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Short communication

Simultaneous determination of urinary cystathionine, lanthionine, *S*-(2-aminoethyl)-L-cysteine and their cyclic compounds using liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization

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Abstract

A measurement system for cystathionine (Cysta) lanthionine (LT), and *S*-(2-aminoethyl)-L-cysteine (AEC), and reduced products of their ketimines, perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), 1,4-thiomorpholine-3,5-dicarboxylic acid (TMDA) and 1,4-thiomorpholine-3-carboxylic acid (TMA) in the urine samples of a patient with cystathioninuria and normal human subjects has been developed, using column liquid chromatography–mass spectrometry. The recoveries were about 90–105% for Cysta, LT and AEC, and about 77–87% for PHTZDC, TMDA and TMA after ion-exchange treatment. The concentrations of Cysta and PHTZDC in the urine of a patient with cystathioninuria were much higher compared with those in the urine of normal human subjects. The concentrations of AEC and TMDA were almost the same. LT and TMA could not be detected in the urine samples by this method. This method proved useful for the determination of sulfur-containing amino acids and their cyclic compounds in biological samples. © 1997 Elsevier Science B.V.

Keywords: Cystathionine; Lanthionine; *S*-(2-Aminoethyl)-L-cysteine; Perhydro-1,4-thiazepine-3,5-dicarboxylic acid; 1,4-Thiomorpholinedicarboxylic acid

1. Introduction

Cystathioninuria is an autosomal recessive hereditary disorder and phenotypical homozygotes lead to persistent excretion of large amounts of cystathionine in the urine due to cystathionine γ-lyase deficiency [1]. Several papers from our laboratory previously reported [2–7] that the following unusual sulfur-containing amino acids were excreted in the urine of a cystathioninuric patient: *S*-(3-hydroxy-3-carboxypropyl)cysteine (HCPC), *S*-(2-hydroxy-2-carboxyethyl)homocysteine (HCEHC), *S*-(2-carboxyethyl)cysteine (β-CEC), *N*-monoacetylcystathionine (NAc-cysta), perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), *N*-acetyl-*S*-(3-hydroxy-3-carboxypropyl)cysteine (NAc-

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HCPC), *N*-acetyl-*S*-(2-carboxyethyl)cysteine (NAc-β-CEC), *S*-(3-oxo-3-carboxypropyl) cysteine (OCPC), *S*-(2-oxo-2-carboxyethyl)homocysteine (OCEHC), cystathionine ketimine (CK), cystathionine sulfoxide (CystaSO) and NAc-cystathionine sulfoxide (NAc-cystaSO).

The cyclic ketimine derivatives of cystathionine (Cysta), lanthionine (LT), and *S*-(2-aminoethyl)-L-cysteine (AEC) have been identified as products of the enzymatic monodeamination of the parent amino acids by a transaminase activity occurring in liver, kidney and brain [8–10]. The reduced forms of cystathionine ketimine (CK), lanthionine ketimine (LK), and *S*-(2-aminoethyl)-L-cysteine ketimine (AECK), perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), 1,4-thiomorpholine-3,5-dicarboxylic acid (TMDA) and 1,4-thiomorpholine-3-carboxylic acid (TMA) as shown in Scheme 1, have been identified in bovine brain [11,12], normal human urine [13,14] and a cystathioninuric patient urine [4,15]. LT was thought to be not of endogenous origin and normally not occurring in human, so far only one paper [16] reported that LT was detected incidentally in the urine of a patient with an abdominal pseudo-tumor, the concentrations were 0.2 and 0.3 mmol/l (for meso-LT and L- and/or D-LT), respectively.

We have previously reported [17] a method for the determination of Cysta and PHTZDC in the urine of a patient with cystathioninuria using liquid chromatography–mass spectrometry with a atmospheric pressure chemical ionization (LC–APCI–MS) interface system, but contents of AEC, LT, TMA and TMDA in the urine of cystathioninuric patients have not been determined by this method. In this paper, we present simultaneous determination of contents of these sulfur-containing amino acids in the urine samples of a cystathioninuric patient and normal subjects using LC–APCI–MS.

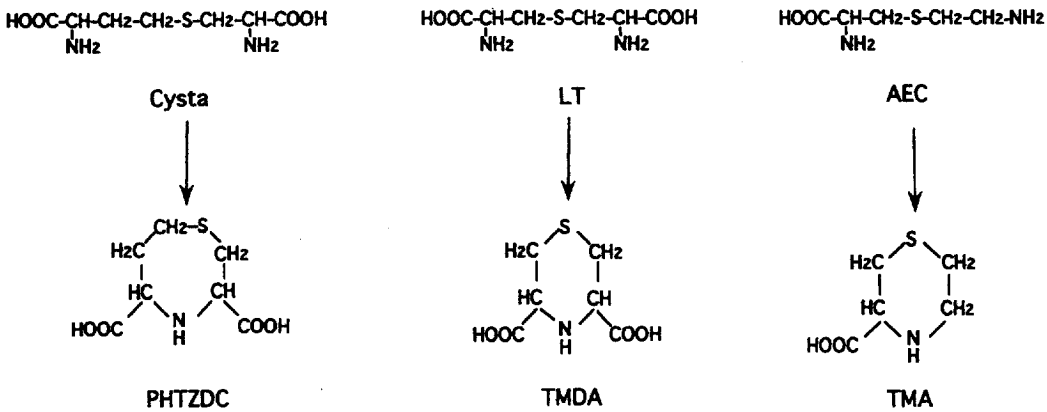
2. Experimental

2.1. Materials

Authentic Cysta and LT were obtained from Sigma (St. Louis, MO, USA); AEC was obtained from Wako Pure Chemical Industries (Osaka, Japan); PHTZDC, TMDA and TMA were prepared according to Refs. [18,19]. All other chemicals used were of analytical grade.

2.2. Preparation of urine samples

Urine samples from normal human subjects were obtained from laboratory personnel; the urine sample from a patient with cystathioninuria was obtained from an elder sister as reported in a previous paper [2]. Urine samples were stored at −20°C. Sulfur-containing amino acids and their cyclic compounds were isolated as follows: 10 ml of urine sample was adjusted to pH 5 with 2 *M* CH_3COOH, applied into



Scheme 1. Cyclic derivatives (PHTZDC, TMDA and TMA) of Cysta, LT and AEC.

a column containing 10 ml of Diaion SK-1(H-form of sulphonated cation-exchanger, 100 mesh, 10×1 cm I.D. column, Mitsubishi, Tokyo, Japan), and then eluted with 100 ml of water and 30 ml of 2 *M* NH_4OH respectively. The 2 *M* NH_4OH fraction was evaporated to dryness under reduced pressure at 45°C and the residue was dissolved in 0.5 ml of water for the determination of AEC, LT and Cysta. Part of the 2 *M* NH_4OH fraction was applied into a column containing 10 ml of Diaion SA-100 (OH-form of anion-exchanger, 100 mesh, 10×1 cm I.D. column, Mitsubishi, Tokyo, Japan), and washed with 30 ml of water, and then eluted with 50 ml of 5 *M* CH_3COOH. The eluate was dried under reduced pressure at 45°C, and the residue was dissolved in 0.5 ml of water for the determination of TMA. The water fraction eluted from the Diaion SK-1 (H-form) column was concentrated to about 10 ml under reduced pressure at 45°C, the solution was applied into a Diaion SA-100 (OH-form of anion-exchanger, 100 mesh, 10×1 cm I.D. column), and washed with 30 ml of water and 30 ml of 1 *M* CH_3COOH, and eluted with 30 ml of 1 *M* HCl. The eluate was evaporated to dryness under reduced pressure at 45°C and dissolved in 0.5 ml of water for the determination of PHTZDC and TMDA.

In order to identify these sulfur-containing amino acids in urine samples by mass spectra, the 2 *M* NH_4OH fraction, containing AEC and Cysta, was applied into a column containing 10 ml of Diaion SA-100 (OH-form), and washed with 30 ml of water, and eluted with 30 ml of 0.2 *M* CH_3COOH. The eluate was dried under reduced pressure at 45°C. The 0.2 *M* CH_3COOH fraction containing AEC and Cysta, and the 1 *M* HCl fraction containing TMDA and PHTZDC collected from 100 ml of urine sample was separately applied into a long column (40×1.5 cm) containing 100 ml of Diaion SK-1 (H-form). For the identification of AEC and Cysta by mass spectra, the column was washed with 500 ml of water, and eluted with 800 ml of 2 *M* HCl. Portions (50 ml) of the effluent were evaporated under reduced pressure to dryness; for the identification of TMDA and PHTZDC by mass spectra, the column was eluted with 600 ml of water. Portions (50 ml) of the effluent were evaporated under reduced pressure to dryness. Each sample of water fractions and 2 *M* HCl fractions were analyzed using LC–APCI–MS.

2.3. Instrumentation

The apparatus used was a Hitachi L-6200 high-performance liquid chromatography (HPLC) instrument, equipped with a 5-μm Inertsil ODS-2 packed column (150×4.6 mm I.D.) from Gasukuro Kogyo (Tokyo, Japan), connected to a Hitachi M80B mass spectrometer-computer system through the APCI interface. The nebulizer and vaporizer temperatures were 260 and 400°C, respectively. Authentic compounds and urine samples were analysed using a mobile phase of 50 m*M* CH_3COONH_4–CH_3CN (90:10, v/v) at a flow-rate of 0.9 ml/min.

The amino acid analyzer used was a Hitachi Model 835 liquid chromatograph.

3. Results and discussion

Mass chromatograms and spectra of authentic AEC (*m/z* 165), LT (*m/z* 209), Cysta (*m/z* 223), TMA (*m/z*,148), PHTZDC (*m/z* 206) and TMDA (*m/z* 192) are shown in Fig. 1. In the LC–APCI–MS, quasi-molecular ions $[M+H]^+$ of these sulfur-containing amino acids were observed as base peaks in addition to the ions of $[(M+H)\text{-}CO_2$ (or $H_2O)]^+$ and $[(M+H)+NH_3]^+$.

The standard curves for different concentrations of AEC, LT, Cysta, TMA, PHTZDC and TMDA were linear over the concentration ranges from 100 to 800 ng. The recoveries of authentic Cysta, AEC, LT, TMA, PHTZDC, and TMDA after treatment with ion-exchange resins were determined as shown in Table 1. These results indicate that this method is reliable for the determination of these sulfur-containing amino acids and their cyclic compounds.

The mixed solution of the authentic sulfur-containing amino acids and their cyclic compounds was treated by ion-exchange resins as described under the preparation of urine samples. The 2 *M* NH_4OH fraction eluted from Diaion SK-1 (H-form) and the 1 *M* HCl fraction eluted from the Diaion SA-100 (OH-form) of the water fraction from Diaion SK-1 column, were analysed using LC–APCI–MS. AEC (*m/z* 165), LT (*m/z* 209), Cysta (*m/z* 223) and TMA (*m/z* 148) were detected in the 2 *M* NH_4OH fraction, PHTZDC (*m/z* 206) and TMDA (*m/z* 192) in the 1 *M* HCl fraction (Fig. 2A,B). The retention times of

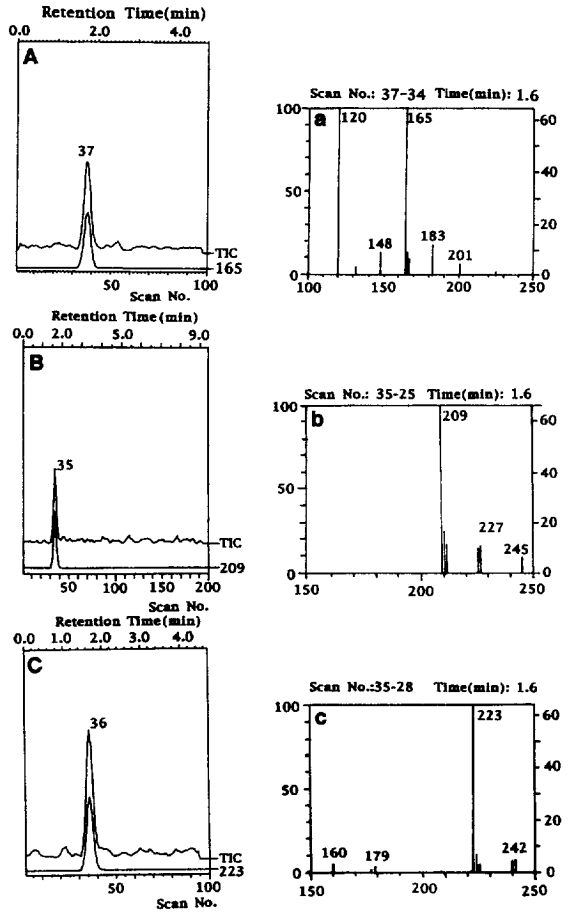


Fig. 1. Mass chromatograms and mass spectra of authentic AEC (A,a; *m/z* 165), LT (B,b; *m/z* 209), Cysta (C,c; *m/z* 223), TMA (D,d; *m/z* 148), PHTZDC (E,e; *m/z* 206) and TMDA (F,f; *m/z* 192). Mass spectra were scanned at the peak top of the mass chromatograms. The chromatographic conditions were: mobile phase, 50 m*M* CH_3COONH_4–CH_3CN (90:10, v/v); flow-rate, 0.9 ml/min. The mass spectrometer was scanned from *m/z* 100 to 300 at a rate of 4 s per scan.

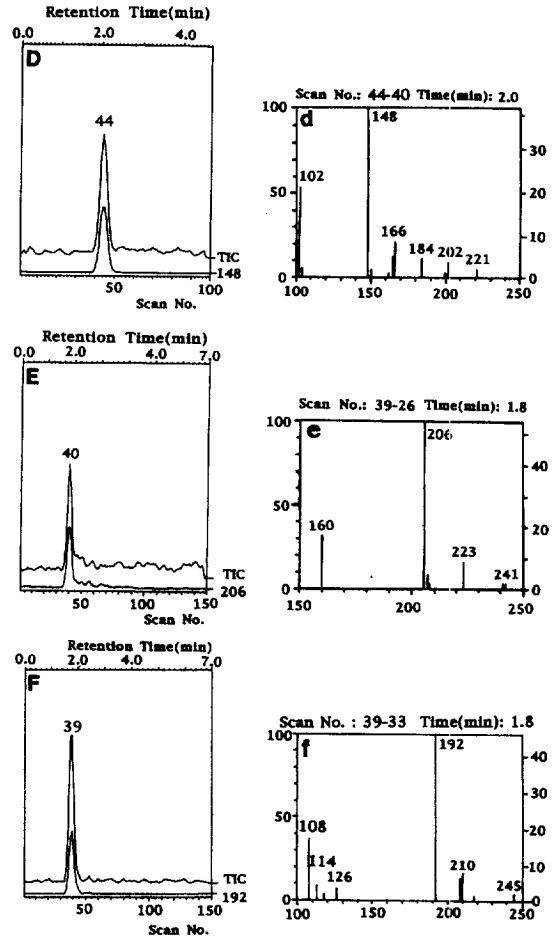


Fig. 1. (*continued*)

these sulfur-containing amino acids were almost the same on mass chromatograms, but their peaks were clearly detected because of the different quasi-molecular ions. We found that there were other substances interfering with the determination of TMA in the 2 *M* NH_4OH fraction of urine. Therefore we extracted TMA using the Diaion SA-100 column after treatment with the SK-1 column, and achieved a good separation.

Mass chromatograms of 2 *M* NH_4OH eluted from Diaion SK-1, 1 *M* HCl eluted from Diaion SA-100 of the water fraction from SK-1, and 5 *M* CH_3COOH fractions, from the urine samples of a cystathioninuric patient and a normal human subject after treatment by ion-exchange resins, as described in Section 2.2, are shown in Fig. 3(A,B,C,a,b,c). Cysta and PHTZDC were detected as large peaks in the urine of a cystathioninuric patient (A, *m/z* 223; B, *m/z* 206), but as small peaks in a normal human urine (a, *m/z* 223; b,*m/z* 206); AEC and TMDA were detected in urine samples both of a patient with cystathioninuria and a normal human (A,a, *m/z* 165; B,b, *m/z* 192); LT and TMA were not detected in 10 ml of urine samples both of a patient with cystathioninuria and a normal human subject (A,a,

Table 1
Recoveries of Cysta, AEC, LT, PHTZDC, TMDA and TMA

Name	Amount added (ng/10 μl)	Amount detected (range) (ng/10 μl)	Recovery (mean±S.D.) (%)
Cysta	300	246.5–300.8	90.98±7.28
AEC	400	361.9–395.7	95.70±3.31
LT	300	286.7–328.8	105.83±4.89
PHTZDC	600	480.8–528.7	82.60±2.26
TMDA	600	434.1–479.9	76.93±3.03
TMA	300	246.5–273.3	87.73±3.73

Note: values are from six experiments for AEC and LT ($n=6$), five experiments for Cysta, PHTZDC and TMDA ($n=5$) and three experiments for TMA ($n=3$).

m/z 209; C,c, m/z 148). For the identification of Cysta, AEC, PHTZDC and TMDA in the urine sample by mass spectra, a 0.2 *M* CH_3COOH fraction (eluted from Diaion SA-100) and a 1 *M* HCl fraction (eluted from Diaion SA-100) collected from 100 ml of urine were separately applied into a long column of Diaion SK-1 (H-form), and eluted with 2 *M* HCl and water, respectively. Cysta appeared at between 450 and 500 ml of the 2 *M* HCl fraction and AEC between 550 and 600 ml of the 2 *M* HCl fraction; TMDA appeared between 200 and 250 ml of the water effluent and PHTZDC between 300 and 350 ml of the water effluent. Mass chromatograms and spectra both of the 2 *M* HCl and water fractions from the urine samples of a cystathioninuric patient (A,a; B,b; C,c; D,d) are shown in Fig. 4. In the LC–APCI–MS, quasi-molecular ions $[M+H]^+$ and some ions of $[(M+H)\text{-}CO_2$ (or $H_2O)]^+$ and $[(M+H)+$

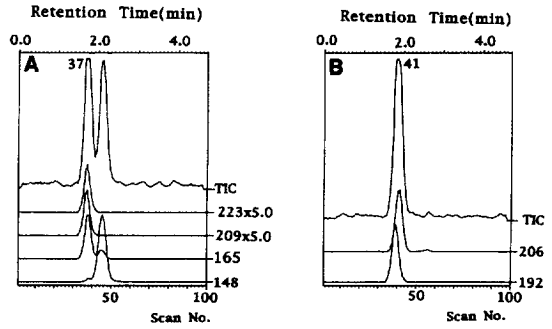


Fig. 2. Mass chromatograms of a 2 *M* NH_4OH fraction eluted from Diaion SK-1 (A) and a 1 *M* HCl fraction eluted from Diaion SA-100 of water fraction from Diaion SK-1 (B) of authentic compounds of AEC (m/z 165), LT (m/z 209), Cysta (m/z 223), TMA (m/z 148), PHTZDC (m/z 206) and TMDA (m/z 192) after treatment with ion-exchange resins, as described in Section 2.2. The chromatographic conditions were the same as in Fig. 1.

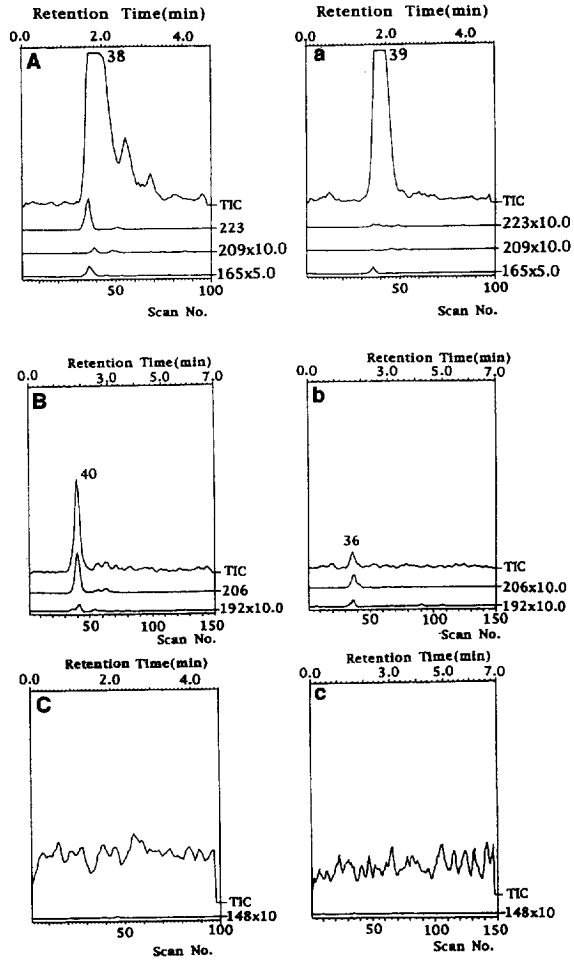


Fig. 3. Mass chromatograms of 2 *M* NH_4OH eluted from Diaion SK-1, 1 *M* HCl eluted from Diaion SA-100 of a water fraction from Diaion SK-1 and a 5 *M* CH_3COOH (Diaion SA-100) fraction of urine samples from a patient with cystathioninuria (A,B,C) and a normal human subject (a,b,c) after treatment with ion-exchange resins, as described in Section 2.2. The chromatographic conditions were the same as in Fig. 1.

Table 2
Contents of sulfur-containing amino acids and their cyclic compounds in urine samples

Name	Patient (μg/mg creatinine)	Control urine (μg/mg creatinine)
Cysta	1154.73	3.03±2.16
AEC	16.46	12.70±3.46
PHTZDC	194.14	1.22±0.82
TMDA	3.68	1.98±1.38
LT	ND[a]	ND
TMA	ND	ND

Note: the contents represent the mean obtained from urine from a cystathioninuric patient ($n=4$) and mean±S.D. from urine samples from normal subjects ($n=7$).
[a]ND, the amino acid was not detected.

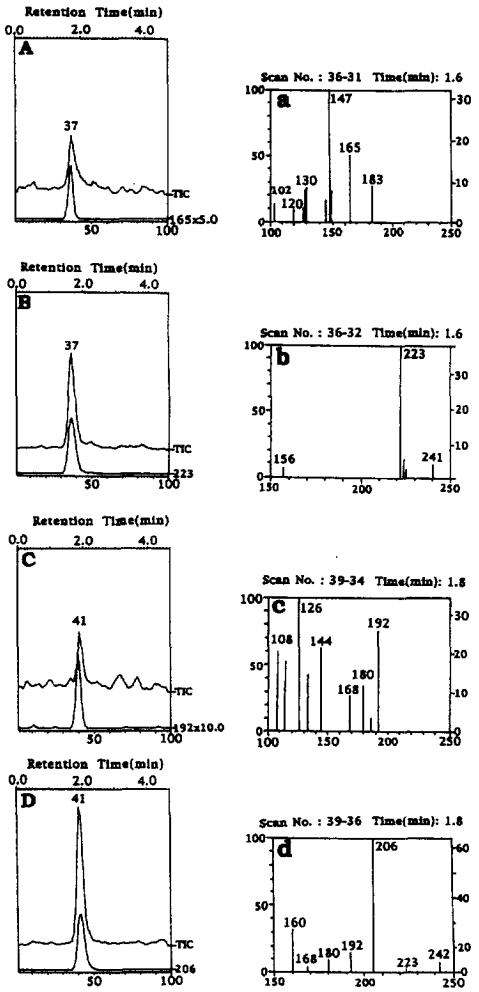


Fig. 4. Mass chromatograms and mass spectra of AEC (550–600 ml) and Cysta (450–500 ml) in 2 *M* HCl fractions, TMDA (200–250 ml) and PHTZDC (300–350 ml) in water fractions of urine samples of a patient with cystathioninuria (A,a; B,b; C,c; D,d) after treatment with a long column containing 100 ml of Diaion SK-1 (H-form), as described Section 2.2. The chromatographic conditions were the same as in Fig. 1.

$NH_3]^+$ were observed in each authentic compound, and also detected in the urine samples, and demonstrated the presence of these sulfur-containing amino acids in the urine samples both of a patient with cystathioninuria and of a normal human subject (data not shown).

To provide further support for the presence of AEC, the final urine extracts of 2 *M* HCl fraction after treatment with ion-exchange resins were also analyzed by amino acid analyser. AEC was detected in the urine samples both of a cystathioninuric patient and normal human subject(data not shown).

Concentrations of Cysta, AEC, PHTZDC and TMDA in the urine samples of a cystathioninuric patient and normal human subjects, based on peak areas, are shown in Table 2. The values of Cysta in the patient urine coincided with that reported previously [17]. There is a significant difference of Cysta and PHTZDC contents between patient urine and normal urine, but there is no significant difference of AEC, and TMDA contents.

This report indicates that LC–APCI–MS is very useful for the detection and quantitative analysis of these sulfur-containing amino acids and their cyclic compounds, and for studies of the metabolism of these compounds in biological materials.

Acknowledgments

We dedicate this paper to Dr. Doriano Cavallini on the occasion of his 80th birthday, in recognition of a long and distinguished career in the area of the intermediate metabolism of sulfur-containing compounds and of his recent pioneering work on ketimines and related compounds.

References

[1] H.S. Mudd, H.L. Levy, F. Skovby, in: C.R. Scriver, A.L. Beaudet, P. Valle (Eds.), The Metabolic Basis of Inherited Disease, vol. 1, McGraw-Hill, New York, 6th ed., 1989.

[2] H. Kodama, K. Yao, K. Kobayashi, K. Hirayama, Y. Fujii, H. Mizuhara, H. Haraguchi, M. Hirosawa, Physiol. Chem. Phys. 1 (1969) 72.

[3] H. Kodama, S. Ohmori, M. Suzuki, S. Mizuhara, Physiol. Chem. Phys. 2 (1970) 287.

[4] H. Kodama, Y. Ishimoto, M. Shimomura, T. Hirota, S. Ohmori, Physiol. Chem. Phys. 7 (1975) 147.

[5] H. Watanabe, Y. Fujita, K. Sugahara, H. Kodama, S. Ohmori, Biol. Mass Spectrom. 20 (1991) 602.

[6] T. Okada, T. Takechi, H. Wakiguchi, T. Kurashige, K. Sugahara, H. Kodama, Arch. Biochem. Biophys. 305 (1993) 385.

[7] J. Zhang, N. Masuoka, T. Ubuka, H. Kodama, J. Mass Spectrom. 30 (1995) 1296.

[8] M. Costa, B. Pensa, M. Fontana, M. Foppoli, D. Cavallini, Biochim. Biophys. Acta 881 (1986) 314.

[9] G. Ricci, M. Nardini, G. Federici, D. Cavallini, Eur. J. Biochem. 157 (1986) 57.

[10] M. Costa, B. Pensa, B. Di Costanzo, R. Coccia, D. Cavallini, Neurochem. Int. 10 (1987) 377.

[11] D. Cavallini, L. Pecci, R.M. Matarese, G. Ricci, M. Achilli, J. Biol. Chem. 260 (1985) 15577.

[12] D. Cavallini, R.M. Matarese, L. Pecci, G. Ricci, FEBS Lett. 192 (1985) 247.

[13] R.M. Matarese, L. Pecci, G. Ricci, M. Nardini, A. Antonucci, D. Cavallini, Proc. Natl. Acad. Sci. USA 84 (1987) 5111.

[14] R.M. Matarese, S.P. Solinas, G. Montefoschi, G. Ricci, D. Cavallini, FEBS Lett. 250 (1989) 75.

[15] H. Kodama, K. Sasaki, H. Mikasa, D. Cavallini, G. Ricci, J. Chromatogr. 311 (1984) 183.

[16] S.K. Wadman, P.K. De Bree, J.P. Kamerling, Clin. Chim. Acta 82 (1978) 281.

[17] K. Sugahara, M. Takemura, H. Kodama, J. Chromatogr. 579 (1992) 318.

[18] G. Ricci, L. Santoro, M. Achilli, R.M. Matarese, M. Nardini, D. Cavallini, J. Biol. Chem. 258 (1983) 10511.

[19] G. Ricci, M. Nardini, A.M. Caccuri, G. Federici, Biochim. Biophys. Acta 748 (1983) 40.