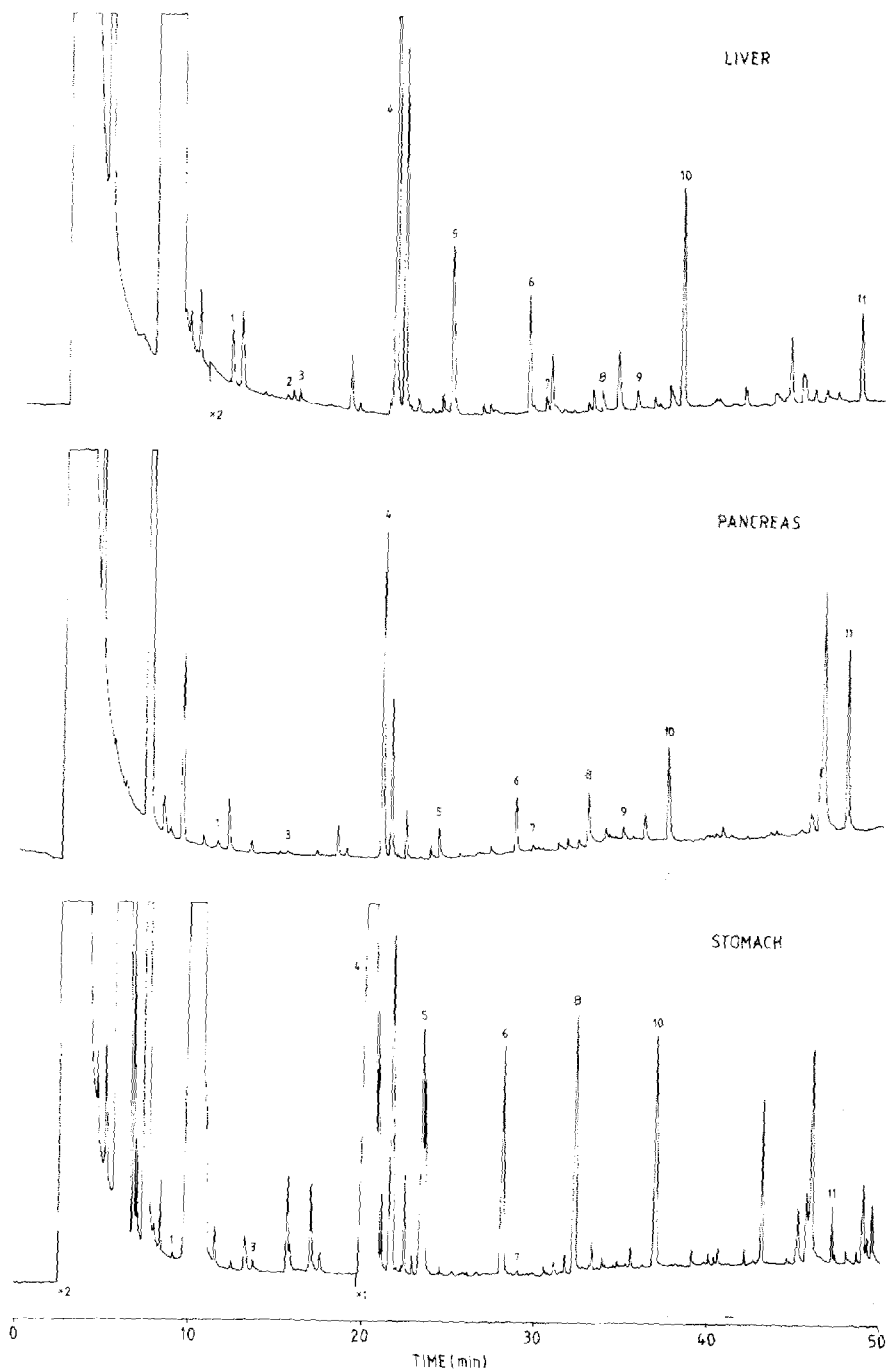
Fig. 2. GC analysis of carboxylic acids in rat tissues. O-Methyloximes were prepared and the carboxylic acids were isolated and analysed as *tert.*-butyldimethylsilyl derivatives using a fused-silica capillary column (CP Sil 5 CB) at 90°C for 10 min and then temperature programmed (4°C/min) to 250°C. The following compounds were identified: pyruvate (1), acetoacetate (2 and 3), lactate (4), 3-hydroxybutyrate (5), succinate (6), fumarate (7), phosphate (8), 2-oxoglutarate (9), malate (10) and citrate (11). The lactate peak contained an unknown contaminant.

TABLE II

MASS SPECTRA OF MO-tBDMS DERIVATIVES OF ORGANIC ACIDS

Compound	Principal ions (except isotope peaks), m/z (relative intensity)							Others above m/z 70 of more than 10% relative intensity
	[M − 15]+	[M − 57]+	189	147	115	75	73	
Lactate	303(2)	261(59)	(52)	(100)	(13)	(27)	(60)	233(30), 159(20)
3-Hydroxybutyrate	317(1)	275(72)	(55)	(39)	(16)	(100)	(65)	233(33), 159(89)
Malate	461(6)	419(86)	(32)	(54)	(68)	(59)	(100)	287(63)
Citrate	633(2)	591(57)	(22)	(52)	(20)	(100)	(79)	459(82), 431(28), 357(31), 269(22), 149(15)
Pyruvate	216(3)	174(100)	—	—	(21)	(21)	(9)	99(10), 89(14), 74(10)
Acetoacetate	230(1)	188(100)	—	—	(10)	(51)	(22)	116(11), 89(47)
Oxaloacetate	374(2)	332(48)	(14)	(53)	(13)	(84)	(100)	98(42)
2-oxoglutarate	388(2)	346(49)	—	(27)	(11)	(100)	(48)	317(16), 244(19), 98(34)
Fumarate	329(4)	287(100)	—	(5)	—	(17)	(26)	
Succinate	331(5)	289(100)	(11)	(67)	(12)	(32)	(85)	

bromide was present. Excess imidazole was removed by water extraction [6] and chromatography on Sephadex LH-20 [13], since it otherwise condensed at the beginning of the capillary column.

The derivatives of Krebs cycle and related acids and of remaining phosphate were completely separated by capillary GC (Fig. 2). The isomers of derivatized acetoacetate formed two peaks.

Mass spectra of MO-tBDMS derivatives of carboxylic acids

The mass spectra of the hydroxyacids (lactate, 3-hydroxybutyrate, malate and citrate, Table II) agreed with those previously described [6]. Perdeuterated lactate, 3-hydroxybutyrate and malate were prepared, and the tBDMS derivatives were analysed. The ions at M − 57 and M − 15 were shifted by the number of ^2H atoms, confirming that these ions are formed by cleavage of $C(CH_3)_3$ [5] and CH_3 from the tBDMS group with no exchange of hydrogens. The peak at m/z 159 in the mass spectrum of the tBDMS derivative of lactate was shifted to m/z 163 to an extent of 80% in the corresponding deuterated compound. This is in agreement with the double origin of this ion as the two halves of the molecule formed by α-cleavage [6]. The similar origin of the ion at m/z 159 in the mass spectrum of the derivative of 3-hydroxybutyrate could also be confirmed, since it was shifted to m/z 163 in the hexadeuterated compound. The ion at m/z 287 in the mass spectrum of the tBDMS derivative of malate was shifted two mass units in the derivative of trideuterated malate, confirming that this ion is formed by loss of $C(CH_3)_3$ and $C(CH_3)_3Si(CH_3)_2OH$ from tBDMS groups [6], with the extra hydrogen originating from the methylene group. The ions at m/z M − 57 − 28 in the mass spectra of the tBDMS derivatives of lactate (m/z 233) and malate (m/z 391, 6% relative intensity) were shifted by the number of ^2H atoms in the perdeuterated compounds, confirming their origin by loss of $C(CH_3)_3$ and CO [6]. The ion at m/z 233 in the tBDMS derivative of 3-hydroxybutyrate was shifted to m/z 237 in the hexadeuterated compound, as expected from its suggested origin by loss of $C(CH_3)_3$ and CH_2CO [6].

Mass spectra of methoxime tBDMS derivatives of oxoacids have not previously been reported. The spectra of the derivatives of pyruvate, acetoacetate, oxaloacetate and 2-oxoglutarate showed intense ions at M − 57 and weak ions at M − 15 (Table II). The origin of these ions by cleavage in the tBDMS group was demonstrated by analysis of the corresponding acids fully deuterated at the carbon atom adjacent to the carbonyl group.

The mass spectra of tBDMS derivatives of fumarate and succinate were similar and were dominated by intense ions at M — 57 (Table II).

In several mass spectra of tBDMS derivatives, ions were seen at m/z 189, 147, 115, 75 and 73 (Table II). The spectra of the deuterated compounds indicated that these ions were formed from the tBDMS group with no hydrogens from the rest of the molecule, supporting the interpretations given by Mawhinney [8].

In all deuterated compounds, the ion at M — 57 appeared to contain all the deuterium. Thus, this ion should be suitable for measurements of the deuterium content in quantitative studies and in metabolic studies using deuterium.

ACKNOWLEDGEMENTS

REFERENCES

1 L. Marai and A. Kuksis, J. Chromatogr., 268 (1983) 447.
2 N.J. Haskins, Biomed. Mass Spectrom., 9 (1982) 269.
3 S. Blomberg and T. Cronholm, Eur. J. Biochem., 101 (1979) 111.
4 E.J. Corey, J. Amer. Chem. Soc., 94 (1972) 6190.
5 G. Phillipou, Lipids, 10 (1975) 714.
6 A.P.J.M. de Jong, J. Elema and B.J.T. van de Berg, Biomed. Mass Spectrom., 7 (1980) 359.
7 M.A. Quilliam and J.B. Westmore, Anal. Chem., 50 (1978) 59.
8 T.P. Mawhinney, J. Chromatogr., 257 (1983) 37.
9 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
10 H. Determann, Gel Chromatography, Springer, Berlin, Heidelberg, New York, 1968, p. 133.
11 L.M. Hall, Anal. Biochem., 3 (1962) 75.
12 G.R. Bartlett, J. Biol. Chem., 234 (1959) 466.
13 R.W. Kelly and P.L. Taylor, Anal. Chem., 48 (1976) 465.
14 R.H. Horrocks, E.J. Hindle, P.A. Lawson, D.H. Orrell and A.J. Poole, Clin. Chim. Acta, 69 (1976) 93.
15 S. Holm and E. Lundgren, Anal. Biochem., 136 (1984) 157.
16 L. Kesner, T.-T. Yao and R.B. Dell, Clin. Chem., 19 (1973) 593.
17 V.P. Williams, D.K. Ching and S.D. Cederbaum, Clin. Chem., 25 (1979) 1814.
18 S.H. Sterri and F. Fonnum, Eur. J. Biochem., 91 (1978) 215.
19 M. Schröder-Nielsen, Acta Pharm. Suec., 13 (1976) 133.