ELSEVIER

Journal of Chromatography B, 713 (1998) 427–432

JOURNAL OF CHROMATOGRAPHY B

Short communication

Urinary organic acid screening by solid-phase microextraction of the methyl esters

H.M. Liebich*, E. Gesele, J. Wöll

Medizinische Universitätsklinik, D-72076 Tübingen, Germany

Received 7 November 1997; received in revised form 17 March 1998; accepted 17 March 1998

Abstract

We developed a new sample preparation method for profiling organic acids in urine by GC or GC–MS. The method includes derivatisation of the organic acids directly in the aqueous urine using trimethyloxonium tetrafluoroborate as a methylating agent, extraction of the organic acid methyl esters from the urine by solid-phase microextraction, using a polyacrylate fiber with a thickness of 85 μm and transfer of the methyl esters into the GC or the GC–MS instrument. Desorption of the analytes takes place in the heated injection port. The proposed sample preparation is very simple. There is no need for any evaporation step and for the use of an organic solvent. The risk of contamination and the loss of analytes are minimized. The total sample preparation time prior to GC or GC–MS analysis is about 40 min, and therefore more rapid than other sample preparation procedures. The urinary organic acids are well separated by GC and 29 substances are identified by GC–MS.

Keywords: Organic acids

1. Introduction

The analysis of urinary organic acids can be of great importance to diagnose certain diseases, in particular inborn errors of metabolism connected with organic acidurias. The analysis of the profile of the organic acids can decisively support diagnosis [1]. In the case of inherited acidurias increased concentrations of organic acids in urine are characteristic. Also in other metabolic diseases abnormally high concentrations of certain organic acids in urine can be found. In patients with diabetes mellitus an increased excretion of hydroxycarboxylic acids and dicarboxylic acids in urine is observed [2].

Organic acids are usually analysed by gas chromatography (GC) or GC–mass spectrometry (GC–MS) after preceding sample preparation [3–5]. Due to the wide range in structure and polarity of the organic acids in urine derivatisation is absolutely necessary. The most common derivatives are silyl products and methyl esters. As compared to the silyl products the methyl esters have the advantage, that their mass spectra are often easier to interpret and that the substances are more stable, especially against moisture.

All the sample preparations when analysing urinary organic acids have in common that they require isolation of the organic acids from the urine matrix prior to derivatisation, because the conventional derivatisation procedures require an organic medium

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00156-X

for the reaction. Usually liquid–liquid extraction or liquid–solid-phase extraction are chosen [3,5].

We proposed a simplification of the sample preparation by omitting the time consuming extraction step of the organic acids in urine. Derivatisation to form methyl esters of the organic acids is performed directly in the aqueous urine by using trimethyloxonium tetrafluoroborate (TMO) as the methylating agent [6]. The substance was first synthesized and described by Meerwein et al. [7]. The proposed derivatisation takes place under mild conditions at room temperature, requiring only 15 min for complete reaction.

In this paper we first describe solid-phase microextraction (SPME) for isolation of the methyl esters out of the aqueous urine. The SPME technique has been mainly developed by Pawliszyn and coworkers [8,9] and has been used primarily for environmental analysis of ground water and other samples [10]. For the analysis of biological samples some applications have already been described, for example, the analysis of amphetamines in urine [11,12] or the analysis of anoretic compounds in urine [13]. The analysis of organic acids in urine has not been performed before. Organic acids cause some problems in their extraction from an aqueous solution because of their polarity. Pawliszyn succeeded in extracting some relatively unpolar fatty acids by SPME out of the water and chromatographed them without any derivatisation. Other fatty acids could be analysed by the headspace technique [14]. Furthermore a procedure is described for derivatisation of fatty acids on the SPME fiber in the gaseous phase using 1-pyrenyldiazomethane [14]. With our procedure, which includes the extraction of methyl esters of the organic acids by SPME, we propose a relatively simple way to analyse organic acids with a wide range of structure and polarity.

2. Materials and methods

2.1. Chemicals

TMO was purchased from Aldrich (Steinheim, Germany), sodium carbonate and sodium hydrogen carbonate were received from Merck (Darmstadt, Germany).

2.2. SPME equipment

The SPME holder and fiber assembly for manual sampling was obtained from Supelco (Deisenhofen, Germany). A fiber carrying a polyacrylate coating with a film thickness of 85 μm was used as the stationary phase. Before measurements the SPME fiber was conditioned for 2 h at 300°C in the GC-injection port until no peaks were detected in a blank analysis. Before every new analysis of a urine sample, the fiber was heated for 5 min at 280°C in the GC-injection port to ensure that there was no contamination adsorbed on the stationary phase.

2.3. Sample preparation

Two ml of urine are transferred into a 3 ml round bottom reaction vial with a magnetic stirring bar. While stirring the solution, about 20 mg of sodium carbonate are added at room temperature. Derivatisation with TMO is performed in five steps. Within 4 min approximately 30 mg of TMO are added in five portions. After 1 min the solution is neutralized with about 15 mg of sodium hydrogen carbonate. This procedure of adding 30 mg of TMO followed by neutralization with sodium hydrogen carbonate is repeated twice. After that, a further 30 mg of TMO are added in portions, this time followed by an alkalisation of the mixture with sodium carbonate (about 20 mg). Finally within 4 min another 30 mg of TMO are added and the solution is neutralized with sodium hydrogen carbonate and incubated for 2 min at 100°C.

2.4. Extraction conditions

The sample is stirred at room temperature in the glass vial by a magnetic stirring bar. The SPME fiber, coated with the polyacrylate phase is pushed out of the needle of the manual SPME instrument. The fiber is held into the sample for 20 min in the way that the coating is entirely beneath the liquid surface. The metallic needle remains outside the solution. After the adsorption the fiber is taken out of the solution and drawn back into the shielding needle.

2.5. Desorption conditions

The SPME instrument with the organic acid methyl esters adsorbed at the fiber with the polyacrylate coating is transferred to the GC system. The needle is punched entirely through the septum of the injector. Afterwards the fiber is pushed out of the needle for a length of 2.5 cm. In this position the instrument is held for 4 min. At an injection port temperature of 280°C the analyte is desorbed from the fiber. Finally the fiber is drawn back into the shielding needle, and the SPME instrument is removed from the injection port.

2.6. GC- and GC–MS conditions

GC instrumentation, Vega 6130 (Carlo Erba, Hofheim, Germany), equipped with flame ionization detection; column, 25 m×0.25 mm fused-silica column, coated with OV1701; carrier gas, helium; head pressure, 102 kPa; injector temperature, 280°C; detector temperature, 300°C; temperature was programmed from 40°C to 280°C at 2°C/min; split, 1:3. GC–MS instrumentation, TSQ 70 (Finnigan MAT Bremen, Germany); injector temperature, 280°C; ionization mode, electron impact (EI); temperature of ion source, 150°C; temperature of manifold, 70°C; pressure, 10 mTorr; emission current, 200 μA; multiplier, 1400 V; mass scan from m/z 40 to m/z 400; scan time, 0.7 s; split, 1:3.

3. Results and discussion

3.1. Derivatisation directly in the urine

The SPME technique, applied to liquid samples, requires an aqueous solvent, which may contain a maximum of 10% of an organic solvent. Therefore it can be easily combined with the derivatisation procedure using TMO, where the organic acids are directly methylated in the aqueous urine. TMO was first synthesized and characterized by Meerwein and coworkers [7,15]. Meerwein and coworkers found in their studies, that water does not react instantaneously with TMO. This is the reason why it is possible to perform the methylation of the organic acids directly in the aqueous phase.

We first alkalize the urine with sodium carbonate in order to form the anions of the organic acids. To minimize reaction with water, we work at room temperature and perform derivatisation in five steps by adding the TMO in small portions. The yield of the reaction with TMO has not been determined for the broad spectrum of organic acids in urine, because the present study is qualitative in nature. For quantitative analysis using calibration curves on the basis of external standards for each component, the yield would also not be required for calculation.

In the reaction a methyl group of the oxonium ion combines with the anion of the organic acid to form the organic acid methyl ester:

$$RCOO^- + CH_3\text{-}O\text{-}(CH_3)_2^+ \rightarrow RCOOCH_3 + CH_3\text{-}O\text{-}CH_3$$

The reaction between water and TMO, which does not take place as fast as the reaction between the organic acid anions and TMO, occurs only to a small extent. There are other side reactions, also occurring to a minor extent, for example the reaction of the hydroxide ion or the carbonate ion with a methyl group. The extent of such reactions is lowered by the protons, which are produced during the hydrolysis of the tetrafluoroborate ion. Fluoride ions, which are also resulting from the hydrolysis and the following reactions, will not combine with a methyl group, as Meerwein and coworkers found in their studies [15].

After several additions of TMO the reaction medium turns acidic due to the hydrolysis products of the tetrafluoroborate ion. Therefore we neutralize with sodium hydrogen carbonate and include an alkalisation step. At the end of the reaction we neutralize the solution because at pH = 7 the SPME coating has a long lifetime. Heating up to 100°C ensures that all the remaining reagents, especially the product boron trifluoride, which originates from the tetrafluoroborate ion, will be hydrolysed. It is known, that even small traces of boron trifluoride can damage a capillary column irreversibly.

3.2. SPME technique

For the extraction of the analyte from the urine the relatively new SPME technique is applied, developed mainly by Pawliszyn and coworkers [8,9]. It allows

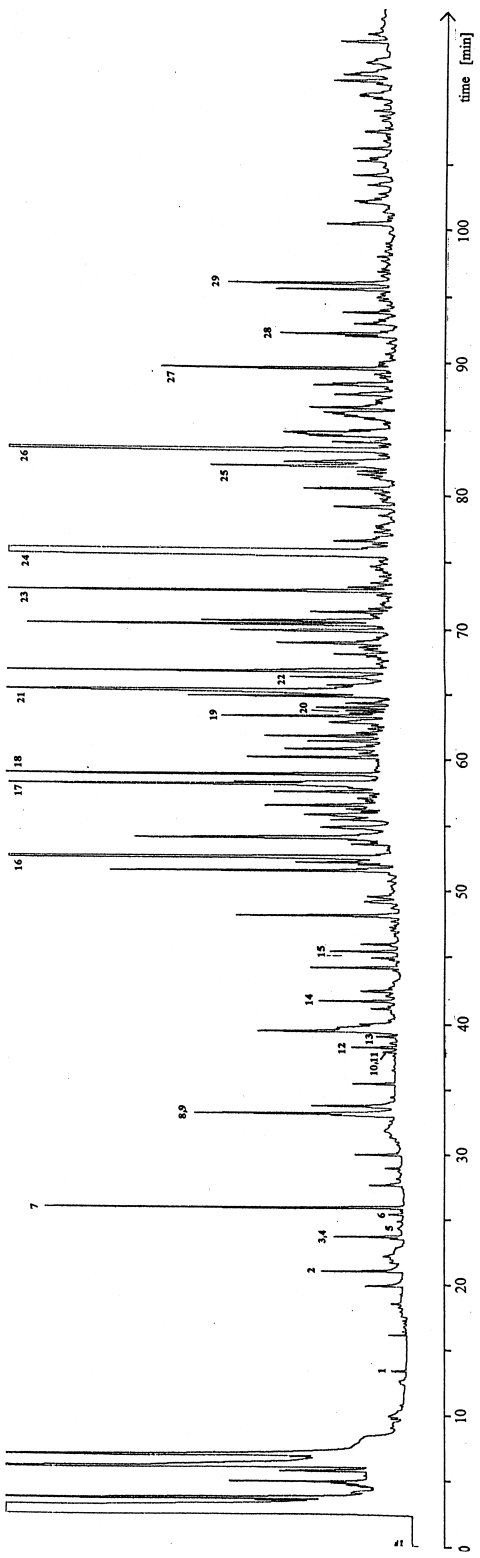

Fig. 1. Gas chromatogram of the organic acid methyl esters extracted from a normal urine by SPME.

the extraction of organic substances out of an aqueous solution in a very simple way: a coated fiber of about 2 cm in length is held into the solution and, while agitated to enhance partitioning, the analyte is adsorbed by the fiber material. After adsorption, the fiber can be brought into the injection port of a GC or a GC–MS system where thermal desorption of the substances takes place.

The coating of the SPME fiber consists of polyacrylate with a film thickness of 85 μm. Its polarity is similar to that of the relatively polar organic acid methyl esters in the urine sample. In order to avoid determination of time–sorption profiles for the large number of urinary acids, a relatively long adsorption time of 20 min is chosen to reach an equilibrium in the two phase system. Optimal desorption conditions are observed when the fiber is pushed out for 2.5 cm from the shielding needle to bring the coating of the fiber to the hottest region of the injection port of the GC or GC–MS. Desorption temperature for the organic acid methyl esters should be 280°C. The SPME fiber is kept for 4 min in the injection port. This delay is recommended because the fiber is not heated instantaneously to the high temperature of the injection port.

Table 1
Substances identified by mass spectrometry: the numbers indicate the peaks in the chromatogram in Fig. 1

1	Malonic acid
2	Phosphoric acid
3	Succinic acid
4	Ethylmalonic acid
5	Maleinic acid
6	Methylsuccinic acid
7	Benzoic acid
8	Phenylacetic acid
9	3-Methylglutaric acid
10	3-Methylglutaconic acid
11	Methoxysuccinic acid
12	3-Hydroxy-3-methylglutaric acid
13	Adipic acid
14	3-Methyladipic acid
15	3,4-Methyleneadipic acid
16	Methoxyphenylacetic acid
17	Citric acid
18	Azelaic acid
19	Furoylglycine
20	Hydroxymandelic acid
21	4-Hydroxyphenylacetic acid
22	Homovanillic acid
23	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid
24	Hippuric acid
25	3-Carboxy-4-methyl-5-pentyl-2-furanpropionic acid
26	3-Indoleacetic acid
27	Methoxyhippuric acid
28	Isomer to 27
29	Methoxyindoleacetic acid

3.3. GC and MS

Fig. 1 demonstrates a profile of the urinary organic acids. Most of the acids occur in high concentrations. Therefore for these components no problems arise with the detection limit. The components are well separated and we could identify 29 organic acid methyl esters (Table 1). Although there are still some unidentified peaks, interferences from contaminants or other endogenous substances with peaks of organic acid methyl esters occurring in urine are not observed, as concluded from the MS data.

4. Conclusion

We demonstrate the application of the SPME technique to the GC and GC–MS analysis of organic acids in urine. The sample preparation is simple and requires only about 40 min. As the SPME technique requires an aqueous solution with a maximum content of 10% of an organic solvent, we derivatise the organic acids directly in the aqueous urine by using TMO as the methylating agent. The resulting gas chromatogram shows a well separated profile of the urinary organic acids. We propose that the described method is applicable to other aqueous samples as well.

References

[1] P.T. Ozand, G.G. Gascon, J. Child Neurol. 6 (1991) 196.
[2] H.M. Liebich, J. Chromatogr. 379 (1986) 347.
[3] H.M. Liebich, C. Först, J. Chromatogr. 525 (1990) 1.
[4] I.M. Bengtsson, D.C. Lehotay, J. Chromatogr. B 685 (1996) 1.
[5] Y. Mardens, A. Kumps, C. Planchon, C. Wurth, J. Chromatogr. 577 (1992) 341.
[6] H.M. Liebich, E. Gesele, 21st International Symposium on Chromatography, Stuttgart, Germany, 1996.

[7] H. Meerwein, G. Hinz, P. Hofmann, E. Kroning, E. Pfeil, J. Prakt. Chem. 147 (1937) 257.
[8] C.L. Arthur, J. Pawliszyn, Anal. Chem. 62 (1990) 2145.
[9] Z. Zhang, M.J. Yang, J. Pawliszyn, Anal. Chem. 66A (1994) 844.
[10] D.W. Potter, J. Pawliszyn, J. Chromatogr. 625 (1992) 247.
[11] H.G. Ugland, M. Krogh, K.E. Rasmussen, J. Chromatogr. A 701 (1997) 29.
[12] F. Centini, A. Masti, I. Barni-Comparini, 18th International Symposium on Capillary Chromatography, Riva del Garda, Italy, 1996.
[13] M. Chiarotti, S. Strano-Rossi, R. Marsili, 18th International Symposium on Capillary Chromatography, Riva del Garda, 1996.
[14] L. Pan, M. Adams, J. Pawliszyn, Anal. Chem. 67 (1995) 4396.
[15] H. Meerwein, E. Battenberg, H. Gold, E. Pfeil, G. Willfang, J. Prakt. Chem. 154 (1939) 83.