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Short Communication

Use of a thick-film capillary column for the analysis of organic acids in body fluids

Wolfram Meier-Augenstein* and Georg F. Hoffmann

Abteilung Neuropädiatrie, Universitäts-Kinderklinik, Im Neuenheimer Feld 150, 6900 Heidelberg (Germany)

Bonnie Holmes, Jill L. Jones and William L. Nyhan

Department of Pediatrics and Institute of Molecular Genetics 0609-A, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0609 (USA)

Larry Sweetman

Biochemical Genetics Laboratory, Children's Hospital, University of Southern California, Los Angeles, CA 90027 (USA)

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ABSTRACT

An improved method for the identification and quantification of organic acids in body fluids employing capillary gas chromatography–mass spectrometry has been developed. A thick-film capillary column, that combines the properties of a capillary column with those of a megabore column, has been successfully introduced into an existing method. Analysis over a concentration range from 1 μmol/l to 500 μmol/l body fluid is possible. This permits the assay of samples that are usually obtained in small volumes, *e.g.* cerebrospinal fluid.

INTRODUCTION

The analysis of organic acids by gas chromatography–mass spectrometry (GC–MS) is a widely used method for the separation and identification of organic acids in body fluids. Exact determination of organic acids in body fluids is of paramount importance for a definitive diagnosis and therapeutic control of inborn errors of amino acid and organic acid metabolism.

During the past ten years this matter has been approached in two different ways. One method put emphasis on high resolution, and therefore, it employed thin-film capillary columns with a film thickness of 0.25 μm and an internal diameter (I.D.) <0.32 mm [1–3]. However, this resulted in a limited capacity. The other approach aimed to encompass the wide range of concentrations of organic acids found in body fluids. Therefore it employed megabore columns with a film thickness of 1.5 μm and an I.D. of 0.53 mm [4,5], gaining capacity but losing resolution. In this paper

* Corresponding author.

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we report the use of a thick-film capillary column with a film thickness of 1 μm and an I.D. of 0.25 mm that combines the high resolution of capillary columns with the good capacity of megabore columns.

EXPERIMENTAL

Chemicals and reagents

Silicic acid (100–300 mesh) and O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride were obtained from Sigma (St. Louis, MO, USA). The silylating reagents bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and Tri-Sil® were purchased from Pierce (Rockford, IL, USA).

Authentic compounds were obtained from either Sigma or Aldrich (Milwaukee, WI, USA). Compounds that could not be purchased were synthesized. 5-Hydroxycaproic acid was prepared from 2-methylcyclopentanone by oxidation with 3-chloroperbenzoic acid to form the δ-caprolactone [6], which was converted into potassium 5-hydroxycaproate by refluxing the lactone in 4 *M* KOH solution. 3-Hydroxyadipic acid was synthesized by reducing 3-oxoadipic acid with $NaBH_4$ in alkaline solution. To a solution of 100 mg of 3-oxoadipic acid in a mixture of 10 ml of water and 1.27 ml of 1 *M* NaOH, 18 mg of $NaBH_4$ were added in one portion. The reaction mixture was stirred at room temperature for 1 h and 1.27 ml of 1 *M* HCl were added dropwise. The resulting mixture was immediately frozen in liquid nitrogen and subsequently lyophilized to yield 3-hydroxyadipic acid quantitatively. Any delay in freezing the acidified reaction mixture would result in the formation of 2-hexenedioic acid.

Sample preparation

Sample preparation was carried as described in detail by Sweetman [5], except for the following modifications. Sample volumes were 0.5–1 ml for plasma and cerebrospinal fluid (CSF). Sample volumes for urine contained 1 μmol of creatinine. The column silicic acid and of sample silicic acid amounts were 0.5 and 0.4 g, respectively.

Apparatus

GC–MS analysis was carried out on a DB5 fused-silica capillary column (30 m × 0.25 mm I.D., film thickness 1 μm, J & W, Rancho Cordova, CA, USA). The GC–MS system consisted of a Hewlett-Packard HP 5890 Series II gas chromatograph, an HP 7673 autoinjector, an MSD 5971 mass spectrometer, and an HP Vectra 386/25 computer equipped with HP G1034B MS Chem Station software operating under MS Windows 3.0.

The following chromatographic parameters were finally used: the injector was operated in the splitless mode at 280°C; the injected sample volume was 1 μl; the temperature was programmed from an initial temperature of 80°C, kept for 5 min, to 180°C at 3.8°C/min. After 5 min at 180°C, the temperature was programmed to 200°C at 3.9°C/min and to 300°C at 4°C/min and was kept at 300°C for the final 25 min; helium was used as carrier gas with a column head pressure of 49 kPa; the carrier gas velocity was set at 30.41 cm/s corresponding to a flow-rate of 3.58 ml/min.

The mass spectrometer was operated in the scan mode; the temperature of the transfer line was kept at 290°C; scan range from 40 to 650 a.m.u.; solvent delay, 12 min; data were acquired utilizing electron impact (EI) at 70 eV and at an electron multiplier voltage (EMV) of 1900 V.

RESULTS AND DISCUSSION

Starting with the chromatographic conditions of the megabore column method (*i.e.* temperature, 5 min isothermal at 80°C, 80 to 300°C at 4°C/min, 25 min isothermal at 300°C, helium as carrier gas at 27.8 cm/s [4,5]), conditions were systematically changed in order to achieve the separation of former coeluting compounds and the separation of deuterated internal standards from the corresponding unlabelled compounds.

A total ion chromatogram of a standard mixture containing 97 authentic compounds at a concentration of 150 n*M* is shown in Fig. 1. This mixture also contained [$^2H_4$]4-nitrophenol and 2-oxocaproic acid (concentration = 250 n*M*) as internal standards as well as a small volume (0.5 μl

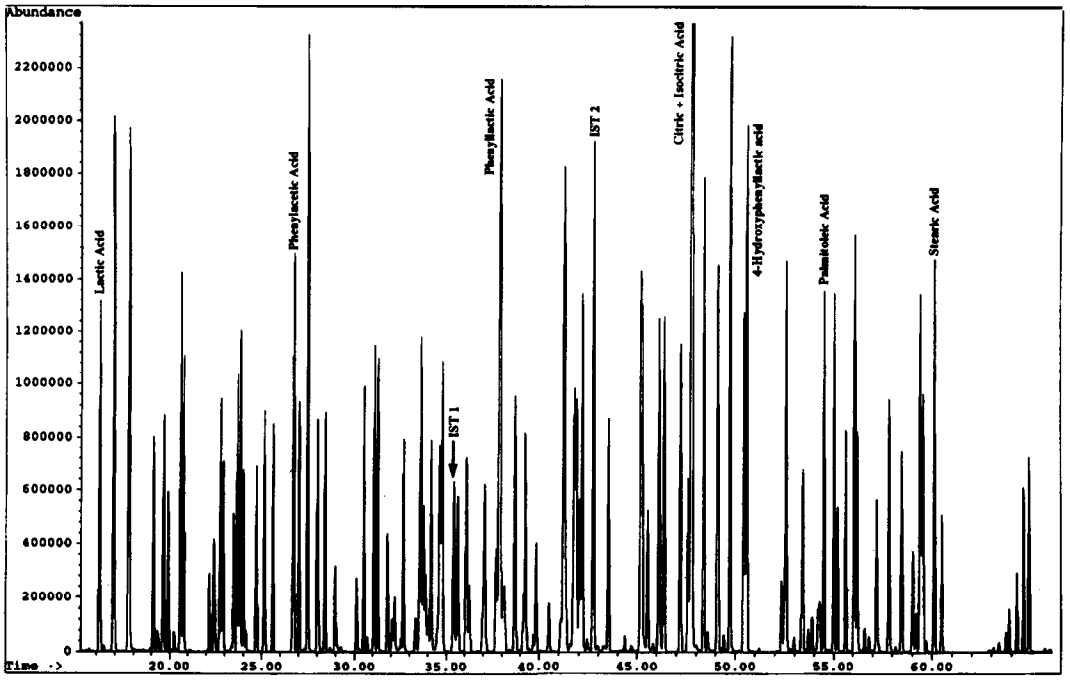

Fig. 1. Total ion chromatogram (TIC) of a standard mixture containing 97 authentic organic acids and glycine conjugates of organic acids. The carrier gas velocity was set at 30.41 cm/s. The temperature was programmed as described in the Experimental section. Compounds were transformed into their corresponding trimethylsilyl and pentafluorobenzyloxime derivatives prior to analysis. The abbreviations IST 1 and IST 2 refer to the internal standards [$^2H_4$]4-nitrophenol and 2-oxocaproic acid, respectively.

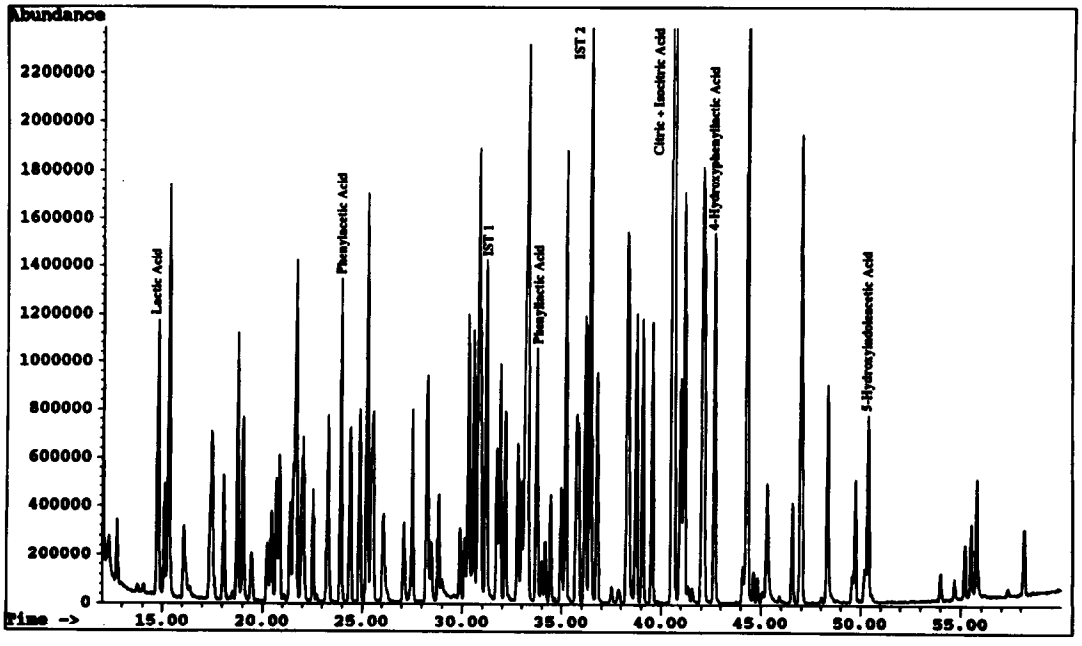

Fig. 2. Total ion chromatogram of the same standard mixture as shown in Fig. 1, analysed on a DB5 megabore column. The temperature was programmed from an initial 80°C, kept for 5 min, to 300°C at 4°C/min and was kept at 300°C for the final 25 min. Helium was used as carrier gas and its velocity was set at 27.8 cm/s.

TABLE I

CAPACITY FACTORS OF ORGANIC ACIDS

Compounds were analysed as their corresponding trimethylsilyl (TMS) derivatives. Oxo groups were transformed into their corresponding O-(2,3,4,5,6-pentafluorobenzyl)oximes (PFBO). Capacity factors are the mean of five analyses using the standard mixture in five different concentrations each.

Compound	Capacity factor (k')	
	Capillary column	Megabore column
Lactic acid	8.93	7.26
Glycolic acid	9.39	7.38
Hexanoic acid	9.42	7.49
2-Hydroxybutyric acid	10.73	8.67
3-Hydroxypropionic acid	11.00	8.99
3-Hydroxybutyric acid	11.64	9.37
3-Hydroxyisobutyric acid	11.66	9.38
2-Hydroxyisovaleric acid	11.75	9.51
2-Methyl-3-hydroxybutyric acid[a]	12.61	10.19
2-Methyl-3-hydroxybutyric acid[a]	12.75	10.32
Malonic acid	12.83	10.30
$C_{12}H_{26}$	12.93	10.18
3-Hydroxyisovaleric acid	12.98	10.44
Methylmalonic acid	13.06	10.53
2-Ethyl-3-hydroxypropionic acid	13.41	10.82
2-Hydroxyisocaproic acid	13.53	10.96
3-Hydroxyvaleric acid	13.62	10.98
4-Hydroxybutyric acid	13.64	10.92
2-Hydroxy-3-methylvaleric acid	13.74	11.14
Benzoic acid	14.17	11.20
Octanoic acid	14.43	11.46
Ethylmalonic acid	14.71	11.87
Phenylacetic acid	15.41	12.22
Succinic acid	15.58	12.50
Methylsuccinic acid	15.86	12.75
Glyceric acid	15.88	12.95
Uracil	16.20	13.07
Fumaric acid	16.45	13.12
5-Hydroxyhexanoic acid	16.57	13.38
Propionylglycine (monoTMS)	16.79	13.39
Isobutyrylglycine (monoTMS)	17.49	14.01
Glutaric acid	17.74	14.22
$C_{14}H_{30}$	17.84	14.14
Propionylglycine (diTMS)	18.04	14.49
Isobutyrylglycine (diTMS)	18.08	14.50
3-Methylglutaric acid	18.19	14.62
3-Methylglutaconic acid (*Z*)	18.52	14.93
2-Methylglutaconic acid (*Z*)	18.68	15.29
Glutaconic acid	18.71	15.17
Pyruvic acid (PFBO syn)	18.97	15.75
2-Methylbutyrylglycine (monoTMS)	19.47	15.76
3-Methylglutaconic acid (*E*)	19.56	15.65
Malic acid	19.63	15.90

TABLE I *(continued)*

Compound	Capacity factor (k')	
	Capillary column	Megabore column
Glyoxylic acid (PFBO anti)	19.72	15.74
2-Methylglutaconic acid (*E*)	19.79	15.99
2-Methylbutyrylglycine (diTMS)	19.80	15.86
Isovalerylglycine (monoTMS)	19.88	15.75
Adipic acid	20.26	16.11
Isovalerylglycine (diTMS)	20.33	16.26
Pyruvic acid (PFBO anti)	20.34	16.24
2-Oxobutyric acid (PFBO syn)	20.60	16.37
[$^2H_4$]4-Nitrophenol (I.S. 1)[b]	20.74	16.25
5-Oxoproline	20.87	16.55
3-Methyladipic acid	21.15	16.62
2-Oxoisovaleric acid (PFBO syn)	21.31	16.80
2-Oxobutyric acid (PFBO anti)	21.46	16.84
Tiglylglycine (diTMS)	21.69	16.86
Mevalonic acid	21.77	17.27
2-Oxoisvaleric acid (PFBO anti)	21.98	17.12
3-Methylcrotonylglycine (diTMS)	22.12	17.32
3-Methylcrotonylglycine (monoTMS)	22.21	17.18
2-Hydroxyglutaric acid	22.25	17.38
2-Hydroxyphenylacetic acid	22.26	17.31
3-Hydroxyglutaric acid	22.28	17.38
Tiglylglycine (monoTMS)	22.34	17.25
Acetoacetic acid (PFBO syn)	22.58	17.45
Phenyllactic acid	22.76	17.65
2-Methylacetoacetic acid (PFBO syn)	23.02	17.71
3-Hydroxy-3-methylglutaric acid	23.10	17.90
Acetoacetic acid (PFBO anti)	23.28	17.89
$C_{16}H_{34}$	23.36	17.70
2-Oxoisocaproic acid (PFBO syn)	23.39	18.02
2-Methylacetoacetic acid (PFBO anti)	23.46	18.02
2-Oxoisocaproic acid (PFBO anti)	24.06	18.51
Hexanoylglycine (monoTMS)	24.22	18.33
Hexanoylglycine (diTMS)	24.31	18.36
4-Hydroxyphenylacetic acid	24.34	18.44
N-Acetylaspartic acid (diTMS)	24.58	18.74
Lauric acid	24.64	18.56
2-Oxocaproic acid (PFBO syn) (I.S. 2)[b]	24.70	18.80
2-Hydroxyadipic acid	24.90	18.98
3-Hydroxyadipic acid	25.22	19.19
2-Oxocaproic acid (PFBO anti) (I.S. 2)[b]	25.23	19.13
Suberic acid	25.71	19.33
Orotic acid	26.68	20.20
Aconitic acid	26.77	20.15
4-Hydroxyphenylpropionic acid	27.30	20.31
Homovanillic acid	27.43	20.59
Azelaic acid	27.97	20.86
Hippuric acid (diTMS)	28.20	21.32
$C_{18}H_{38}$	28.21	20.88
Isocitric acid	28.29	21.42

(Continued on p. 132)

TABLE I *(continued)*

Compound	Capacity factor (k')	
	Capillary column	Megabore column
Citric acid	28.31	21.45
Methylcitric acid (2*S*,3*S* + 2*R*,3*R*)	28.69	21.76
Methylcitric acid (*meso*)	28.82	21.87
Hippuric acid (monoTMS)	29.13	21.69
Myristic acid	29.14	21.64
3-Methoxy-4-hydroxymandelic acid	29.52	22.25
Sebacic acid	29.96	22.36
4-Hydroxyphenyllactic acid	30.04	22.58
2-Oxoglutaric acid (PFBO syn)	31.11	23.36
2-Oxoglutaric acid (PFBO anti)	31.26	23.48
Phenylpyruvic acid (PFBO syn)	31.38	23.62
Phenylpropionylglycine (diTMS)	31.77	23.72
Phenylpyruvic acid (PFBO anti)	31.94	23.92
$C_{20}H_{42}$	32.06	23.76
Phenylpropionylglycine (monoTMS)	32.26	24.26
Palmitoleic acid	32.44	–
Palmitic acid	32.76	24.65
2-Oxoadipic acid (PFBO anti)	32.77	24.71
3-Oxoadipic acid (PFBO syn)	32.87	24.91
3-Hydroxydecanoic acid	32.90	24.82
3-Oxoadipic acid (PFBO anti)	33.13	25.02
Dodecanedioic acid	33.40	25.27
N-Acetyltyrosine (triTMS)	33.49	25.31
Transcinnamoylglycine (diTMS)	33.73	25.51
N-Acetyltyrosine (diTMS)	34.09	25.76
Indoleacetic acid (triTMS)	34.87	26.49
Transcinnamoylglycine (monoTMS)	35.20	26.57
$C_{22}H_{46}$	35.32	26.41
5-Hydroxyindoleacetic acid	35.44	26.89
Oleic acid	35.53	26.88
Stearic acid	35.89	27.13
Suberylglycine (diTMS)	36.11	27.39
4-Hydroxyphenylpyruvic acid (PFBO syn)	36.15	27.50
4-Hydroxyphenylpyruvic acid (PFBO anti)	36.44	27.88
Succinylacetone (diPFBO)[a]	38.08	29.21
$C_{24}H_{50}$	38.19	28.86
Succinylacetone (diPFBO)[a]	38.42	29.49
Succinylacetone (diPFBO)[a]	38.63	29.66
Succinylacetone (diPFBO)[a]	38.80	29.83
$C_{26}H_{54}$	40.87	31.19

[a] Absolute configuration was not determined.
[b] I.S. = internal standard.

per 100 μl of derivatized sample) of a mixture of even-numbered alkanes (C_{12}–C_{26}) serving as time reference compounds.

In comparison with the established method the separation was appreciably improved (Table I, Fig. 2). For instance, pyruvic acid and internal standard 1, [$^2H_4$]4-nitrophenol, were satisfactorily separated on the capillary column. However, some compounds could not be separated satisfactorily: a separation factor $\alpha < 1.0008$ was ob-

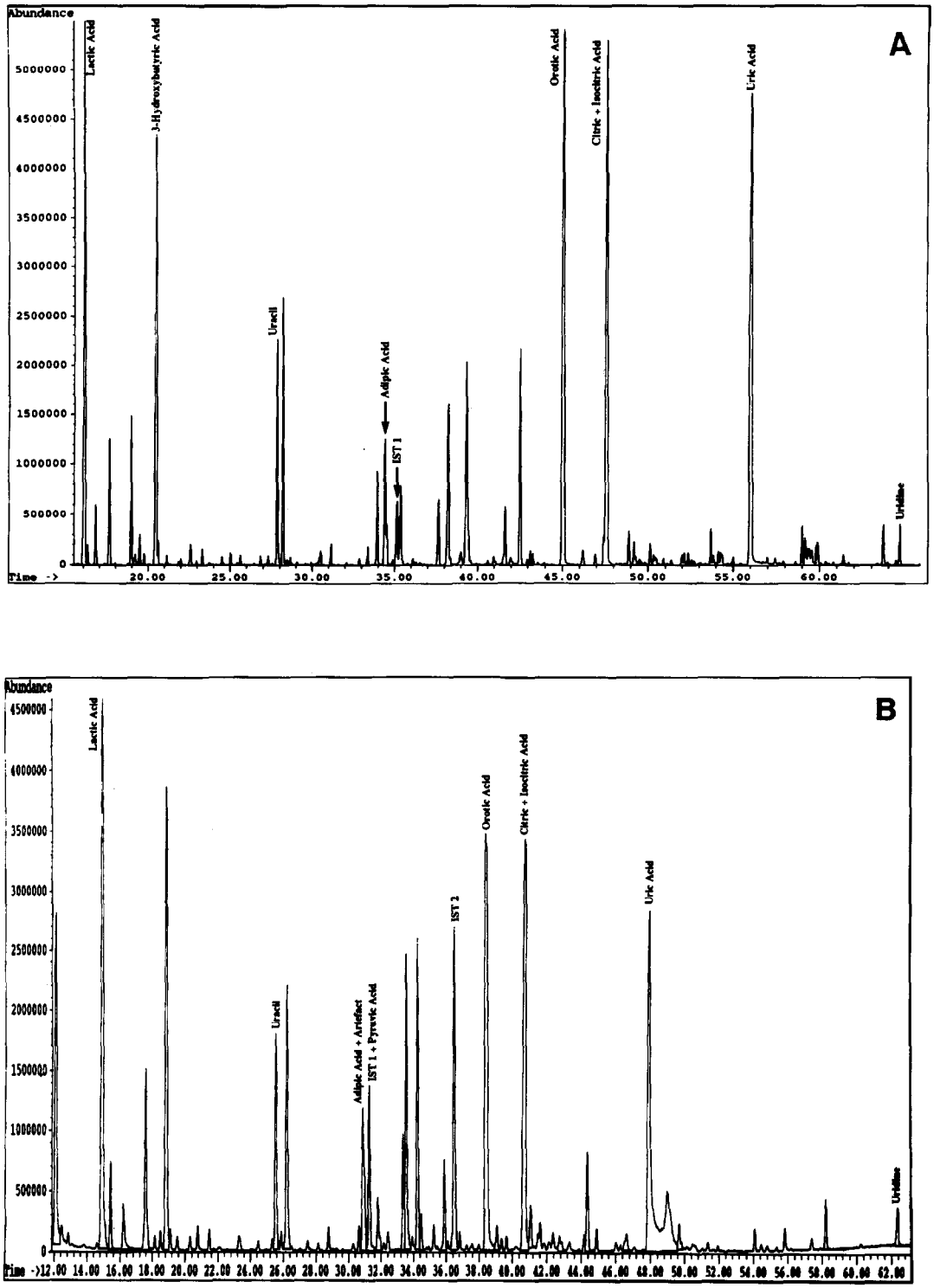

Fig. 3. (A) Total ion chromatogram of a urine sample obtained from a thirteen-year-old boy with OTC deficiency. Chromatographic conditions were as in Fig. 1 but the carrier gas velocity was 31.50 cm/s. (B) Total ion chromatogram of the urine sample obtained from the same patient as in (A) analysed on a DB5 megabore column. Chromatographic conditions were as in Fig. 2.

TABLE II

SEPARATION OF DEUTERATED COMPOUNDS FROM THEIR UNLABELLED ANALOGUES

Compounds are analysed as their corresponding TMS derivatives. Capacity factors are the mean of five runs with a deviation of ± 0.02.

Compound	Capacity factor (k')	Separation factor (α)
[$^2H_4$]Lactic acid	8.86	1.006
Lactic acid	8.93	
[$^2H_4$]Glutaric acid	17.64	1.005
Glutaric acid	17.74	
[$^2H_4$]3-Hydroxy-3-methylglutaric acid	23.02	1.003
3-Hydroxy-3-methylglutaric acid	23.10	
[$^2H_4$]4-Hydroxy-phenylacetic acid	24.25	1.003
4-Hydroxyphenylacetic acid	24.34	
[$^2H_4$]Citric acid	28.24	1.002
Citric acid	28.31	
[$^2H_2$]Hippuric acid	29.09	1.001
Hippuric acid	29.13	
[$^2H_4$]Sebacic acid	29.87	1.003
Sebacic acid	29.96	

served in the case of 2-hydroxyglutaric acid, 2-hydroxyphenylacetic acid and 3-hydroxyglutaric acid, and also in the case of hippuric acid and myristic acid. These compounds usually do not occur simultaneously in biological samples.

A total ion chromatogram of a urine sample obtained from a thirteen-year-old boy suffering from ornithine transcarbamylase (OTC) deficiency is shown in Fig. 3A. The large peak of orotic acid and the elevated peak of uridine assist in the diagnosis and management of this condition. Fig. 3 illustrates the separation of pyruvic acid and [$^2H_4$]4-nitrophenol. This is important because in the presence of a large peak of pyruvic acid, [$^2H_4$]4-nitrophenol could coelute making quantification impossible. In comparison to Fig. 3A, Fig. 3B shows the megabore column analysis of the above-mentioned urine sample.

Using five-point calibration curves most compounds could be quantified with an average error of ±9% whereas for the glycine conjugates the error was of the order of ±14%.

In the case of the deuterated internal standards and the corresponding unlabelled compounds good separations were achieved with an average separation factor α of 1.004 (Table II). The quality of this separation is comparable to that of enantiomeric compounds on a capillary column coated with a chiral stationary phase like heptakis(2,3,6-tri-O-propyl)-β-cyclodextrin in OV 1701-OH [7].

Better separation permitted the identification of artefacts that coeluted with authentic compounds on the megabore column, *e.g.* trisiloxane-1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy] coeluted with 2-hydroxyisovaleric acid. There is evidence that, under the conditions of trimethylsilylation, a hitherto undescribed trimethylsilylated elimination product of hippuric acid is formed.

At concentrations above 500 μmol/l in body fluid, front tailing of some peaks can be observed requiring dilution of the sample to avoid overloading of the column. Nevertheless the use of a thick-film capillary column is an excellent alternative for a megabore column. Sample volumes as small as 0.5 ml are sufficient. This permits the investigation of material that is usually available in small amounts only, such as CSF. Furthermore, the separations achieved for the deuterated and undeuterated compounds will permit their use as internal standards, which should result in better quantification.

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REFERENCES

1 K. J. Ng, B. D. Andresen and J. R. Bianchine, *J. Chromatogr.*, 228 (1982) 43.
2 M. Tuchman, L. D. Bowers, K. D. Fregien, P. J. Crippin and W. Krivit, *J. Chromatogr. Sci.*, 22 (1984) 198.
3 M. Y. Tsai, C. Oliphant and M. W. Josephson, *J. Chromatogr.*, 341 (1985) 1.
4 G. F. Hoffmann, S. Aramaki, E. Blum-Hoffmann, W. L. Nyhan and L. Sweetman, *Clin. Chem.*, 35 (1989) 587.
5 L. Sweetman, in F. A. Hommes (Editor), *Techniques in Diagnostic Human Biochemical Laboratories: A Laboratory Manual*, Wiley-Liss, New York, 1991, Ch. 11, p. 143.
6 C. Lüthy, P. Konstantin and K.G. Untch, *J. Am. Chem. Soc.*, 100 (1978) 6211.
7 W. Meier-Augenstein, B. V. Burger, H. S. C. Spies and W.J.G. Burger, *Z. Naturforsch. B*, 47 (1992) 877.