



ELSEVIER

Journal of Chromatography B, 693 (1997) 499–502

JOURNAL OF
CHROMATOGRAPHY B

Technical note

Simple approach for analysis of plasma oxo-, hydroxy- and dicarboxylic acids

Petr Hušek*, Petr Matucha

Institute of Endocrinology, Národní třída 8, CZ-11694 Prague 1, Czech Republic

Received 7 October 1996; revised 23 December 1996; accepted 2 January 1997

Abstract

Following deproteinization of plasma with organic solvents the supernatant was shaken with hexane and cation-exchange resin in an Eppendorf tube to remove fatty and amino acids and the medium was subjected to direct treatment with ethyl chloroformate under catalytic influence of pyridine. A subsequent extraction of the immediately formed ethyl esters with a drop of chloroform enabled us to subject the sample to gas chromatographic (GC) analysis. Since ketocarboxylic acids do not require a preliminary oximation the total time of sample workup and analysis takes only several minutes.

Keywords: Ketocarboxylic acids; Hydroxycarboxylic acids; Dicarboxylic acids; Carboxylic acids

1. Introduction

An important metabolic role is now recognized for the α -keto analogues of the branched-chain amino acids leucine, isoleucine and valine, i.e., 2-ketoisovaleric, 2-keto-3-methylvaleric and 2-ketoisocaproic acid. These keto acids are normally in equilibrium with their parent amino acids and consequently contribute to their free pools [1]. It is essential to develop a rapid microquantitative determination procedure of various keto acids in serum for differential diagnosis of metabolic disorders associated with acidemia. Measurement of lactate and pyruvate in blood is valuable in critical-care medicine and exercise physiology [2,3]. Rapid monitoring of plasma levels of hydroxycarboxylic acids

renders a useful tool in evaluation of pathophysiological state especially in diabetic subjects [4,5]. The use of plasma lactate to assess metabolic or circulatory impairment requires definition of critical preanalytical and analytical parameters, as glycolysis continues in blood after phlebotomy and the resulting decrease in blood glucose is paralleled by increase in lactate concentrations [6,7].

Among procedures aiming at rapid determination of the mentioned classes of organic acids, the recent one using chloroformates [8] is especially valuable. By treating plasma supernatant with ethyl chloroformate (ECF) the capillary GC profile of plasma acidic metabolites with [9] or without [10] amino acids can be reached in less than 1 h including sample workup.

This report offers a further simplification of the previous procedure [10] and a more rapid analysis of keto-, hydroxy- and dicarboxylic acids provided that

*Corresponding author.

one is not interested in determination of fatty and amino acids.

2. Experimental

The only tool necessary for plasma sample pre-treatment is an Eppendorf tube in which the amino and fatty acids are removed from the plasma supernatant by shaking with cation-exchanger and hexane.

The supplied chemicals, equipment and apparatus (Shimadzu GC 14A gas chromatograph with FID and split-splitless injector) were essentially the same as in our earlier report [10]. Blood from six healthy volunteers was gathered in heparinized tubes containing sodium fluoride (2 mg/ml blood) and after centrifugation the pooled plasma was processed as follows: to 0.2 ml of acetonitrile and 0.1 ml of ethanol were added 2 μ l of aqueous solution of malonic acid (10 mmol/l) and 0.1 ml of plasma. Following vortex-mixing the precipitated proteins were centrifuged down at 2000 *g* for 10 min and the supernatant (>0.3 ml) was transferred into Eppendorf tube together with 10 mg of dry AG 50W-X8 cation-exchange resin (100–200 mesh) and 0.5 ml of *n*-hexane. The content was subjected to 15 s vortex-mixing, the hexane layer was aspirated off and this step was repeated after addition of another portion of hexane. Following a brief centrifugation aiming at sedimentation of the resin, 0.25 ml of the aqueous phase, acidified during shaking to pH<3 due to liberated protons from the exchanger, were transferred into a silanized 1 ml screw-vial and treated with 10 μ l ECF and 20 μ l pyridine. After few seconds of gentle shaking 0.15 ml of chloroform (or dichloromethane) and 0.25 ml of approximately 1 *M* sodium bicarbonate-carbonate (2:1, w/w) solution were added and following vortex-mixing and 1 min standing to reach phase equilibrium the upper aqueous layer was aspirated and the organic phase dried over sodium sulphate. The volume of the extractant was reduced to approximately one half by nitrogen blowing at room temperature and a 2 μ l volume injected onto the column via split-splitless mode (1:30) in such a way that the split vent was closed for the initial 5 s. By this arrangement both the reproducibility and sensitivity were improved markedly.

The same procedure was carried out for the chromatographic standards (equimolar mixture with 10 nmoles of each compound) by addition of 0.1 ml water instead of plasma. The analysis succeeded on the same fused-silica capillary column as used previously [10], i.e. 15 m \times 0.25 mm of DB-225 (0.25 μ m film thickness; J&W Scientific, Folsom, CA, USA) in a temperature range of 60–200°C at 10°C/min. The carrier hydrogen was used at a head pressure of 30 kPa, the injector and detector temperatures were maintained at 240°C and the attenuation \times 32.

3. Results and discussion

The chromatogram of standards and plasma acidic metabolites is shown in Fig. 1. The results shown in Table 1 concerning the within-day reproducibility and analytical recovery of organic acids in spiked and non-spiked plasma samples are in a close agreement with literature values and our previous study [10]. As stated and documented previously, lactic acid was even here, being always the predominant compound in the profile of controls, accompanied by side-reaction products, a phenomenon generally occurring with chloroformate-treated 2-hydroxycarboxylic acids [11,12]. These side-reaction products are marked in the figure by asterisks and do not interfere with the other analytes of interest under the chosen analytical conditions. All other remarks concerning the procedure and analysis are comprehensively given in the preceding study [10].

This concerns also employment of malonic acid as internal standard. During profiling of hundreds of samples from patients with inherited and non-inherited disorders we did not record its occurrence in plasma at detectable amounts, which is in agreement with many papers dealing with profiling. However, in some very rare metabolic defects (e.g., malonyl CoA decarboxylase deficiency) its occurrence was noticed. We suggest, therefore, to spike plasma with two internal standards, the second being 2-hydroxyvaleric acid, which would elute, under the conditions used, behind HIV in a vacant area of the chromatogram. We have tested some other possible standards, e.g., tricarballylic acid, but its esterification does not

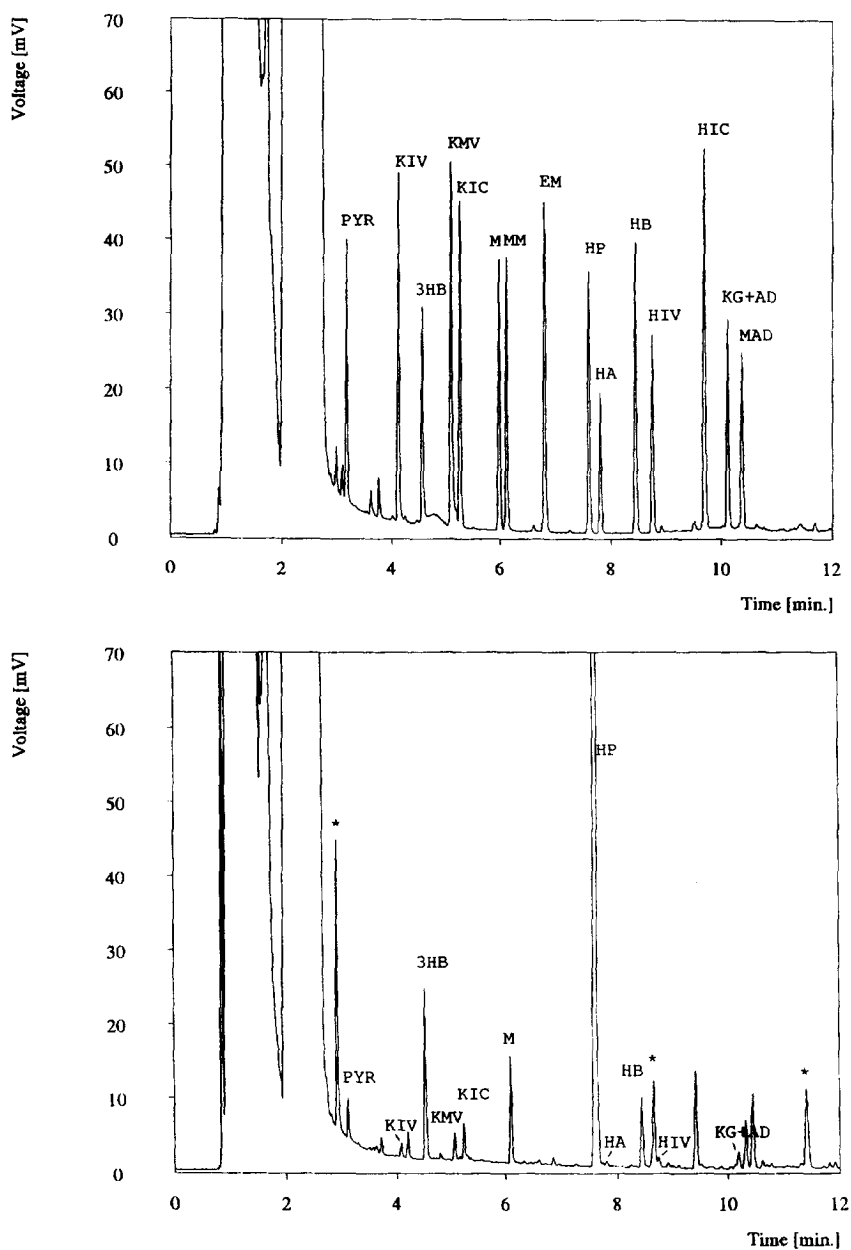


Fig. 1. Equimolar mixture of chromatographic standards (above; 10 pmol of each) and profile of acidic metabolites in plasma (below) treated according to the text. Analyzed on DB-225 with malonic acid (M) added as internal standard. Abbreviations of carboxylic acids are given in Table 1; the peaks marked by an asterisk represent side-reaction products of lactic acid.

proceed smoothly in a medium with prevailing acetonitrile.

To conclude, regarding simplicity and rapidness, this procedure offers itself as a useful tool for routine screening in clinical laboratory.

Acknowledgments

This study was supported by the Internal Grant Agency of the Ministry of Health of the Czech Republic, Grant No. 2733-3, which is acknowledged.

Table 1
 Within-day reproducibility of plasma organic acid profiling and analytical recovery of organic acids added to plasma

Carboxylic acid		Plasma		Spiked plasma	
Trivial name	Abbreviation	Concentration (nmol/ml)	C.V. (%) (n=6)	Recovery (%)	C.V. (%) (n=6)
Pyruvic	PYR	106.4	3.9	102	6.7
2-Ketoisovaleric	KIV	15.7	3.2	106	6.9
3-Hydroxybutyric	3HB	161.3	2.9	116	9.4
2-Ketomethylvaleric	KMV	24.5	2.3	103	8.1
2-Ketoisocaproic	KIC	33.2	3.7	96	8.2
Malonic (I.S.)	M	[100]			
Methylmalonic	MM	0.0		82	5.2
Ethylmalonic	EM	0.0		86	4.8
2-Hydroxypropionic(lactic)	HP	1942.8	4.9	96	3.4
2-Hydroxyacetic (glycolic)	HA	3.6	8.7	98	7.5
2-Hydroxybutyric	HB	48.8	5.1	105	3.3
2-Hydroxyisovaleric	HIV	8.7	6.4	119	3.0
2-Hydroxyisocaproic	HIC	0.0		102	4.7
2-Ketoglutaric+Adipic	KG+A	17.1	7.1	109	6.1
Methyladipic	MAD	0.0		93	7.2

References

- [1] L.J. Hoffer, A. Taveroff, L. Robitaille, O.A. Mamer and L.J. Reimer, *J. Nutr.*, 123 (1993) 1513.
- [2] K. Koike, Y. Urata and N. Hiraoka, *J. Clin. Biochem. Nutr.*, 9 (1990) 151.
- [3] M. Panteghini and F. Pagani, *Clin. Chem.*, 39 (1993) 908.
- [4] S. Nissen, M.V. Koeving and D. Webb, *Anal. Biochem.*, 1 (1990) 17.
- [5] A. Avogaro and D.M. Bier, *J. Lipid Res.*, 30 (1989) 1811.
- [6] R. Astles, C.P. Williams and F. Sedor, *Clin. Chem.*, 40 (1994) 1327.
- [7] Y.D.I. Chen, B.B. Varasteh and G.M. Reaven, *Diabetes Metab.*, 19 (1993) 348.
- [8] P. Hušek, *LC-GC Int.*, 5(9) (1992) 43.
- [9] P. Hušek, *J. Chromatogr. B*, 669 (1995) 352.
- [10] P. Hušek and H.M. Liebich, *J. Chromatogr. B*, 656 (1994) 37.
- [11] H.M. Liebich, M. Gesele, H.G. Wahl, C. Wirth, J. Woll and P. Hušek, *J. Chromatogr.*, 626 (1992) 289.
- [12] P. Hušek, *J. Chromatogr.*, 630 (1993) 429.