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Analysis of Organic Acids in Human Fluids by Capillary Gas Chromatography: A New Approach

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Organic acids analysis is a powerful technique in the diagnosis of inborn errors of metabolism. Carboxylic acids in plasma or urine can be conveniently determined by capillary gas chromatography (GC), following treatment with ethyl chloroformate (ECF). We propose a rapid, simple methodology for routine analysis of human fluids to detect eleven organic acids related to metabolic disease. With this methodology, isolation of the compounds of interest is not necessary. The run time is 14 minute.

Key words: Organic acids, heritable disorders, methylmalonic acidemia, screening, capillary gas chromatography, diagnostic aids

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Introduction

Organic acids occurring in physiological fluids comprise key metabolites of virtually all pathways of intermediary metabolism and may also be derived from exogenous sources. Exact determination by capillary gas chromatography of organic acids in body fluids is of paramount importance for a definitive diagnosis and therapeutic control of inborn errors of amino and organic acid metabolism (Tanaka ***et al.***, 1980). Earlier used methodology for quantitative analysis of organic acids in fluids is based on oximation of ketoacids with pentafluorobenzylhydroxylamine (2h at pH 2-3), lyophilization of the sample, isolation of the acids by liquid partition chromatography on silica, evaporation of the eluate and subsequent silylation of the dry residue with trisilil-N,O-bis(trimethylsilyl)trifluoroacetamide (2h at 60°C), (Hoffman ***et al.***, 1989; Sweetman, 1991; Bonham ***et al.***, 1994; Duez ***et al.***, 1996). The use of ECF as derivatizing agent presents a completely new approach to profiling organic acids in biological fluids (Husek and Liebich, 1994; Husek, 1995).

This report shows a marked simplification of the two ways: in sample preparation and derivative formation. It's shown that oximation of ketoacids is not necessary since the biological sample, plasma and urine deproteinized by mixed organic solvent can be treated by ethylchloroformate (ECF) ***in situ*** (Husek and Liebich, 1994; Husek, 1997).

The aim of this study was to shown our experience in application of this methodology to patients with suspicion diagnosis of organic acidemia (aciduria). We described a simple practical capillary gas chromatographic method of plasma and urine organic acid analysis, which can be used in organic acidemia screening programs.

Materials and Methods

Organic solvents such as acetonitrile, ethanol, n-hexane, chloroform, pyridine and ethyl chloroformate were the highest available grade and all other chemicals, including the standards of carboxylic acids were obtained from Sigma-Aldrich.

Sample preparation

Samples used in the study were plasma (anticoagulant heparine) and urine. The pooled plasma and urine was processed immediately or stored at -20°C.

After centrifugation the pooled plasma was processed. Four hundred µL of acetonitrile and 200 µL of ethanol were added in 10 µL of aqueous solution of malonic acid (10 $mmolL^{-1}$) and 200 µL of plasma. The mixture was briefly shaken and the content was then centrifuged. The supernatant (600 µL) was transferred into another tube, alkalinized by addition of 2-3 µL NaOH and extracted two times with 500 µL n-hexane. The hexane layer was aspirated off and 500 µL aqueous phase were treated with 20 µL ECF and 40 µL pyridine. Following the addition of 250 µL of chloroform and 500 µL carbonate-bicarbonate solution (1 $molL^{-1}$, pH 10), the stoppered tube was shaken and left to reach phase equilibrium by standing for a 2-3 min. Finally, aqueous layer was aspirated off and the organic phase was dried by adding 50-80 mg of sodium sulphate. The volume was reduced to approximately 60 to 100 µL in nitrogen stream at room temperature (Husek and Liebich, 1994).

Procedure for urine samples is different in following steps: 200 µL urine (approximate creatinine content of 8 µmolL^{-1}) was supplemented with 10 µL of malonic acid used as internal standard (10 mmolL^{-1} in water), 200 µL ethanol and 400 µL of acetonitrile. If a precipitate was formed during 10-15 min of standing, it was subsequently centrifuged. To remove amines and amino acids the medium was pushed through a bed of AG 50W-X8 cation exchange resin (100-200 mesh, H^+ form; 25 mg). 500 µL of the acidified eluent were treated with 20 µL of ECF and 40 µL of pyridine (Husek, 1997). The next steps were the same as steps in procedure for preparation of plasma. The same procedure was carried out for the chromatographic standards by addition of 200 µL of water instead of sample (plasma, urine). A 3 µL aliquot was injected onto the column in the split-splitless mode (1:30).

Gas chromatography

The analysis succeeded on capillary column HP5 30 m × 0.32 mm I.D. (0.25 µm film thickness; crosslinked 5% phenylmethyl siloxane, Hewlett Packard, USA), in a temperature range of 60-200°C at 6°C/min. The carrier nitrogen was used at head pressure of 30 kPa, the injector and detector temperatures were set at 240 and 280°C. Instrument was used HP6890 gas chromatograph with a flame ionization detector (Hewlett Packard, USA).

Results and Discussion

The analysis of organic acids in human body fluids (plasma, urine, cerebrospinal fluid) is necessary for diagnosis of inborn errors of metabolism amino and organic acid metabolism. The large number of organic acids are present in body fluids, because of that it's separation and quantitation is very difficult. Five hundred plasma and urine samples of ill children with suspicion diagnosis of organic acidemia (aciduria) were analyzed. Five of them have a diagnosis of methylmalonic acidemia (1%).

Retention times (RT) and response factors (RF) of the individual organic acids on the HP5 column were determined with respect to malonic acid. This compound unlike some other dicarboxylic acids can be converted to diethylester with a good and reproducible yield. During profiling hundreds of samples from patients with inherited and non-inherited disorders we did not record its occurrence in plasma or urine at detectable amounts, which is in agreement with many papers dealing with profiling. The overall reproducibility of the procedure, expressed as percent variation from the mean, was determined from six independent analyses of the standards (Table 1). The average CV of the within day reproducibility for standard mixture was 7%. As shown in chromatogram in Fig. 1, 12 different organic acids listed in Table 1 were separated on HP5 column on conditions described in Material and Methods. Chromatogram of the standard mixture shows that separation power of the polar phenylmethyl siloxane phase for this kind of derivatives is excellent. In Table 2 and 3 are given concentration of organic acids in plasma and urine, within day reproducibility and analytical recovery of organic acids added

Table 1: Chromatographic parameters of derivatized standards of organic acids on HP5 column

Elution order	Organic acids	Abbreviation	RRT	RRF	CV (%) n=6
1	3-Hydroxybutyric	3-HB	0.780	0.577	6.54
2	2-Ketoisovaleric	KIV	0.804	0.265	7.47
3,4	2-ketomethylvaleric/2-ketoisocaproic	KMV/KIC	0.958	0.733	7.76
5	Malonic	M	1.000	1.000	4.94
6	Methylmalonic	MM	1.052	0.836	5.13
7	2-Hydroxyacetic (glycolic)	HA	1.146	0.291	8.49
8	2-Hydroxypropionic (lactic)	2-HP	1.180	0.467	7.19
9	2-Hydroxybutyric	2-HB	1.314	0.571	6.93
10	2-Hydroxyisovaleric	2-HIV	1.388	0.357	9.72
11	Adipic	AD	1.549	0.150	7.51
12	2-Hydroxycaproic	2-HC	1.603	1.271	7.80

Table 2: Concentration of organic acids in plasma, reproducibility and analytical recovery of organic acids added to plasma profile

Organic acids	Plasma concentration (µM)	CV (%) (n=6)	Spiked plasma recovery (%)
3-HB	115.60	3.14	104.2
KIV	0	/	90.0
KMV/KIC	0	/	95.0
M	10.00		/
MM	171.40	1.45	105.3
HA	0	/	82.5
2-HP	1347.30	5.65	100.9
2-HB	0	/	92.5
2-HIV	0	/	97.5
AD	0	/	85.0

Table 3: Concentration and reproducibility of organic acid in urine

Organic acids	Concentration in urine (mmol/mol creatinine)	CV (%) (n=6)
3-HB	0	/
KIV	0	/
KMV/KIC	0	/
M	10.00	
MM	422.90	0.82
HA	15.36	8.57
2-HP	13.62	/
2-HB	0	4.00
2-HIV	0	/
AD	0	/

to plasma profile. Reproducibility of the plasma and urine profiling and the recovery of the acids from the spiked plasma was surprisingly good; slightly lower values were found for 2-hydroxyacetic and adipic acid (Table 2 and 3). A pathological plasma profile of organic acids and urine is shown in Fig. 2 and 3. Identification of the analyses was based on comparison of the retention times with those of standard solutions. The structure of the others peaks remains unknown. As shown in Fig. 2 and 3 in samples we detected three organic acids but only abnormal peak belong to methylmalonic acid. When we compared

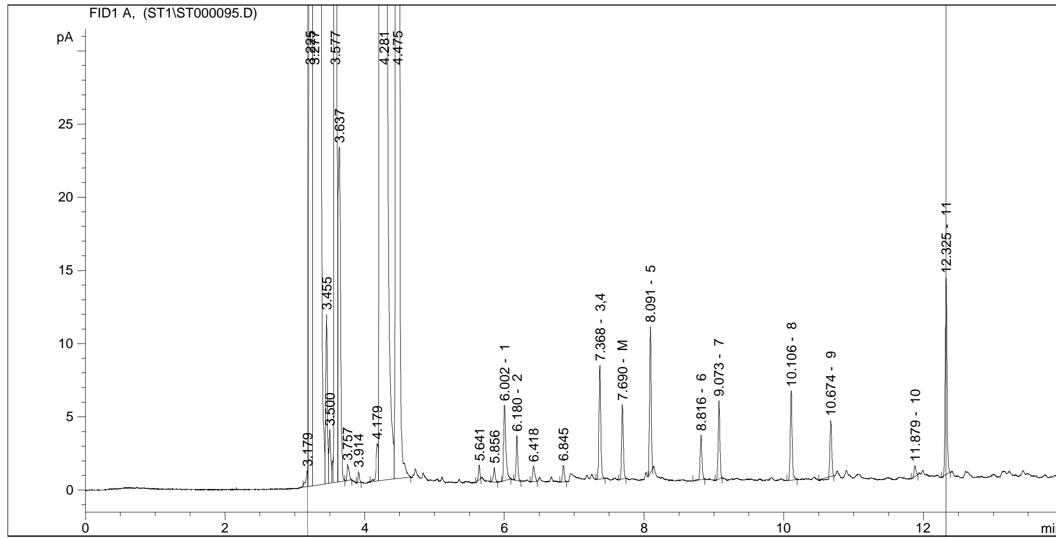

Fig. 1: Chromatogram of organic acids analyzed on the HP5 column as an equimolar standard mixture
Injected amount 0.46 nmol at 1:30 split ratio
1, 3-hydroxybutyric acid; 2, 2-ketoisovaleric acid
3,4, 2-ketomethylvaleric/2-ketoisocaproic; 5, methylmalonic acid; 6, 2-hydroxyacetic acid
7, 2-hydroxypropionic acid; 8, 2-hydroxybutyric acid; 9, 2-hydroxyisovaleric acid
10, adipic acid; 11, 2-hydroxycaproic; M added as an internal standard

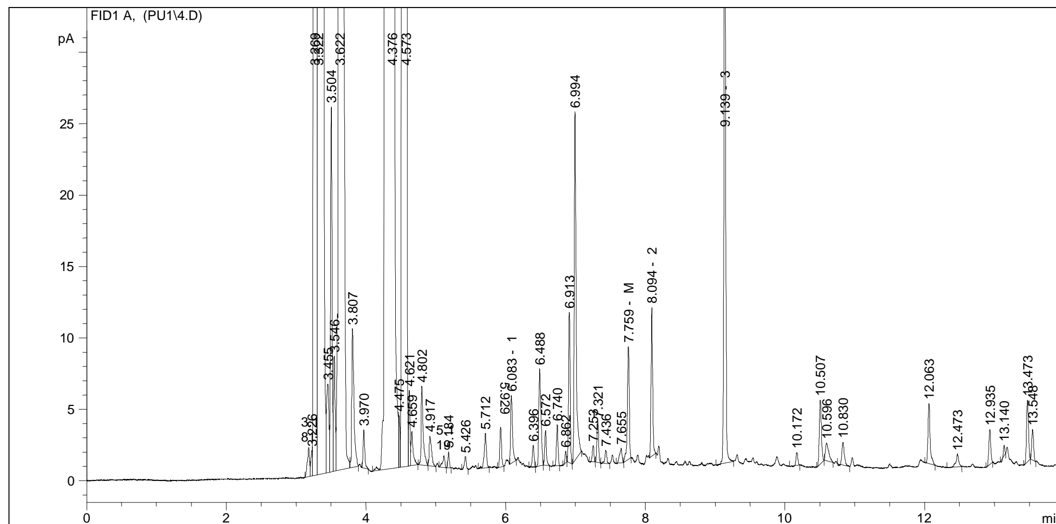

Fig. 2: Plasma organic acids from a patient with methylmalonic acidemia
1, 3-hydroxybutyric acid; 2, methylmalonic acid; 3, 2-hydroxypropionic acid; M added as an internal standard

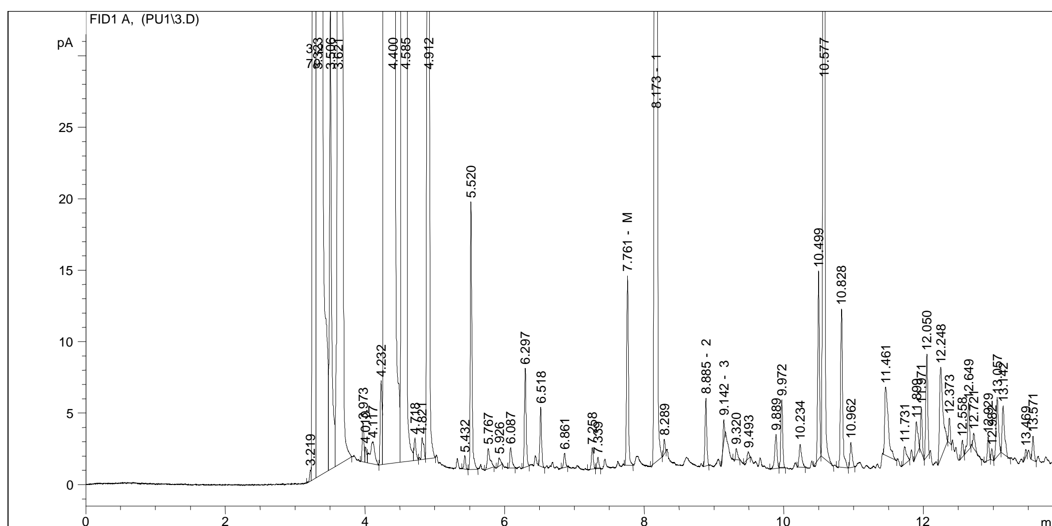

Fig. 3: Urinary organic acids from a patient with methylmalonic acidemia
1, methylmalonic acid; 2, 2-hydroxyacetic acid; 3,2-hydroxypropionic acid; M added as internal standard

chromatogram of standard mixture (Fig. 1) with chromatograms of the plasma and urine samples (Fig. 2 and 3) we can observe peak of methylmalonic acid in samples. Presence of this peak in plasma and urine appears on methylmalonic acidemia, one the most frequently diagnosed organic acidemias. Methylmalonic acidemia results from the deficiency of the cobalamin-dependent enzyme methylmalonyl-CoA mutase (EC 5.4.99.2) (Lehotay and Clarke, 1995). Defective activity of this enzyme may result from defect of apoenzyme or deficiency his coenzyme 5'-deoxyadenosylcobalamin (AdoCb1). Consequence of this defect is accumulation of methylmalonic acid in plasma and enormous excretion of this acid (intermediary metabolite of catabolic pathways Ile, Val, Thr I Met) which is not detectable in plasma and urine of healthy persons.

Described methodology is fast, require small amount of biological material, preparation of the sample is simple and reliability is high. Also, the cost of analyse is lower. Rapidity and reliability of analysis are crucial because the therapy in the first weeks or months is life saving.

The proposed method allows the operator to become familiar with the patterns of nonpathological samples and to recognize immediately "true abnormal profiles" in biological samples of children. It could be a valuable tool in the routine diagnostic system, mainly in newborns, applied to severe diseases that need a specific and early treatment.

References

Bonham, J.R., M. Downing, R. Pollitt, N.J. Manning, K.H. Carpenter and S.E. Olpin, 1994. Quality ass of urinary organic acid analysis. Ann. Clin. Biochem., 31: 129-133.

Duez, P., A. Kumps and Z. Mardens, 1996. GC-MS profiling of urinary organic acids evaluated as a quantitative method. Clin. Chem., 42: 1609-1615.

Hoffman, G.F., S. Aramaki, E. Blum-Hoffmann, W.L. Nyhan and L. Sweetman, 1989. Quantitative analysis for organic acids in biological samples: Batch isolation followed by gas chromatographic-mass spectrometric analysis. Clin. Chem., 35: 587-595.

Husek, P. and H.M. Liebich, 1994. Organic acid profiling by direct treatment of deproteinized plasma with ethyl-chloroformate. J. Chromatog., 656: 37-43.

Husek, P., 1995. Simultaneous profile analysis of plasma amino and organic acids by capillary gas chromatography. J. Chromatog. B., 669: 352-357.

Husek, P., 1997. Urine organic acid profiling by capillary gas chromatography after a simple sample pretreatment. Clin. Chem., 43: 1999-2001.

Lehotay, D.C. and J.T. Clarke, 1995. Organic acidurias and related abnormalities. Crit. Rev. Clin. Lab. Sci., 32: 377-429.

Sweetman, L., 1991. Organic acids analysis. Techniques in diagnostic human biochemical genetics. A laboratory manual. In: Hommes F.A. (Ed.), Wiley-Liss, New York, pp: 143-176.

Tanaka, K., A. West-Dull, D.G. Hine, T.B. Lynn and T. Lowe, 1980. Gas-chromatographic method of analysis for urinary organic acids. II. Description of the procedure and its application to diagnosis of patients with organic acidurias. Clin. Chem., 26: 1847-1853.