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Short communication

Simultaneous profile analysis of plasma amino and organic acids by capillary gas chromatography

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Abstract

A simultaneous GC analysis of more than 20 amino and nearly 30 non-amino organic acids abundant in plasma is for the first time possible. Isolation of the analytes from the plasma matrix is not necessary, keto acids do not require a preliminary oximation. An instantaneous derivatization of the acids with ethyl chloroformate takes place directly in the medium after deproteinization. Less than 30 min are required to prepare a plasma sample for the GC analysis.

1. Introduction

In the series of recent papers Hoffmann et al. [1–3] substantially contributed to both the methodology and the acquisition of data in organic acid analysis in human fluids by capillary gas chromatography (GC). The authors stated that an "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[1] metabolism" [1]. Nevertheless, "a very limited information is available on amounts of organic acids in body fluids other than urine" [2]. For quantitative organic acid analysis in cerebrospinal fluid and plasma [2,3] the same methodology was used as described by the authors earlier [4–6]. It is based on oximation of keto acids with pentafluorobenzylhydroxylamine (2 h at pH 2–3), lyophilization of the sample, isolation of the acids by liquid partition chromatography on silica, evaporation of the eluate (30 ml of 42% 2-methyl-2-butanol in chloroform) and subsequent silylation of the dry residue with Trisil-N,O-bis(trimethylsilyl)trifluoroacetamide (2 h at 60°C). Even when tedious and time-consuming it is in accordance with state-of-the-art procedures, which require oximation, isolation of the analytes from the matrix and derivatization at higher temperatures in common.

The use of ethyl chloroformate (ECF) as derivatizing agent [7] presents a completely new approach to profiling of organic acids in biological fluids [8]. A marked simplification of the methodology is emphasized in three ways: sample preparation, derivative formation and more comprehensive analysis. In a previous report [8] we have shown that oximation of keto acids and isolation of the analytes from plasma are not necessary since the plasma —deproteinized by a

[1] The used connection *amino and organic acids* is habitual rather than exact since even the amino acids belong to the organic acids.

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mixed organic solvent— can be treated by ECF in situ. This report shows an additional advantage consisting of simultaneous analysis of amino acids. The possibility of a simultaneous treatment of amino acids along with other organic acids was already brought forth in one of our previous reports concerning analysis of biogenic amines together with their precursors and catabolites [9].

2. Experimental

As far as the *Experimental* is concerned, see our previous report [8]. Instead of malonic acid, the *p*-chlorophenylalanine (Cl-PHE) as internal standard (I.S.) for plasma was used. Unlike the previous sample workup the step for amino acid uptake on cation exchanger was omitted. The simplified procedure was done as follows: to 0.4 ml of acetonitrile and 0.2 ml of ethanol were added 2 μl of I.S. solution (containing 10 mmol/l of Cl-PHE in 50 mmol/l HCl) and 0.2 ml of plasma. The mixture was briefly shaken to precipitate the proteins and the content was then centrifuged at 2000 *g* for 10 min. The supernatant (0.6 ml approximately) was transferred into another tube, alkalinized to pH > 8 by addition of 2–3 μl of 2 mol/l NaOH and extracted two times with 0.5 ml of *n*-hexane (shaking for about 1 min) to remove neutral lipids. The hexane layer was aspirated off and 500 μl of the aqueous phase were treated in a silanized glass tube with 20 μl ECF and 40 μl pyridine while shaking the content for a few seconds to let the liberated carbon dioxide escape. Following the addition of 0.25 ml of chloroform and 0.5 ml of carbonate–bicarbonate solution (1 mol/l, pH ca. 10), the stoppered tube was shaken for ca. 10 s and left to reach phase equilibrium by standing for 2–3 min. Finally, the upper aqueous layer was aspirated off by means of pipette-tips and the organic phase was dried by adding 50–80 mg of sodium sulphate. The volume was reduced to approximately 80 to 100 μl by blowing nitrogen across the surface of the solvent at room or slightly elevated temperature (40°C) within 2–3 min. An aliquot of 2–3 μl was then injected onto the fused-silica capillary column 30 m × 0.25 mm of DB-17HT type (0.15 μm film thickness of 50% phenylmethyl silicone; J&W Scientific, Folsom, CA, USA) in the split mode (1:20). The temperature was programmed from 60°C at 6°C/min to 300°C (5 min hold), head pressure of the carrier hydrogen was 50 kPa, the injector and detector temperatures were set at 240°C and 280°C, respectively. The used instrument was GC 14A gas chromatograph with a flame ionization detector (Shimadzu, Vienna, Austria).

3. Results and discussion

Relative retention times (RRT) and response factors (RRF) of the individual organic acids on the DB-17HT column were related to *p*-chlorophenylalanine, an amino acid, used already as I.S. in our previous studies on amino acid derivatization [10]. The overall reproducibility of the procedure, expressed as percent variation from the mean, was determined from six independent analyses of the standards (Table 1). With exception of GLU and GLN, the C.V. of which were higher than 10, the reproducibility was in general satisfactory. Response of some amino acids (ILE, TYR) was slightly lower and that of GLU substantially lower (ASP disappeared at all) when compared with their responses in the medium free of acetonitrile [10]. Even under the used reaction conditions ARG was not derivatized fully and its elution from the column failed. The chosen stationary phase of DB-17 type was found as optimum, especially for separation of C_{16} and C_{18} fatty acids (on the optional DB-1701 phase coelution of fatty acids with PHE and Cl-PHE occurred). Despite of the failed resolution of KMV/KIC pair and marginal resolution of some couples (e.g., THR/SER, ILE/12:0, 18:0/18:1), the separation ability of the thin-film-coated and high-temperature (max. at 340°C) column was remarkable (Figs. 1 and 2). Unlike the previous report where a more polar silicone phase of DB-225 type was used, pyruvic acid occurs on the tail of the solvent peak and cannot be evaluated in this case. The used I.S. was eluted in a vacant area of the

Table 1
Chromatographic parameters of derivatized standards of organic acids on DB-17HT column

Elution order	Organic acid (amino acid)	Abbreviation	RRT	RRF	C.V. (%) ($n = 6$)
1	3-Hydroxybutyric	3HB	0.193	0.34	8.8
2	2-Ketoisovaleric	KIV	0.201	0.40	8.0
3,4	2-Ketomethylvaleric/2-ketoisocaproic	KMV/KIC	0.256	1.08	8.3
5	Malonic	M	0.302	0.45	5.4
6	Methylmalonic	MM	0.312	0.46	6.6
7	Ethylmalonic	EM	0.364	0.54	7.5
8	2-Hydroxyacetic (glycolic)	HA	0.382	0.25	8.1
9	2-Hydroxypropionic (lactic)	HP	0.391	0.38	8.1
10	2-Hydroxybutyric	HB	0.449	0.48	7.9
11	Alanine	ALA	0.461	0.46	4.6
12	2-Hydroxyisovaleric	HIV	0.477	0.35	9.0
13	Glycine	GLY	0.483	0.37	5.1
14	3-Methylglutaric	MG	0.499	0.27	9.7
15	2-Aminobutyric	AB	0.516	0.49	4.1
16	2-Hydroxyisocaproic	HIC	0.534	0.60	5.5
17	Adipic	AD	0.538	0.51	6.7
18	Valine	VAL	0.545	0.52	5.0
19	3-Methyladipic	MAD	0.559	0.49	6.8
20	3-Hydroxylactic (glyceric)	HL	0.574	0.47	9.6
21	Leucine	LEU	0.595	0.70	5.3
22	Isoleucine	ILE	0.607	0.40	7.2
23	Lauric	12:0	0.610	1.01	5.5
24	Threonine	THR	0.652	0.34	4.4
25	Serine	SER	0.654	0.26	4.6
26	Glutamic	GLU	0.671	0.10	12.4
27	Proline	PRO	0.681	0.49	5.3
28	Asparagine	ASN	0.692	0.38	4.7
29	Hydroxysuccinic (malic)	HS	0.718	0.11	9.0
30	Myristic	14:0	0.738	1.17	3.7
31	Methionine	MET	0.818	0.63	3.3
32	4-Hydroxyproline	HYP	0.847	0.39	5.6
33	Palmitic	16:0	0.852	1.37	5.4
34	Palmitooleic	16:1	0.858	1.35	5.2
35	Phenylalanine	PHE	0.898	1.02	2.9
36	Cysteine	CYSH	0.925	0.23	9.8
37	Stearic	18:0	0.959	1.57	4.9
38	Oleic	18:1	0.961	1.55	5.2
39	Linoleic	18:2	0.970	1.54	4.3
40	Linolenic	18:3	0.986	1.52	6.2
41	*p*-Chlorophenylalanine (I.S.)	Cl-PHE	1.000	1.00	–
42	Glutamine	GLN	1.013	0.24	10.3
43	Ornitine	ORN	1.051	0.67	3.3
44	Arachidic	20:0	1.064	1.62	4.1
45	Arachidonic	20:4	1.070	1.60	3.7
46	Lysine	LYS	1.104	0.75	3.7
47	Histidine	HIS	1.148	0.37	7.8
48	Docosahexanoic	22	1.178	1.67	3.6
49	Tyrosine	TYR	1.201	0.84	3.1
50	Nervonic	24:1	1.231	1.70	5.4
51	Tryptophan	TRP	1.307	1.08	3.7
52	Cystine	CYS	1.384	0.28	7.5

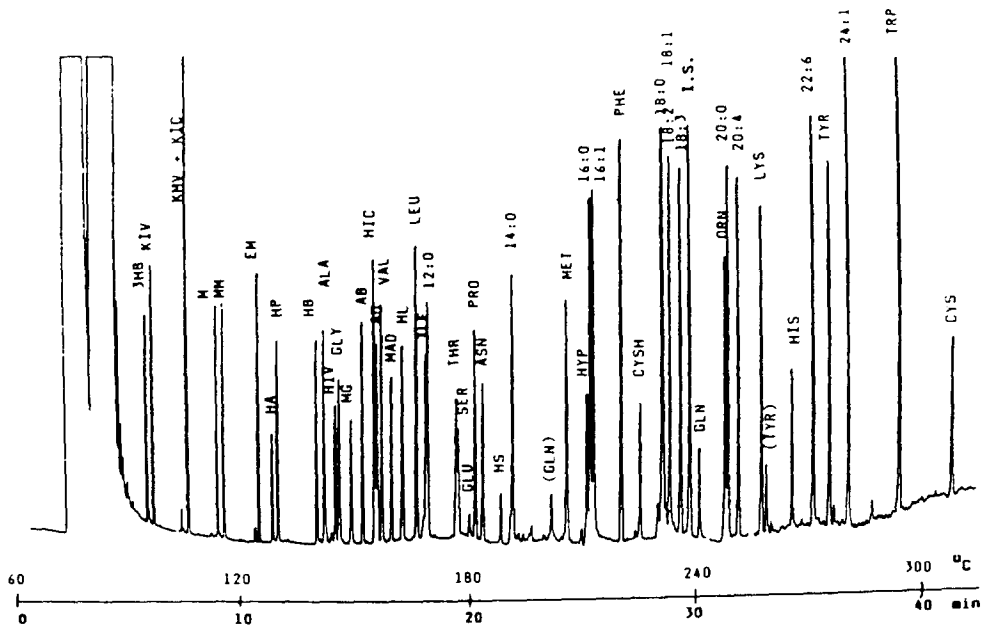

Fig. 1. Chromatographic standards analyzed on the DB-17HT column as an equimolar mixture (fatty acids at half molarity). Derivatized amount 20 nmol of each, injected amount 0.5 nmol at 1:20 split ratio. Attenuation × 32.

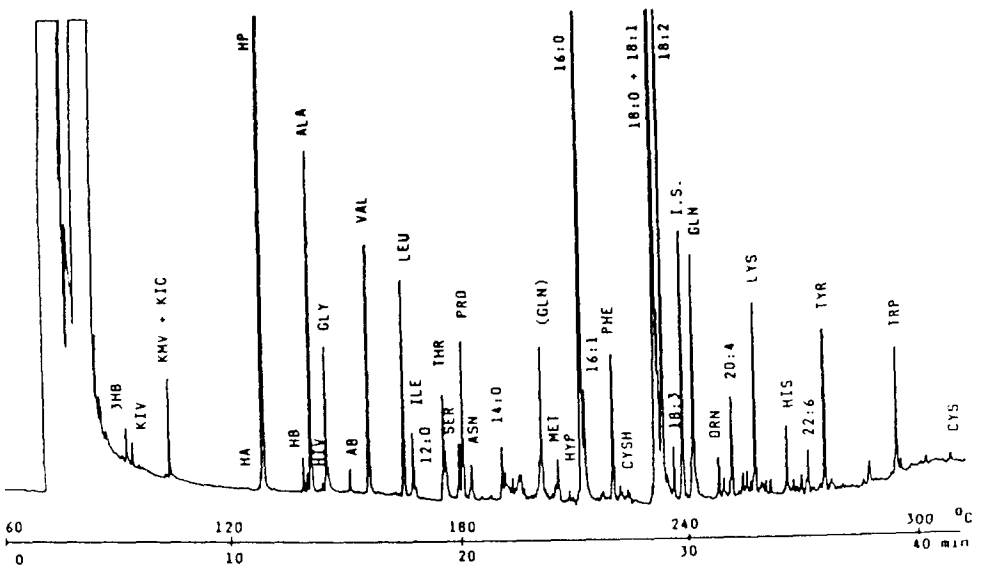

Fig. 2. Organic acid profile corresponding to 0.125 ml of plasma. I.S. added to a concentration of 10 nmol/0.1 ml of plasma. Processed under the same conditions as in Fig. 1.

plasma chromatogram; however, a double I.S. labelling (in connection with e.g., malonic acid as in the previous report) would be more provident as pathophysiological samples with high abundance of acidic metabolites might occur. An improved resolution of the analytes can be achieved by using a usual DB-17 phase at 0.25-μm coating and the same column parameters since the maximum operating temperature of 300°C suffices.

The within-day reproducibility of the plasma profiling and the recovery of the acids added to plasma (20 to 100 nmol of the particular standard added to 0.2 ml of plasma) are given in Table 2. The C.V. values are higher for acids of low plasma abundance, and further for some fatty acids as in the previous report and for some basic amino acids (GLN, ORN, HIS, TRP) and even TYR. In the case of TRP a further study should reveal if the findings of Upton and Hindmarsh [11] concerning the used blood anticoagulant (using EDTA as anticoagulant, a very low and

Table 2
Within-day reproducibility of plasma organic acid profiling and analytical recovery of organic acids added to plasma

Peak No.	Acid	Plasma		Spiked plasma recovery (%)
		Concentration (μmol/l)	C.V. (%) ($n = 6$)	
1	3HB	15.8	13.0	110
2	KIV	19.6	6.1	127
3,4	KMV/KIC	57.5	5.5	116
9	HP	1008.0	6.0	96
10	HB	16.0	5.7	100
11	ALA	298.0	6.4	102
12	HIV	12.1	7.5	104
13	GLY	188.0	5.0	106
15	AB	12.1	7.8	114
18	VAL	152.0	4.4	121
21	LEU	102.0	5.2	106
22	ILE	52.0	4.4	115
24	THR	80.0	7.6	88
25	SER	98.0	9.2	96
27	PRO	125.0	4.4	103
28	ASN	32.0	8.5	86
30	14:0	9.5	9.2	94
31	MET	27.0	5.9	116
32	HYP	17.0	15.1	126
33	16:0	139.0	7.9	96
34	16:1	11.0	10.9	102
35	PHE	53.0	6.6	110
37	18:0	75.0	11.8	97
38	18:1	137.0	9.1	104
39	18:2	46.0	12.7	100
40	18:3	5.0	8.3	99
42	GLN	419.0	15.3	91
43	ORN	34.0	10.1	87
45	20:4	5.1	18.1	90
46	LYS	145.0	6.4	84
47	HIS	57.0	13.0	95
48	22:6	4.8	17.5	93
49	TYR	52.0	12.9	103
51	TRP	30.0	15.4	83

variable recovery of TRP was found in comparison with heparinized blood) are valid in general or were connected only with the technique used by the authors (HPLC of plasma-derived PTC-amino acids). EDTA was used as anticoagulant in this and the previous study since with the heparinized blood some extraneous peaks, coeluting with some fatty acids, appeared at the end of the chromatogram.

The advantage of using fused-silica capillaries with chemically bonded phases that can be washed with solvents after a prolonged use with biological material can be fully appreciated at this markedly simplified approach. Signs of column contamination can be easily determined by an enhanced tailing of derivatives with underivatized hydroxyl group, i.e. 3HB and SER, and of some others (HA, GLY, GLN). In such a case washing of the column from the detector-end with aqueous ethanol followed by acetonitrile proved to be useful in restoring the original column performance. A better solution was found using retention gap, i.e. a precolumn consisting of a piece (1 m × 0.32 mm I.D.) of uncoated fused silica connected with the separation capillary by quick coupling (Inner Lok connector, Polymicro Technologies, Phoenix, AZ, USA). The precolumn was washed as mentioned after about 30 to 40 injections of plasma samples.

To conclude, regarding the simplicity of sample preparation together with maximum of data acquired by the simultaneous analysis of the specified classes of organic acids this procedure has no equivalent among the present ones. The next step is to verify the usefulness of this approach in routine clinical analysis.

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References

[1] W. Meier-Augenstein, G.F. Hoffmann, B. Holmes, J.L. Jones, W.L. Nyhan and L. Sweetman, J. Chromatogr., 615 (1993) 127.
[2] G.F. Hoffmann, C.K. Seppel, B. Holmes, L. Mitchell, H.J. Christen, F. Hanefeld, D. Rating and W.L. Nyhan, J. Chromatogr., 617 (1993) 1.
[3] G.F. Hoffmann, W. Meier-Augenstein, S. Stockler, R. Surtees, D. Rating and W.L. Nyhan, J. Inher. Metab. Dis., 16 (1993) 648.
[4] G.F. Hoffmann, S. Aramaki, E. Blum-Hoffmann, W.L. Nyhan and L. Sweetman, Clin. Chem., 35 (1989) 587.
[5] G.F. Hoffmann and L. Sweetman, Clin. Chim. Acta, 199 (1991) 237.
[6] L. Sweetman and F.A. Hommes (Editors), Techniques in Diagnostic Human Biochemical Laboratories: A Laboratory Manual, Wiley-Liss, New York, 1991, p. 143.
[7] P. Hušek, LC/GC, 5 (1992) No. 9, 43.
[8] P. Hušek and H.M. Liebich, J. Chromatogr. B, 656 (1994) 37.
[9] P. Hušek, J. Microcol. Sep., 5 (1993) 101.
[10] P. Hušek, J. Chromatogr., 552 (1991) 289.
[11] J.D. Upton and P. Hindmarsh, Clin. Chem., 36 (1990) 157.