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Quantitative organic acid analysis in cerebrospinal fluid and plasma: reference values in a pediatric population

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ABSTRACT

Quantitative reference values for the concentrations of organic acids in cerebrospinal fluid (CSF) and plasma, as well as ratios of individual organic acids between CSF and plasma, were determined in twenty-three pairs of samples from pediatric patients. Twenty-six organic acids were present and quantifiable in all or the majority of plasma and CSF specimens (limit of detection 1 μmol/l). There were substantial differences between subgroups of organic acids, best reflected by the ratios of individual acids between CSF and plasma. Metabolites related to fatty acid oxidation were present in CSF in substantially lower amounts than in plasma. Organic acids related to carbohydrate and energy metabolism and to amino acid degradation were present in CSF in equal or slightly lower amounts than in plasma. Finally, some organic acids were found in substantially higher amounts in CSF than in plasma, *e.g.* glycolate, glycerate, 2,4-dihydroxybutyrate, citrate and isocitrate. Quantitation of organic acids in CSF and plasma should aid diagnosis and monitoring of treatment of patients with organic acid disorders.

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INTRODUCTION

Very limited information is available on amounts of organic acids in body fluids other than urine [1]. We have reported normal ranges of organic acids in amniotic fluid from sixteen to nineteen weeks of gestation and in plasma and urine of adults [2]. Reference values for urine in children were subsequently reported [3]. Quantitative data are not available for organic acids in plasma of children or in cerebrospinal fluid (CSF) of any age group. It was the aim of this investigation to employ an improved method for quantitation of organic acids in physiological fluids [2,3] to establish reference values for these compounds in CSF and plasma in a pediatric population. The study of CSF appeared especially important, as it has become increasingly apparent that disorders of organic acid metabolism are responsible for a wide spectrum of neurological disease [4].

EXPERIMENTAL

Patient samples

From 1984 to 1989 more than 1000 pairs of specimens of CSF and plasma were obtained simultaneously from pediatric patients, who were undergoing a diagnostic lumbar puncture during the investigation of suspected lyme disease in northern Germany [5]. Whenever possible, CSF and plasma samples were put aside for investigation after completing the immunological studies. We used 23 paired specimens of CSF and plasma for compiling reference values of organic acids in CSF and plasma. The reference group consisted of 9 girls and 14 boys, aged 1.3 to 14 years. In these patients there was no evidence of neurological disorder, malignant disease or inborn error of metabolism. Immunological studies gave no evidence of lyme disease. The erythrocyte content was $<100/\mu l$, the number of white blood cells was $<10/\mu l$, the protein content was <0.5 g/l, and glucose concentrations were normal.

Reagents

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride, silicic acid (Sil-R Mesh 100+), and most standard compounds were obtained from Sigma (St. Louis, MO, USA). The stable ^{2}H-labelled internal standard 4-nitrophenol was purchased from MSD Isotopes (Pointe-Clare-Dorval, Quebec, Canada). Silylating reagents, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and TriSil, were obtained from Pierce (Rockford, IL, USA). 2-Methyl-2-butanol and chloroform (Burdick & Jackson, Muskegon, MI, USA) were high-purity high-performance liquid chromatography solvent grade.

Preparation of silicic acid

Silicic acid was sieved and the 200–400 mesh fraction dried overnight at 130°C. "Sample" silicic acid was cleaned by mixing 60 g of dried silicic acid with 17.7 ml of 0.1 *M* HCl in methanol, suspending it in pure chloroform and washing it with 700 ml of 2-methyl-2-butanol 40% (v/v) in chloroform in a preparative glass column. Sample silicic acid was redried overnight at 130°C. "Column" silicic acid was prepared by mixing 50 g of sample silicic acid with 30 ml of 0.05 *M* H_2SO_4.

Sample preparation

Sample preparation was identical for CSF and plasma. Samples were stored at −20°C for up to two years until analysis. The methodology has been described in detail [2,3,6,7]. In brief, the samples were thawed, and 0.5–2 ml were taken for analysis. Two internal standards, 2-oxocaproic acid for oxo acids, ketones and aldehydes, and [$^2H_4$]4-nitrophenol for others, were added. The pH was adjusted to 2–3, and 10 mg of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride were added to form oxime derivatives of oxo acids, ketones and aldehydes. After 2 h the samples were titrated back (pH 8–9) to prevent losses during the subsequent lyophilization. The organic acids were quantitatively isolated from the lyophilized samples by liquid partition chromatography on silicic acid. Samples were acidified with 300 μl of 0.25 *M* H_2SO_4 (pH 1), adsorbed on 0.75 g of sample silicic acid, placed on top of 1 g of column silicic acid and eluted

with *ca.* 30 ml of 42% 2-methyl-2-butanol in chloroform (v/v). The exact elution volume must be determined periodically, as the volume that achieves best separation between glyceric acid, the last organic acid to elute, and sulphuric acid. The eluate was titrated with 10 m*M* NaOH to a yellow color of 4-nitrophenol as a measure of the total organic acid content of the sample. As many organic acids contain two or three carboxyl groups, the amount of total organic acid found is reported in mequiv./l per sample of CSF or plasma. The eluate was subsequently dried under nitrogen. Trimethylsilyl derivatives were formed by adding 300 μl of equivolume Trisil–BSTFA to the dry residue and heating the samples for 2 h at 60°C.

Analysis by gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC–MS) was carried out on a DB5 Megabore (30 m × 0.53 mm I.D.) fused-silica capillary column with a 1.5-μm bonded film (J&W, Rancho Cordova, CA, USA) connected to a Hewlett-Packard MSD 5971A mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). The starting column temperature was 80°C, programmed at 4°C/min to 300°C and held there for 15 min for a total run time of 75 min. After the GC–MS run, data were automatically analysed qualitatively and quantitatively using the HP MS ChemStation (DOS series) software (Hewlett-Packard). When metabolites were identified on the basis of retention times and confirming masses, the concentration of each compound was calculated using a linear fit from standard curves. Identification and quantitation of chromatographically unresolved compounds, *e.g.* 3-hydroxyisobutyric and 3-hydroxybutyric or citrate and isocitrate, were based on unique quantitation and confirmation ions as well as their ratios [3]. After completion of computerized identification and quantitation, the chromatograms were visually examined on the data system display. One of the most important aspects of the procedure was the calibration of the method by carrying as many reference standards as possible through the entire procedure. We calibrated the system for a total of over 170 different compounds, using four-point standard curves containing 10, 50, 150, and 250 nmol for most compounds. This mixture contained most of the commercially available metabolites as well as a number of specifically synthesized compounds. A complete list of compounds studied has been provided [3]. The quantitation was checked weekly by analysing a standard mixture containing 150 nmol of all compounds. The calibration was repeated monthly, or weekly if the quantitation report differed from the expected values by more than 20% for more than ten compounds.

RESULTS

The reference values for organic acids in CSF and plasma have been presented in three subgroups. Table I lists acids that were always present in plasma and CSF in sufficient amounts to be reliably quantified. Table II summarizes the results for metabolites that were detectable and quantifiable in the majority of plasma and/or CSF samples, and Table III lists metabolites that were detectable and/or quantifiable in only a few of our reference plasma or CSF samples. An organic acid chromatogram of a sample of control CSF is shown in Fig. 1, and that of a control plasma in Fig. 2. Fig. 3 shows a plasma sample from a patient with propionic acidemia at the beginning of an acute crisis. The total content of organic acids was increased to 26 mequiv./l due to the elevation of the following metabolites: lactate (2800 μmol/l), 3-hydroxypropionate (47 μmol/l), 3-hydroxyisovalerate (27 μmol/l), propionylglycine (30 μmol/l), pyruvate (400 μmol/l) and methylcitrate (3 μmol/l). The branched-chain oxo acids were also borderline-elevated. There was no significant ketosis.

Twenty-six compounds were present and quantifiable in either all or the majority of plasma and CSF control specimens. Analyses of a series of four different sample volumes of pooled plasma and CSF (0.5, 1, 2 and 3 ml) revealed linear responses for all of these compounds, r = 0.98–0.99. As the limit for reliable quantification

TABLE I

CONCENTRATIONS OF ORGANIC ACIDS DETECTABLE IN ALL CONTROL SAMPLES OF CSF AND PLASMA

All values are in mequiv./l for total organic acids, and μmol/l for individual compounds. The number of paired samples of CSF and plasma was 23. The compounds are listed in the order of their chromatographic appearance, *i.e.* in the order of their methylene units. "Trace" reflects values *ca.* 2 μmol/l, around the limit of detection.

Compound	CSF			Plasma			CSF/plasma		
	Mean (S.D.)	Min.	Max.	Mean (S.D.)	Min.	Max.	Mean (S.D.)	Min.	Max.
Total organic acids	8.4 (3.3)	4	13	12.6 (5.4)	5	19	0.67 (0.45)	0.41	2.0
Lactate	1390 (750)	450	2900	2700 (1110)	1120	4300	0.76 (0.50)	0.24	2.1
Glycolate	94 (112)	Trace	352	12 (7.8)	Trace	25	10.9 (11)	0.8	32
2-Hydroxybutyrate	37 (21)	10	76	64 (41)	21	130	0.67 (0.59)	0.26	1.8
3-Hydroxybutyrate	88 (107)	Trace	343	283 (331)	22	1220	0.26 (0.29)	0.02	0.7
3-Hydroxyisobutyrate	14 (8.3)	Trace	34	22 (14)	6	51	0.72 (0.40)	0.25	1.4
Glycerate	18 (8.6)	Trace	37	6.6 (3.4)	Trace	10	2.9 (1.2)	1.5	4.2
Pyruvate	64 (23)	Trace	102	72 (31)	2	117	0.82 (0.42)	0.24	1.3
Pyroglutamate	45 (23)	18	106	61 (54)	11	251	1.2 (1.22)	0.3	4.1
Citrate	340 (160)	80	570	185 (110)	30	410	2.7 (2.5)	0.6	8.2

TABLE II

CONCENTRATIONS OF ORGANIC ACIDS DETECTABLE IN MOST BUT NOT ALL OF CONTROL CSF AND/OR PLASMA SAMPLES

Values are in μmol/l. The values given in parentheses are the percentages of samples in which the compound could be detected and/or quantified (% Occ.). "Trace" reflects values *ca.* 2 μmol/l, around the limit of detection; N.D. indicates not detectable, *i.e.* < 1 μmol/l.

Compound	CSF			Plasma		
	Mean (% Occ.)	Min.	Max.	Mean (% Occ.)	Min.	Max.
Hexanoate	Trace (52%)	N.D.	Trace	7.2 (79%)	N.D.	55
3-Hydroxypropionate	6.4 (44%)	N.D.	9.5	5.4 (40%)	N.D.	8.4
2-Hydroxyisovalerate	4.8 (85%)	N.D.	12	5.7 (90%)	N.D.	17
Succinate	3 (22%)	N.D.	4	19 (73%)	N.D.	57
2,4-Dihydroxybutyrate	144 (40%)	N.D.	460	2 (20%)	N.D.	4
2-Oxoisovalerate	9.8 (83%)	N.D.	16	12 (72%)	N.D.	25
Acetoacetate	10 (44%)	N.D.	17	31 (33%)	N.D.	66
2-Oxo-3-methyl-*n*-valerate	2 (17%)	N.D.	5	14 (66%)	N.D.	26
2-Oxoisocaproate	4.8 (37%)	N.D.	7.4	18 (72%)	N.D.	48
Laurate	2.1 (44%)	N.D.	3.3	12 (100%)	2	39
Isocitrate	10 (72%)	N.D.	18	6 (40%)	N.D.	10
Myristate	Trace (28%)	N.D.	Trace	30 (100%)	3	80
Palmitoleate	N.D. (0%)	N.D.	N.D.	46 (100%)	5	125
Palmitate	11 (87%)	N.D.	30	345 (100%)	109	970
Linoleate	11 (11%)	N.D.	14	171 (100%)	51	460
Oleate	22 (40%)	N.D.	74	644 (100%)	140	2230
Stearate	10 (87%)	N.D.	27	121 (100%)	43	490

TABLE III

CONCENTRATIONS OF ORGANIC ACIDS DETECTABLE IN ONLY TRACE AMOUNTS OR IN LESS THAN 50% OF CONTROL CSF AND/OR PLASMA SAMPLES

Values are in μmol/l. The values given in parentheses are the percentages of samples in which the compound could be detected and/or quantified (% Occ.). "Trace" reflects values *ca.* 2 μmol/l, around the limit of detection; N.D. indicates not detectable, *i.e.* < 1 μmol/l.

Compound	CSF			Plasma		
	Mean (% Occ.)	Min.	Max.	Mean (% Occ.)	Min.	Max.
2-Hydroxyisobutyrate	N.D. (0%)	N.D.	N.D.	9.1 (13%)	N.D.	11
Benzoate	Trace (63%)	N.D.	Trace	Trace (93%)	N.D.	Trace
Octanoate	N.D. (0%)	N.D.	N.D.	2 (22%)	N.D.	3
Fumarate	N.D. (0%)	N.D.	N.D.	1.7 (26%)	N.D.	5
3,4-Dihydroxybutyrate	6.8 (13%)	N.D.	8.8	N.D. (0%)	N.D.	N.D.
Erythronate	5 (30%)	N.D.	21	2 (13%)	N.D.	5
Malate	N.D. (0%)	N.D.	N.D.	15 (40%)	N.D.	43
2-Hydroxyglutarate	2 (35%)	N.D.	4	1.5 (26%)	N.D.	2
Suberate	N.D. (0%)	N.D.	N.D.	10 (4%)	N.D.	10
Aconitate	4 (4%)	N.D.	4	N.D. (0%)	N.D.	N.D.
Azelate	44 (9%)	N.D.	67	52 (13%)	N.D.	72
Hippurate	N.D. (0%)	N.D.	N.D.	4 (17%)	N.D.	6.5

(≤20% coefficient of variation for day-to-day determinations) for most compounds by this method is *ca.* 2–5 nmol, and most metabolites of interest in CSF and plasma occur in the low micromolar range (Tables I–III), the ideal sample volume is 1–2 ml. It is, however, possible to analyse samples down to 0.5 ml of CSF or plasma and still obtain sufficient sensitivity for quantitative evaluation of the major metabolites. A qualitative evaluation of all the commonly present organic acid metabolites listed in Tables I and II is possible down to 0.1 ml of sample. Bearing in mind the very limited number of samples in different age groups, no obvious age or sex dependencies could be detected and the data from all samples were combined. The values for two acids, lactate and glycolate, seemed to decrease after infancy, but not to a statistically significant extent.

In general, concentrations of organic acids in CSF were lower than in plasma. This was reflected in the total content of organic acids, which averaged 8.4 mequiv./l in CSF and 12.6 mequiv./l in plasma. However, there were substantial differences between different subgroups of organic acids. These differences are reflected in the ratios of individual acids between CSF and plasma. Many compounds were present in CSF in substantially lower amounts than in plasma: all the metabolites related to fatty acid oxidation, acetoacetate and 3-hydroxybutyrate, long- and medium-straight-chain fatty acids, as well as acids of the tricarboxylic acid cycle, such as succinate, malate, fumarate and 2-oxoglutarate. Long-chain fatty acids were not regularly detectable in CSF. Citrate and isocitrate were found in higher concentrations in CSF than in plasma.

Lactate and pyruvate were present in CSF in equal or slightly lower amounts than in plasma. Certain organic acids in amino acid catabolic pathways, such as 2-hydroxybutyrate, 3-hydroxypropionate, 3-hydroxyisobutyrate, 2-hydroxyisovalerate, pyroglutamate and the branched-chain oxo acids had ratios of *ca.* 0.6–1.2.

Some organic acids were found in substantially higher amounts in CSF than in plasma. These included glycolate, glycerate, and 2,4-dihydroxybutyrate, as well as citrate and isocitrate. Similar relations seemed true for 3,4-dihydroxybutyrate, erythronate and 2-hydroxyglutarate, which

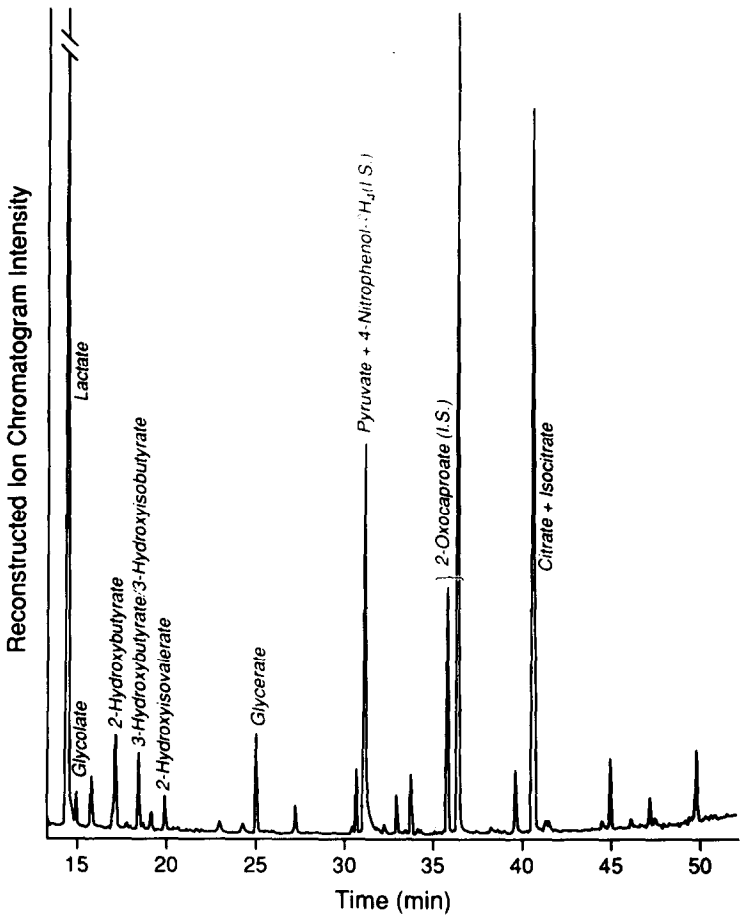

Fig. 1. Reconstructed total-ion GC–MS chromatogram of organic acids as pentafluorobenzyloxime trimethylsilyl derivatives of 1 ml of control CSF.

could, however, not be reliably quantitated in the majority of samples (Table III). These observations are of special interest, because some of these organic acids are known to be intermediates in neurotransmitter pathways. Glycolate and 3,4-dihydroxybutyrate are products of the catabolic pathway of 4-aminobutyrate (GABA) via 4-hydroxybutyrate [8].

In addition to the metabolites listed in Tables I–III, a number of additional known as well as some unknown compounds were detected in substantial amounts in plasma and/or CSF samples of the pediatric control group, and subsequently in some samples of patients under investigation for unexplained neurological diseases. These included drug metabolites, which are also detected in urinary analyses of organic acids of patients, *e.g.* caffeine and derivatives of valproic acid. In general, there were far fewer exogenous compounds present in high concentrations to interfere with organic acid analysis in CSF or plasma than in urine. Some metabolites were also regularly identified and quantified by this method, for which better clinical chemical methods exist and/or for which reliable quantification would require a special handling of the samples, *e.g.* uric acid, ammonia, and cholesterol. These compounds were therefore not included in Table I.

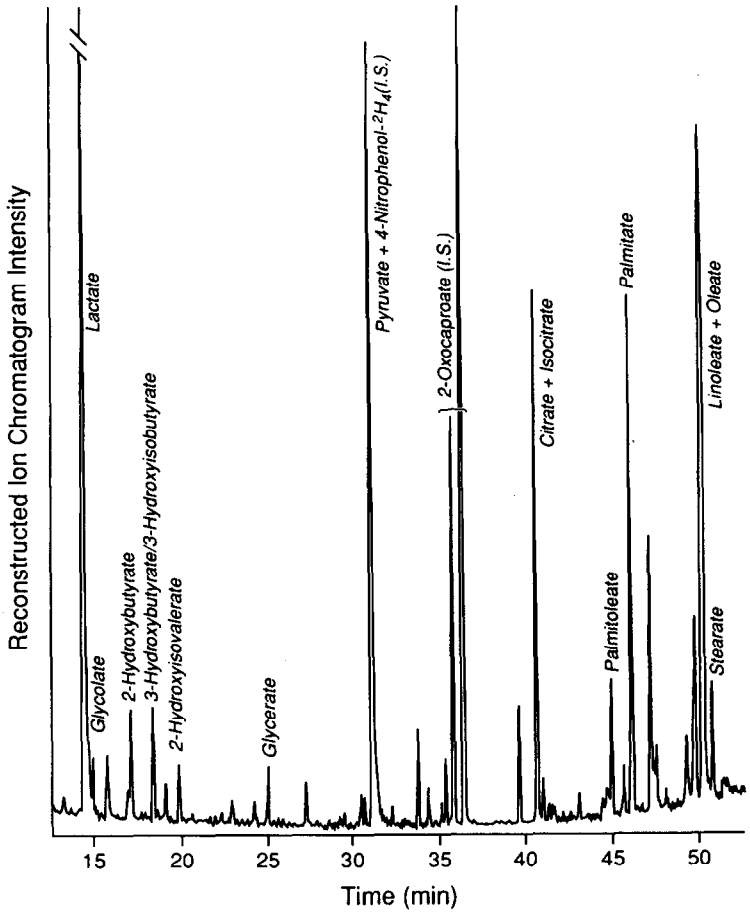

Fig. 2. Reconstructed total-ion GC–MS chromatogram of organic acids as pentafluorobenzyloxime trimethylsilyl derivatives of 1 ml of control plasma.

DISCUSSION

The diagnosis of inborn errors of metabolism is a rapidly expanding area. Organic acids comprise key metabolites in virtually all pathways of intermediary metabolism. Comprehensive quantitative analysis of organic acids in body fluids has therefore the potential of yielding information about the physiological and pathophysiological status of different metabolic pathways, as well as their interrelationships. The very complexity and diversity of organic acids have, however, hampered the quantitative analysis of organic acids. A critical step in organic acid analysis is the extraction of the acids from physiological fluids. This is still usually accomplished by extraction with organic solvents or anion-exchange methods [1], and the results are semi-quantitative. These methods are mainly suited to screen for grossly elevated amounts of organic acids in urine. More subtle abnormalities, such as in partial or vitamin-responsive inborn errors of metabolism, or the methylmalonic aciduria of transcobalamin II deficiency [9], can be easily missed. A quantitation of organic acids in other body fluids, such as plasma, cerebrospinal or amniotic fluid, down to the micromolar range has not been possible. Previous studies on organic acids in CSF reported only four organic acids in control samples: 2-hydroxybutyrate, 3-hydroxy-

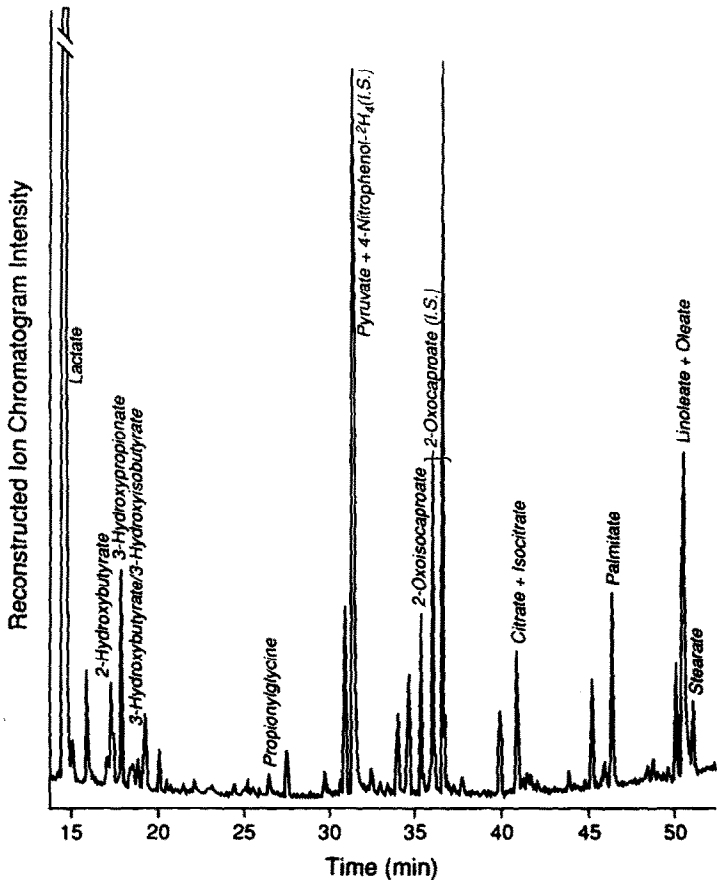

Fig. 3. Reconstructed total-ion GC–MS chromatogram of organic acids as pentafluorobenzyloxime trimethylsilyl derivatives of 1 ml of plasma from a patient with propionic acidemia.

butyrate, palmitate and stearate [10]. Eight additional compounds were found occasionally. These data were obtained using solvent extration, with the limitation of a low and variable recovery of more polar organic acids, such as lactate and citrate, which are the most prominent organic acids in CSF (Table I) [1]. Quantitative analysis of organic acids in CSF and plasma therefore requires quantitative isolation of as wide range of organic acids as possible, which can be achieved by batchwise liquid partition chromatography [2,3].

In interpreting the data presented in this paper, it must be considered that, although care was taken to obtain reference samples that in retrospect appeared to be as normal as possible, all children suffered from illnesses that had been considered to require a diagnostic lumbar puncture. Presenting symptoms included febrile convulsions, headaches, intermittent strabismus and paralysis of an extremity. In some instances, anti-inflammatory, anticonvulsive or other drugs had been administered to the patients prior to lumbar puncture. It may be that the specimens used in this study might reflect a greater variation of some metabolites than would have been found in completely healthy children receiving no medication. Healthy children are not generally considered ethically reasonable subjects for lumbar puncture.

Greater variability of some organic acids was observed in the pediatric control samples in this

study than in the previously reported data on organic acids in plasma of ten healthy adults [2]. The latter samples were collected in a more standardized procedure in the morning and stored until analysis without any other unrelated investigations performed with them. There was an overall higher total content of organic acids in plasma in the pediatric control group. The total content of organic acids in plasma averaged 12.6 mequiv./l in the pediatric control group compared with 6.8 mequiv./l as originally reported for healthy adult controls [2]. The means and ranges for some acids, such as lactate, glycolate, 2-hydroxyisobutyrate, 2-hydroxyisovalerate, glycerate and pyroglutamate, were quite similar in the two groups. Plasma samples from the control children exhibited higher concentrations of metabolites related to fatty acid oxidation, such as acetoacetate and 3-hydroxybutyrate, straight-chain fatty acids, as well as of some dicarboxylic acids not found in adults. There was great variability in the concentrations of these acids, which might reflect different degrees of catabolism or relatively prolonged fasting in moderately ill children.

The methods of obtaining and handling the samples are relevant to the achievment of optimal analytical results. It appeared in this study that substantial loss of some oxo acids had occurred in some control samples. This was most likely the result of inappropriate handling (not immediate freezing) and prolonged storage of the samples at −20°C instead of −80°C. Oxo acids, such as pyruvate, were found to be at lower levels than in previous investigations [2,7].

Indications for quantitation of organic acids in CSF and plasma have not yet been established. A major use of plasma and CSF normal ranges is the possibility of investigating samples from children who were gravely ill and/or deceased and from whom urine could not be obtained after presentation. In most organic acid disorders the elevation of diagnostic metabolites in plasma and CSF are of the order of 10–100 μmol/l rather than in the millimolar range, and so the improved sensitivity and specificity of the discussed method are advantageous for reliable evaluation. Quantitative organic acid analysis in plasma should also be performed to monitor the treatment of organoacidopathies. Concentrations of metabolites in CSF exceeding those of plasma may further signify a special role in cerebral metabolism. An increase of methylmalonate in CSF out of proportion to plasma has been described in patients with cobalamin deficiency [11]. Lactate elevation specific to CSF has been described in biotinidase deficiency [12] and in some mitochondriopathies [13]. In 4-hydroxybutyric aciduria, the key metabolite 4-hydroxybutyrate, which is clearly part of an important pathway in the nervous system as well as a known neuropharmacological agent [15], is found to be increased in CSF out of proportion to plasma [16,17]. In non-ketotic hyperglycinemia the elevated ratio of glycine in the CSF to that of plasma is currently the approach of choice to definitive diagnosis [17]. These observations highlight the need for quantitative analysis of metabolites for diagnosis [12] as well as for the monitoring of therapy [12,15,18].

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