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Analytical derivatization–a tool for determination of orotic acid

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Abstract

Derivatization of orotic acid (OA) into various forms (trimethylsilylderivate, alkyl ester and per-methylated derivate) and their evaluation by GC/MS is described. The tested approach includes ion-exchange SPE clean-up, evaporation and chemical reaction with different types of derivatization agents (N,O-bis-(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane, butanol with acetylchloride and ethereal solution of diazomethane). Derivate originated in the reaction with diazomethane was used for determination of urinary orotic acid by GC/MS. Detection limit of 0.28 μmol l^{-1} was reached using the ion 82 *m/z* in single ion monitoring (SIM) mode. Linearity of the method was tested within the range of 3.4–2503.4 μmol l^{-1} covering physiological and pathological levels of orotic acid in urine sample. Recoveries were within the range 93.7–110.6%. Application of the method on the patient with defect of ornithine transcarbamylase (OTC) was demonstrated as well.

Keywords: Derivatization, GC; Orotic acid

1. Introduction

The determination of the urinary orotic acid (OA) is very important to diagnose some inborn errors of metabolic pathways such as pyrimidine synthesis and urea cycle. Disorder related to pyrimidine synthesis is hereditary orotic aciduria caused by deficiency of uridine monophosphate synthase (UMPS), a bifunctional protein having two catalytic activities: orotate phosphoribosyltransferase (OPRT) and orotidine monophosphate decarboxylase (ODC). A defect in both the activities is classified as orotic aciduria type I while ODC defect is denoted orotic aciduria type II. In most cases, orotic aciduria arises from inherited defects of enzymes involved in the urea cycle after synthesis of intramitochondrial carbamoyl phosphate [1]. Moreover, the high level of urinary orotate occurs also in purine nucleoside phosphorylase deficiency [2], lysinuric protein intolerance [3] and could be induced by drugs such as allopurinol [4,5] and 6-azauridine [6]. Taken together abnormal values of OA are found in 11 diseases making its measurement one of the most important tools to diagnose some metabolic defects. Healthy human urine contains only micromolar levels of OA (adults up to 1.05 mmol mol^{-1} creatinine, children up to 4.1 mmol mol^{-1} creatinine) [7].

Currently, there are several approaches for determination of urinary OA including HPLC with UV [8–12] and MS^2 detection [13–15], GC/MS [16,17], CE [18] and non-selective colorimetric method [19].

The determination of OA by common chromatographic techniques is rather difficult due to the strongly polar character of the molecule resulting in extremely low retention in reversed phase HPLC [11], high retention in ion-exchange or ion-pair chromatography [8–10] and in low volatility for GC. Recently we have reported SPE clean-up and direct loading of the extract into the mass spectrometer for determination of the purine and pyrimidine markers of inherited metabolic disorders [20]. Unfortunately, OA gives a low ionization yields using atmospheric pressure ionizations (API, i.e. electrospray and atmospheric pressure chemical ionization techniques).

Derivatization of OA leads to the significant decrease of high polarity of OA, which is beneficial for majority of chromatographic techniques [12]. Moreover, the substitution of the molecule of OA can be a promising approach for better ionisation in atmospheric pressure ionization

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techniques (e.g. in conjunction with direct infusion into mass spectrometer [20]).

The aim of the paper is to investigate different derivatization approaches (silylation, esterification and reaction with diazomethane) allowing the determination of orotic acid by gas chromatography and potentially by other analytical techniques.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Orotic acid, methyl orotate, N,O-bis-(trimethylsilyl)trifluoroacetamide, trimethylchlorosilane and *N*-methylurea were obtained from Sigma (St. Louis, MO). Deionised water (18 MΩ cm, Millipore, Bedford, USA) was used for preparation of all solutions.

Diazomethane was prepared by alkaline decomposition of *N*-nitroso-*N*-methylurea. *N*-methylurea (11.1 g) and sodium nitrite (10.8 g) were dissolved in 100 ml of water. Then 40 ml of 1.7 mol l^{-1} sulphuric acid was added while mixing and cooling in ice water. After 2 h *N*-nitroso-*N*-methylurea was filtrated, dissolved in 50 ml of diethylether, placed in ice water bath and decomposed by dropwise addition of 70% KOH (20 ml). After 30 min the ethereal solution of diazomethane was decanted and stored in freezer at −20 °C for 1 month without loss of methylation activity.

2.2. Sample preparation

Urine samples from healthy children and patient were stored at −20 °C until analysis. Three aliquots (450 μl) of patient urine were spiked with standard addition of 0, 100 and 200 μmol l^{-1} of OA and analysed as follows.

Samples were purified using SPE ion-exchange columns (SAX) containing quaternary ammonium functional group (Alltech Associates Inc., Laarne, Belgium). Columns were conditioned with 4 ml of water. Urine samples (500 μl) were mixed with 0.1 mol l^{-1} NaOH (500 μl) and applied on the SPE columns. After sample loading, the columns were washed with 1 ml of water and 1 ml of 0.1 mol l^{-1} HCOOH. Finally, OA was eluted with 1 ml of 10 mol l^{-1} HCOOH. The flow rates were about 1 ml min^{-1}. Eluates were dried under nitrogen stream at 40 °C. Sample residues were derivatizated as given further.

Silylation was accomplished by addition of 500 μl of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 50 μl of trimethylchlorosilane (TMCS) and heating at 80 °C for 30 min. Samples (dissolved in excessive derivatization agent) were injected after cooling at room temperature.

Butyl ester was prepared by adding 500 μl of butanol and 55 μl of acetylchloride and heating at 99 °C for 30 min. After cooling at room temperature about 100 mg of $NaHCO_3$ was added in order to neutralize acidic component. Clear solution over the excess of $NaHCO_3$ was injected into GC/MS.

Derivatization with diazomethane was performed by addition of 1 ml of ethereal solution of diazomethane. After 10 min the mixture was dried under nitrogen stream at room temperature and reconstituted in 500 μl of methanol.

2.3. Gas chromatography/mass spectrometry

HP 6890 Series GC system with 5973 N Mass Selective Detector and HP 7683 Series Injector (Agilent, Palo Alto, CA, USA) was used for analysis. HP-5 MS column (30 m × 0.25 mm × 0.25 μm) was operated at a temperature program: 50 °C for 2 min ramped up to 300 °C at 10 °C min^{-1} for 5 min with helium as carrier gas (99.998%, 0.9 ml min^{-1}, SIAD, Bergamo, Italy). Injection volume was 1 μl for all types of samples using pulsed splitless mode.

3. Results and discussion

3.1. Trimethylsilyl derivate of orotic acid

In the mass spectrum of trimethylsilyl derivate of OA (Fig. 1) signal at *m*/*z* 372 corresponds to the molecular ion. Two prominent ions *m*/*z* 254 and 357 represent structures $[M–HCOOTMS]^+$and $[M–CH_3]^+$, respectively. The other peaks at *m*/*z* 147 and 73 correspond to ions $[(CH_3)_2Si{=}O–Si(CH_3)_3]^+$ and $[TMS]^+$, respectively.

The prominent ion *m*/*z* 254 was selected for monitoring and calculation of results. In the Fig. 2 there are overlaid selected ion monitoring (SIM) chromatograms of healthy urine containing 5.1 μmol l^{-1} of OA (determined by standard addition method) and the same sample spiked with 1000 μmol l^{-1} of OA, corresponding to usual pathological value. The linear range of the method sufficiently covers the range of the concentrations expected in clinical samples. The estimated limit of detection (signal-to-noise ratio S/N = 3) was 0.05 μmol l^{-1}. These results confirm that trimethylsilyl derivate of OA is suitable for gas chromatography as already described [16,17].

Generally, trimethylsilyl derivates are very sensitive to air humidity which causes their hydrolytic decomposition [21]. For this reason trimethylsilyl derivate cannot be isolated from a reaction mixture by evaporation of excessive reaction agents. Moreover, sample dissolved in large excess of silylation agent cannot be generally applied for the other analytical purposes (e.g. for the direct loading into API-MS).

3.2. Butyl ester of orotic acid

The addition of butanol in the presence of acetylchloride gives butyl ester of orotic acid. Mass spectrum of butyl ester depicts dominant ion *m*/*z* 68 arising from the cleavage of pyrimidine ring as it is shown in the scheme in the Fig. 3. The analogous fragmentation was observed in the mass spectrum

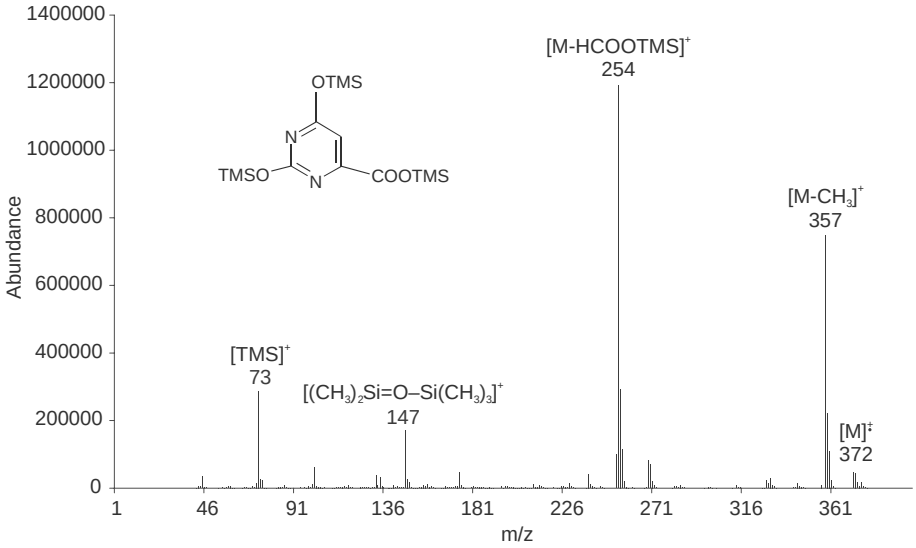


Fig. 1. Mass spectrum of trimethylsilyl derivate of orotic acid.

of methylester of OA as well (data not shown). Ion *m*/*z* 212 corresponds to the molecular ion. The ions *m*/*z* 139 and 157 can be assigned to structures $[M–OC_4H_9]^+$ and $[M–C_4H_7]^+$ ("McLafferty + 1" rearrangement), respectively. Ion *m*/*z* 57 corresponds to $[C_4H_9]^+$ and the signal at *m*/z 41 arises from cleavage of neutral molecule HCN (−27) from ion *m*/*z* 68.

Unfortunately, butyl orotate (as well as methyl orotate, data not shown) gives tailing peak in GC/MS. Its response is too low (detection limit 75 μmol l^{-1} was reached), which is not sufficient for the determination of the orotic acid in healthy urine and clinical samples with slightly increased level of OA (Fig. 4). Supposedly, unblocked keto-enolic tautomerism on pyrimidine ring causes unsuitable behaviour in gas chromatographic system and consequently, low sensitivity of the approach. For these reasons, alkyl esters of OA were excluded from the further GC/MS investigation. On the other hand, alkyl esters are stable enough for their isolation from reaction mixture by evaporating excessive agents. In principle, the facts extend the possibility of exploitation of alkyl orotates in other analytical techniques [12].

3.3. Methylation of orotic acid with diazomethane

Reaction of OA with excessive diazomethane leads probably to methylester of 1,2,3,6-tetrahydro-1,3-dimethyl-2,6-

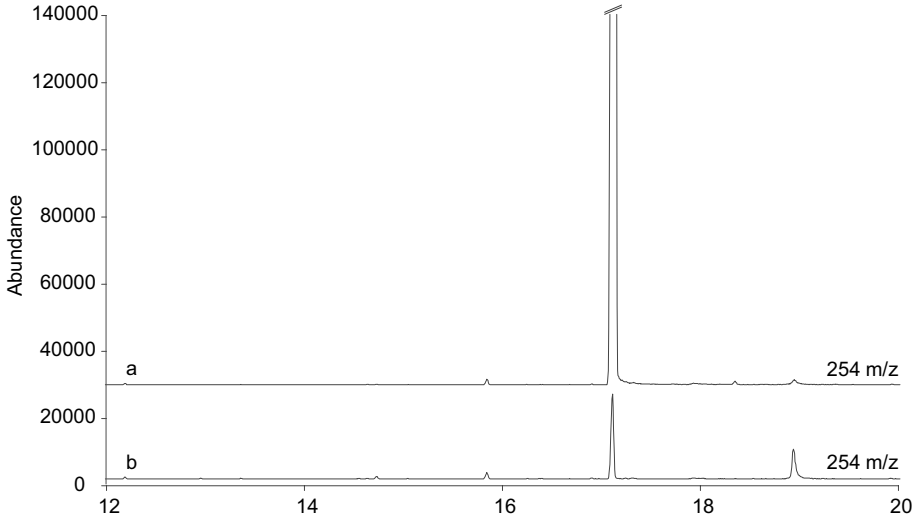


Fig. 2. Selected ion monitoring chromatograms of trimethylsilyl derivate of orotic acid: (a) healthy urine spiked with 1000 μmol l^{-1} of orotic acid; (b) healthy urine (orotic acid concentration determined by standard addition method was 5.1 μmol l^{-1}).

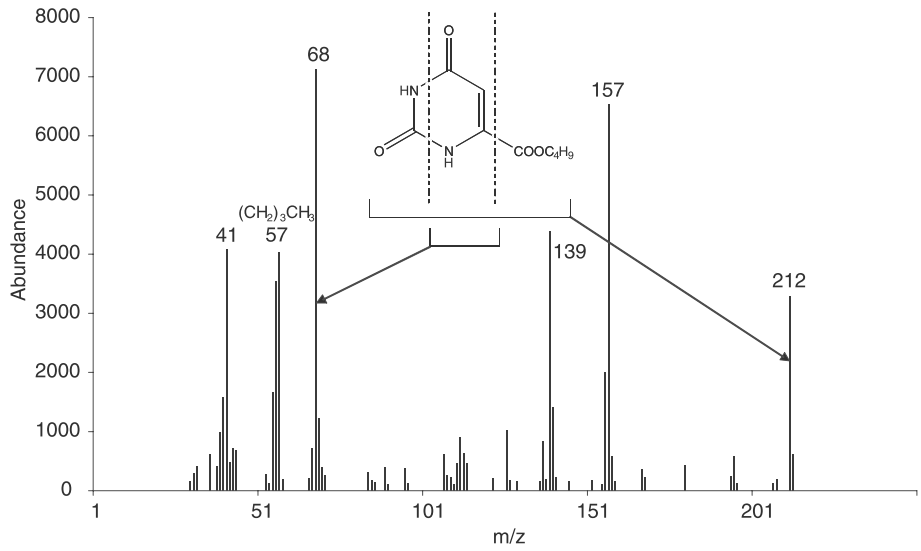


Fig. 3. Mass spectrum of butyl ester of orotic acid.

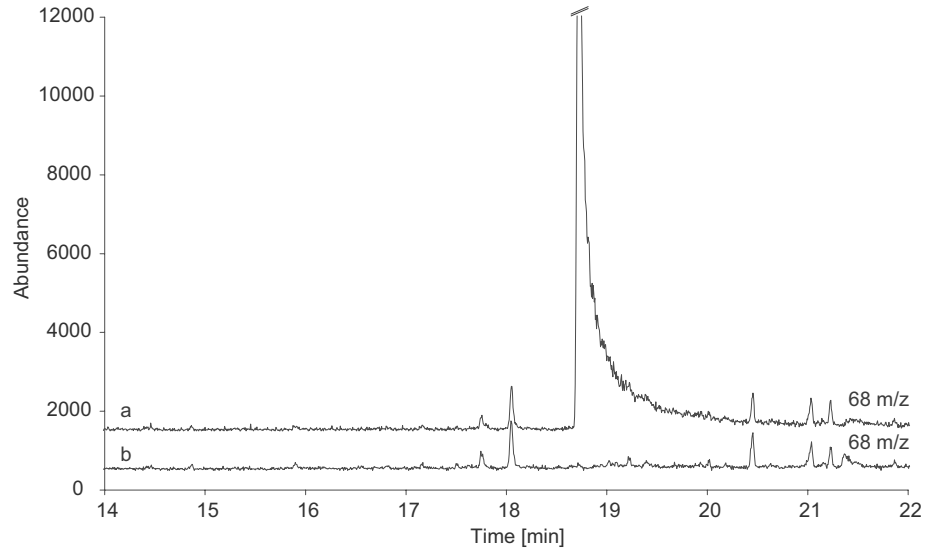
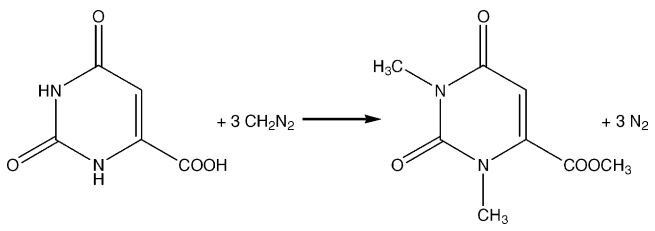


Fig. 4. Selected ion monitoring chromatograms of butyl ester of orotic acid: (a) healthy urine spiked with 1000 μmol l^{-1} of orotic acid; (b) healthy urine (orotic acid was not detected, the same sample as in Fig. 2).

dioxo-4-pyrimidine carboxylic acid (tri-Me-OA) according to equation:



In the mass spectrum of tri-Me-OA there are two main peaks at *m*/*z* 198 and 82 corresponding to the molecular ion and the ion arising from the cleavage of pyrimidine ring (see Fig. 5). Small peaks at *m*/*z* 139 and 167 correspond to ions $[M–COOCH_3]^+$ and $[M–OCH_3]^+$, respectively. The peak at *m*/*z* 55 rises from cleavage of neutral molecule HCN (−27) from ion *m*/*z* 82. So, the fragmentation pathways of methyl derivate and alkyl esters of OA are almost identical. All of them include the cleavage of pyrimidine ring. On the other hand, pyrimidine ring is kept untouched in the case of trimethylsilyl derivate. The different fragmentations suggest different type of substitution on pyrimidine ring in tris(trimethylsilyl) and trimethyl derivate of orotic acid.

The aromatic character of the ring substituted in its enol-form can explain the high stability of the silanized ring. It means, that silylation leads to the O-substituted compounds, which is in agreement with data published previously [17]. Strong tendency for the cleavage of the pyrimidine ring in the tri-Me-OA advocates the non-aromatic character of the ring derivatized in its oxo-form. Oxo-tautomer is generally

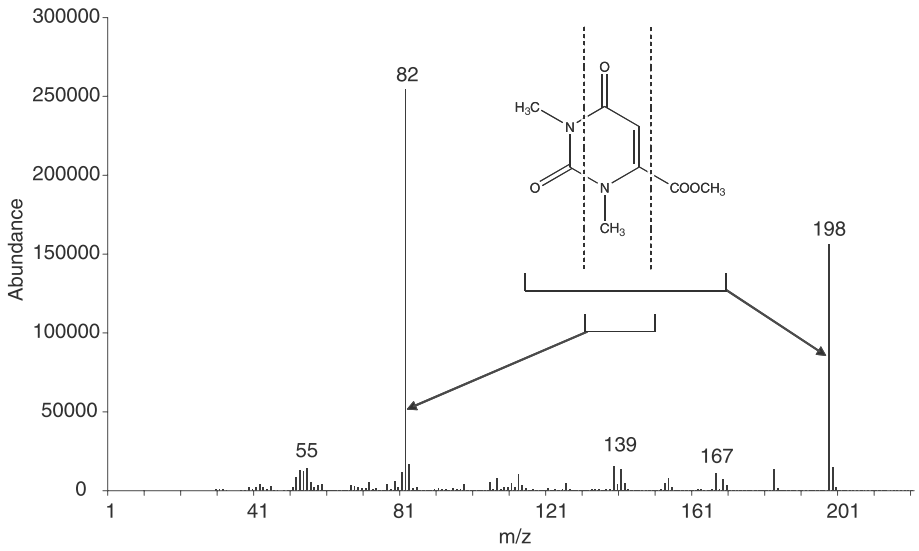


Fig. 5. Mass spectrum of methylated derivate of orotic acid.

considered as the predominant form of the native pyrimidines. For this reason N-substituted formula was adopted for the structure of tri-Me-OA. Paradoxically, the structure of the methylated pyrimidine ring cannot be confirmed from the mass spectrum because of the isobaric nature of the losing fragments CH_3OCN and $OCNCH_3$.

All chromatograms were evaluated in the SIM mode for the ion 82 *m/z*. Fig. 6 shows two overlaid selected ion monitoring chromatograms of healthy urine and the same sample spiked with 1000 μmol l^{-1} of OA, corresponding to the pathological sample. The concentration of OA in the healthy urine was determined 4.9 μmol l^{-1} by the standard addition method.

3.4. Determination of orotic acid

The methylated derivate of OA gives efficient peak allowing sensitive determination. Therefore, derivatization with diazomethane was selected for further investigation and development of GC/MS method. Calibration standards were prepared in pooled paediatric urine ($n = 6$) in which native concentration of OA (3.4 μmol l^{-1}) was determined by standard addition method. Serially diluted solutions of OA were used to achieve final concentrations 3.4, 4.4, 5.9, 8.4, 13.4, 28.4, 53.4, 103.4, 253.4, 503.4, 1003.4, 2503.4 μmol l^{-1}. The samples were cleaned on SPE columns, treated with diazomethane and analysed as

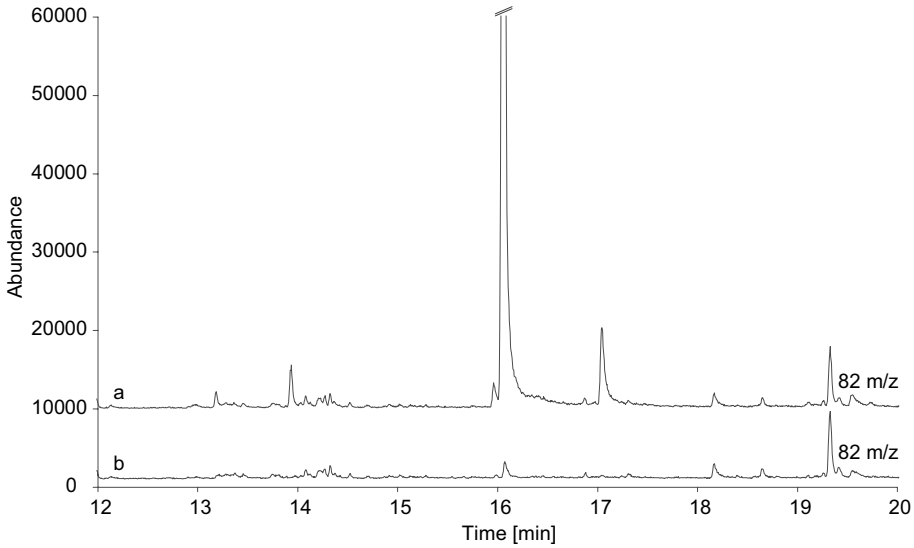


Fig. 6. Selected ion monitoring chromatograms of methylated derivate of orotic acid: (a) healthy urine spiked with 1000 μmol l^{-1} of orotic acid; (b) healthy urine (orotic acid concentration determined by standard addition method was 4.9 μmol l^{-1}, the same sample as in Fig. 2).

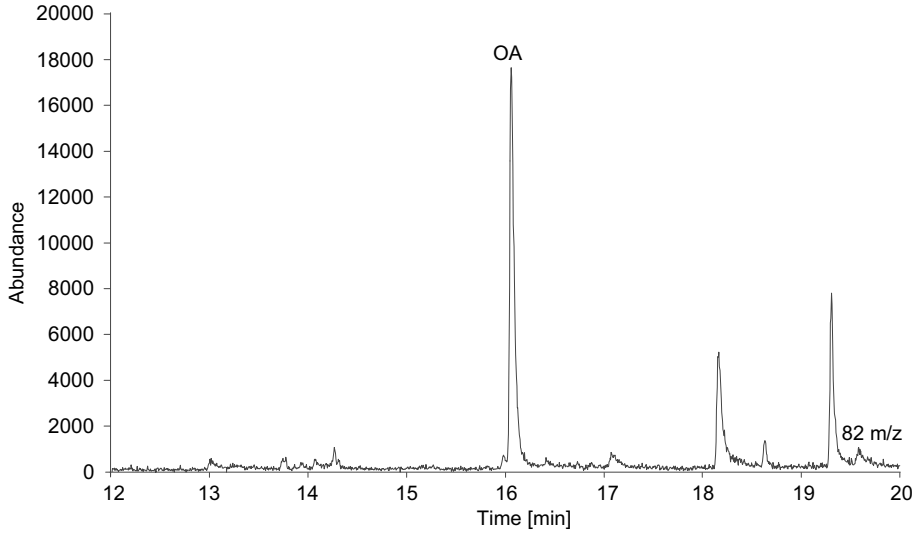


Fig. 7. Selected ion monitoring chromatogram of the urine from a patient with ornithine transcarbamylase deficiency (for detail see Section 3.4).

described in experimental section. The peak-areas were plotted versus OA concentration to construct calibration curves. Linear calibration dependence was obtained in the range of 3.4–2503.4 μmol l^{-1}. For practical purposes, the calibration dependence was divided into two parts covering the intervals from 3.4 to 103.4 μmol l^{-1} and from 103.4 to 2503.4 μmol l^{-1}. The equations of the respective regression lines were $y = 7244.5x + 14192$ ($R^2 = 0.993$) and $y = 7548.4x - 110705$ ($R^2 = 0.998$). Good agreement of both slopes confirms the linearity over the whole range. The estimated limit of detection was 0.28 μmol l^{-1} and limit of quantification (S/N = 10) was 0.93 μmol l^{-1}. The intra-day precisions determined at the concentration levels 53.4 and 503.4 μmol l^{-1} were 6.0 and 4.4%, respectively (relative standard deviation, $n = 5$). The average recoveries determined at the same concentrations were 104.5 and 99.6% (see Table 1). The inter-day precisions determined at the same concentration levels were 7.5 and 6.3%, respectively (relative standard deviation, $n = 5$).

Table 1
Intra-day precision and recovery of OA in urine spiked at two concentrations

	Concentration in urine (μmol l^{-1})	
Target concentration	53.4	503.4
Assay number	Found	Found
1	58.9	528.7
2	56.7	480.6
3	50.0	525.7
4	54.3	478.9
5	59.1	488.6
Average	55.8	500.5
C.V. (%)	6.0	4.4
Recovery (%)	104.5	99.6

Quantitation against calibration curves.

Method was tested on the urine from the patient with defect of ornithine transcarbamylase OTC (Fig. 7). Concentration of orotic acid determined by standard addition method was 103.6 ± 1.3 μmol l^{-1}. The same sample was processed by the calibration curve method, giving the value 98.8 ± 1.0 μmol l^{-1}. Both results are in good agreement with the concentration 100.5 ± 0.3 μmol l^{-1} found by capillary electrophoresis [22]. Mutual agreement of all results confirms the credibility of the GC/MS method.

4. Conclusion

Three derivatization approaches were tested for preparation of derivatives of OA. The suitability of the rising derivative for the GC/MS analysis and possibility of the simple isolation of the product from derivatization agent were used as basic criteria for the consideration. Methylation by diazomethane was found as suitable derivatization procedure for the GC/MS analysis of OA. GC/MS method based on methylated derivative gives satisfactory limit of detection and linear response over the wide range covering the physiological as well as pathological concentrations of OA. Derivative can be easily isolated from the reaction mixture by evaporation of the excessive agent and can be potentially used for other analytical approaches (e.g. HPLC, direct infusion into tandem mass spectrometry and so on).

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References

[1] D.R. Webster, D.M.O. Becroft, D.P. Suttle, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic Basis of Inherited Disease, seventh ed., McGraw-Hill, New York, 1995, pp. 1799–1837.
[2] A. Cohen, G.J. Staal, A.J. Ammann, D.W. Martin, J. Clin. Invest. 60 (1977) 491.
[3] J. Rajantie, Pediatr. Res. 15 (1981) 115.
[4] R.M. Fox, D. Royse-Smith, W.J. O'Sulivan, Science 196 (1970) 861.
[5] T.D. Berdmore, J.S. Cashman, W.N. Kelly, J. Clin. Invest. 51 (1972) 1823.
[6] H.J. Fallon, E. Frei, J. Block, J.E. Seegmuler, J. Clin. Invest. 40 (1961) 1906.
[7] C. Salerno, C. Crifò, J. Chromatogr. B 781 (2002) 57.
[8] S.W. Brusilow, E. Hauser, J. Chromatogr. 493 (1989) 388.
[9] N. Seiler, C.H. Grauffel, G. Therrien, S. Sarhan, B. Knoedgen, J. Chromatogr. B 653 (1994) 87.
[10] S. Ohba, K. Kidouchi, T. Katoh, J. Chromatogr. 568 (1991) 325.
[11] H. Miyazaki, Y. Matsunaga, K. Yoshida, S. Arakawa, M. Hashimoto, J. Chromatogr. 274 (1983) 75.
[12] A. Fioravanti, M. Flaviani, C. Gambelunghe, A. Micheletti, M. Sposito, S. Rufini, J. Chromatogr. B 703 (1997) 263.
[13] T. Ito, A.B.P. van Kuilengurg, A.H. Bootsma, A.J. Haasnoot, A. Van Cruchten, Y. Wada, A.H. van Gennip, Clin. Chem. 46 (2000) 445.
[14] M.S. Rashed, M. Jacob, M. Al-Amoudi, Z. Rahbeeni, A.D. Al-Sayed, L. Al-Ahaidib, A.A. Saadallah, S. Legaspi, Clin. Chem. 49 (2003) 449.
[15] G. la Marca, B. Casetta, E. Zammarchi, Rapid Commun. Mass Spectrom. 17 (2003) 788.
[16] M.T. McCann, M.M. Thompson, I.C. Gueron, M. Tuchamn, Clin. Chem. 41 (1995) 739.
[17] T. Kuhara, Ch. Ohdoi, M. Ohse, J. Chromatogr. B 758 (2001) 61.
[18] J. Ševčík, T. Adam, V. Sázel, Clin. Chim. Acta 259 (1997) 73.
[19] M.L. Harris, V.G. Oberholzer, Clin. Chem. 26 (1980) 473.
[20] P. Fryčák, R. Hušková, T. Adam, K. Lemr, J. Mass Spectrom. 37 (2002) 1242.
[21] V. Miller, V. Pacáková, Chem. Listy (in Czech) 67 (1973) 1121.
[22] D. Friedecký, T. Adam, P. Barták, Electrophoresis 23 (2002) 565.