Identification of Cyclic Cystathionine Sulfoxide and *N*-Acetylcyclic Cystathionine in the Urine of a Patient With Cystathioninuria Using Liquid Chromatography–Mass Spectrometry With an Atmospheric Pressure Chemical Ionization Interface System

Jianying Zhang, Kazunori Sugahara, Yasuhiro Sagara, Kazuko Hashimoto, Noriyoshi Masuoka, and Hiroyuki Kodama

Perhydro-1,4-thiazepine-4,5-dicarboxylic acid sulfoxide (cyclic cystathionine sulfoxide [cyclic cystaSO]) and *N*-acetylperhydro-1,4-thiazepine-3,5-dicarboxylic acid (NAc-cyclic cysta) have been identified in the urine of a patient with cystathioninuria as new metabolites of cystathionine for the first time using liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface system (LC/APCI-MS). The concentrations of cyclic cystaSO and NAc-cyclic cysta in the urine of a patient with cystathioninuria have also been determined for the first time using this method: 18.24 ± 0.79 and 25.23 ± 0.83 mg/g creatinine, respectively.

CYSTATHIONINURIA is an autosomal recessive hereditary disorder, and phenotypical homozygotes have persistent excretion of large amounts of cystathionine in the urine due to cystathionine γ-lyase deficiency.[1] Since we first found cystathioninuric patients in 1969, we have identified a series of cystathionine metabolites in the urine of patients with cystathioninuria.[2-6]

Recently, we have also identified cystathionine sulfoxide (CystaSO) and *N*-acetylcystathionine sulfoxide (NAc-cystaSO) as cystathionine metabolites in the urine of a patient with cystathioninuria.[7] Cystathionine is monodeaminated either by L-amino acid oxidase[8] or by a transaminase[9] exhibiting the properties of glutamine transaminase. Monodeaminated cystathionine mono-oxo acids cycle nonenzymatically, producing cystathionine ketimine (CK).[8] CK is reduced enzymatically in mammalian tissues[10,11] and produces perhydro-1,4-thiazepine-3,5-dicarboxylic acid (cyclic cysta). Cyclic cysta has been found in the urine of patients with cystathioninuria[4] and normal humans,[11] and in bovine brain.[12] Continued studies on cystathionine metabolites in the urine of cystathioninuric patients have led to the finding of three main pathways of degradation of cystathionine in patients with cystathioninuria. The presence of cystaSO and NAc-cysta suggested the existence of cyclic cystaSO and NAc-cyclic cysta in the urine of patients with cystathioninuria. But both compounds have not been identified by any method until now.

In this report, we present the identification of cyclic cystaSO and NAc-cyclic cysta in the urine of a patient with cystathioninuria by liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface system (LC/APCI-MS). The concentration of cyclic cystaSO and NAc-cyclic cysta has also been determined by LC/APCI-MS.

From the Departments of Chemistry and Medical Biology, Kochi Medical School, Kochi; and the Department of Biochemistry, Okayama University Medical School, Okayama, Japan.

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Address reprint requests to Hiroyuki Kodama, PhD, Department of Chemistry, Kochi Medical School, Kohasu, Oko-cho, Nankoku-shi, Kochi 783, Japan.

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MATERIALS AND METHODS

Materials

Synthetic cyclic cysta was kindly supplied by Dr D. Cavallini of the Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza" (Rome, Italy). *S*-(3-oxo-3-carboxy-*n*-propyl)Cysteine was prepared as previously described.[8] All other chemicals used were of analytical grade.

Synthesis of Cyclic CystaSO

Cyclic cysta (23.4 mg) was dissolved in 12 mL water, and *m*-chloroperbenzoic acid (24.0 mg) was added to the solution. The solution was maintained at room temperature for 1.5 hours. The mixture was made acidic with 0.5 mL 2N HCl and washed three times with 20 mL chloroform. The water layer was evaporated to dryness under reduced pressure. The residue was dissolved in 2 mL water, applied to a Diaion SK-I H-form sulfonated cation exchanger (2 × 35 cm, 100 mesh; Mitsubishi, Tokyo, Japan), and eluted with 300 mL water. Each 50-mL fraction was collected and evaporated under reduced pressure to dryness; cyclic cystaSO was obtained from a 50- to 150-mL fraction.

Synthesis of NAc-Cyclic Cysta

Cyclic cysta (100 mg) was dissolved in 1 mL 2-mol/L NaOH with 0.5 mL acetic anhydride. The solution was maintained at 0°C for 20 minutes, applied to a Diaion SK-I (H-form, 1 × 10 cm), and eluted with 100 mL water. Each 20-mL fraction was collected and evaporated to dryness under reduced pressure; NAc-cyclic cysta was contained in a 0- to 20-mL fraction.

Preparation of the Urinary Sample

The urinary sample of a patient with cystathioninuria was obtained via an elder sister as reported previously.[5] It was stored at −20°C for preparation if not analyzed immediately. NAc-cyclic cysta and cyclic cystaSO were isolated as follows: 10 mL urine was applied to a column containing 10 mL Diaion SK-I (H-form, 1 × 10 cm), and eluted with 150 mL water. The water eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 2 mL water and then applied to a column containing 50 mL Diaion SK-I (H-form, 1.5 × 30 cm). The column was eluted with 300 mL water. Each 50-mL fraction was collected and evaporated under reduced pressure to dryness. Fractions of 0 to 50 mL containing NAc-cyclic cysta and 50 to 150 mL containing

cyclic-cystaSO were applied to a column containing 10 mL Diaion SA-100 (OH-form of anion exchanger, 100 mesh), washed with 50 mL water and 0.2 mol/L acetic acid, and eluted with 100 mL 2-mol/L acetic acid. Each 10-mL fraction was collected and evaporated to dryness under reduced pressure. An aliquot of each fraction was analyzed by LC/APCI-MS. An aliquot of the residue was hydrolyzed in 6N HCl at 110°C for 24 hours. The hydrolysate was also analyzed by LC/APCI-MS.

Fractions containing cyclic cystaSO and NAc-cyclic cysta were also detected by paper chromatography. The developing solvent was butanol:acetic acid:H_2O (4:1:2 vol/vol/vol). The acetone solution containing $PtCl_6$ and KI was used for detection of substances containing *S* compounds.[13]

Instrumentation

The apparatus used was a Hitachi L-6200 high-performance liquid chromatography instrument equipped with a 5-μm Inertsil ODS-2 column (150 × 4.6 mm ID) from Gasukuro Kogyo (Tokyo, Japan) connected to a Hitachi M8OB mass spectrometer computer system (Ibargi, Japan) through the APCI interface. The nebulizer and vaporizer temperatures were 255°C and 380°C, respectively. Synthetic samples and urinary samples were analyzed with a mobile phase of 100 mmol/L CH_3COONH_4:CH_3CN(80%:20%) at a flow rate of 0.9 mL/min.

RESULTS AND DISCUSSION

Identification of Cyclic CystaSO

Mass chromatograms and spectra of synthetic cyclic cystaSO (*m/z* 222), cyclic cysta (*m/z* 206), and the hydrolysate of synthetic cyclic cystaSO are shown in Fig 1. In the LC/APCI-MS system, the protonated molecular ion $[M + H]^+$ (*m/z* 222) of synthetic cyclic cystaSO was observed as a base peak, in addition to the ions of $[M + H]^+ + NH_3$ (*m/z* 239), $[M + H]^+ - O$ (*m/z* 206), $[M - CO_2]$ (*m/z* 177), and $[M] - (COOH + O)$ (*m/z* 160) (Fig 1A and a). When synthetic cyclic cystaSO was hydrolyzed, the protonated molecular ion (*m/z* 206) that removed oxygen (mass, 16 daltons) from cyclic cytaSO (*m/z* 222) was observed as a base peak, in addition to the peaks of (*m/z* 223) [(*m/z* 206) + NH_3] and (*m/z* 160) [(*m/z* 206)-CO_2-2H] (Fig 1C and c). The peak of *m/z* 206 from the hydrolysate of synthetic cyclic cystaSO also had the same retention time and mass spectrum as synthetic cyclic cysta (Fig 1B and b). The separation was achieved using 100 mmol/L CH_3COONH_4:CH_3CN (80%:20%) as a mobile phase. The retention times of cyclic cystaSO and cyclic cysta on the mass chromatogram were 1.5 and 1.6 minutes, respectively.

Mass chromatograms and spectra of the unhydrolysate and hydrolysate of the 2-mol/L acetic acid fraction (60 to 70 mL) of the urinary sample from a patient with cystathioninuria are shown in Fig 2. The protonated molecular ion (*m/z* 222) in the unhydrolysate of the 2-mol/L acetic acid fraction (60 to 70 mL) from the patient's urine was observed as a base peak on a mass chromatogram (Fig 2A and a), in addition to the peaks of *m/z* 239, *m/z* 206, and *m/z* 177, the same as that of synthetic cyclic cystaSO.

After hydrolyzation of the 2-mol/L acetic acid fraction (60 to 70 mL) of the patient's urine, the same results as that of synthetic cyclic cystaSO were observed, ie, the peak of protonated molecular ion (*m/z* 222) corresponding to cyclic

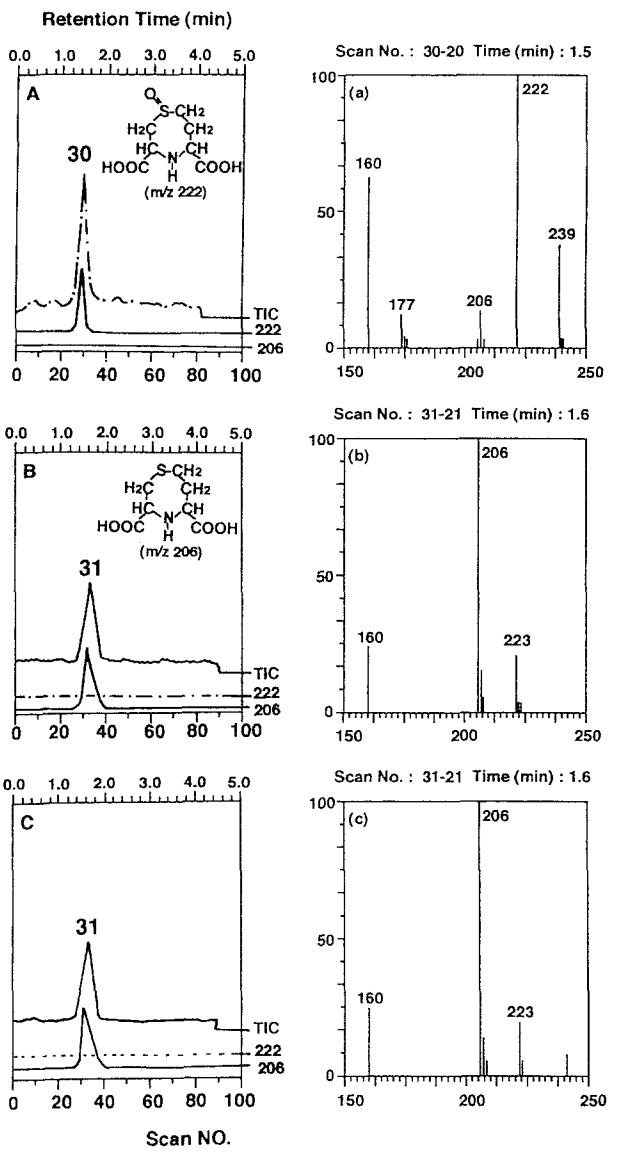


Fig 1. Mass chromatograms and spectra of synthetic cyclic cystaSO (A, a), cyclic cysta (B, b), and the hydrolysate of synthetic cyclic cystaSO (C, c). Chromatographic conditions: mobile phase, 100 mmol/L CH_3COONH_4:CH_3CN (80%:20%); flow rate, 0.9 mL/min.

cystaSO disappeared completely, but the protonated molecular ion of cyclic cysta (*m/z* 206) from which the oxygen (mass of 16 daltons) had been removed from cyclic cystaSO was observed newly as a base peak (Fig 2B and b).

A mixture of synthetic cyclic cysta and the hydrolysate of the 2-mol/L acetic acid fraction (60 to 70 mL) of the urinary sample from a patient with cystathioninuria was also analyzed by LC/APCI-MS. Only one peak was observed on a mass chromatogram of the mixture of synthetic cyclic cysta and urinary hydrolysate. The retention times of the hydrolysates of synthetic cyclic cystaSO and the urinary fraction on mass chromatograms were the same as that of standard cyclic cysta. In the LC/APCI-MS system, the quasi-molecular ion of cyclic cysta (*m/z* 206) was observed as a base peak. When synthetic cyclic cysta was added to the

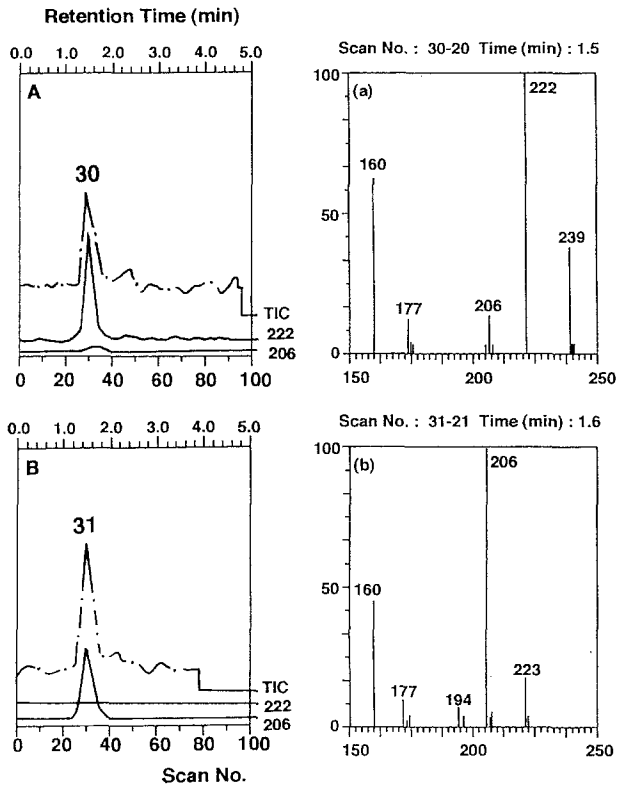


Fig 2. Mass chromatograms and spectra of the unhydrolysate (A, a) and hydrolysate (B, b) of the 2-mol/L acetic acid fraction (60 to 70 mL) from a urinary sample of a patient with cystathioninuria. TIC, total ion current.

hydrolysate of the 2-mol/L acetic acid fraction of the urinary sample from a patient with cystathioninuria, the peak of cyclic cysta in the urinary sample was increased.

The result of paper chromatography of synthetic cyclic cystaSO, hydrolysates of cyclic cystaSO and the urinary sample, synthetic cyclic cysta, and the unhydrolyzed urinary sample coincided with the result of LC/APCI-MS. The spot of the urinary sample containing cyclic cystaSO on the paper chromatogram had almost the same R_f value (0.194) as that of synthetic cyclic cystaSO (R_f 0.193). After hydrolyzation of the urinary sample, the spot of cyclic cystaSO disappeared, and the spot that had the same R_f value (0.357) as synthetic cyclic cysta (0.366) was newly detected. Cyclic cystaSO could not be detected in the normal urine, as reported previously.[14] These results indicate that cyclic cystaSO was present in the urine of the patient with cystathioninuria.

The determination of cyclic cystaSO in the urine from a patient with cystathioninuria has not been reported by any methods until now. Therefore, we determined the content of cyclic cystaSO using LC/APCI-MS. Because both compounds (cyclic cystaSO and OCPC) had the same protonated molecular ion, ie, the protonated molecular ions $[M + H]^+$ of both are 222 and the retention times of both are almost the same,[6] it could not be measured by standard cyclic cystaSO. But the hydrolytic compounds of both were different. The hydrolytic product of cyclic cystaSO is cyclic cysta, but that of OCPC is not cyclic cysta (β-CEC or cysta ketimine). Therefore, we used cyclic cysta as a standard compound and determined the content of cyclic cysta in the hydrolysate and unhydrolysate of the same fraction of the patient's urine. The difference for the content of cyclic cysta between the hydrolysate and unhydrolysate represented the content of cyclic cystaSO in the urine of the patient with cystathioninuria. The standard curves for different concentrations of cyclic cysta was linear over the concentration range from 100 to 1,000 ng. The selected ion monitoring normalized chromatograms of standard cyclic cysta, and the hydrolysate of the 2-mol/L acetic acid fraction (60 to 70 mL) from the urine of a patient with cystathioninuria had the same retention time and was observed clearly. The content of cyclic cystaSO in the urine of the patient with cystathioninuria was 18.24 ± 0.79 mg/g creatinine.

Identification of NAc-Cyclic Cysta

Mass chromatograms and spectra of synthetic NAc-cyclic cysta (m/z 248) and the 2-mol/L acetic acid fraction (0 to 20 mL) of the urine from a patient with cystathioninuria are shown in Fig 3. In the LC/APCI-MS system, the protonated molecular ion (m/z 248) of synthetic NAc-cyclic cysta was observed as a base peak, in addition to the ions of $[M + H]^+ + NH_3$ (m/z 265), $[M + H]^+ - COCH_3$ (m/z 206), and $[M] - (COCH_3 + CO_2)$ (m/z 160). A mass chromatogram (m/z 248) of the urinary fraction (50 to 150 mL) from a patient with cystathioninuria had the same retention time (2.6 minutes) and mass spectrum as the synthetic NAc-cyclic cysta (Fig 3A and B).

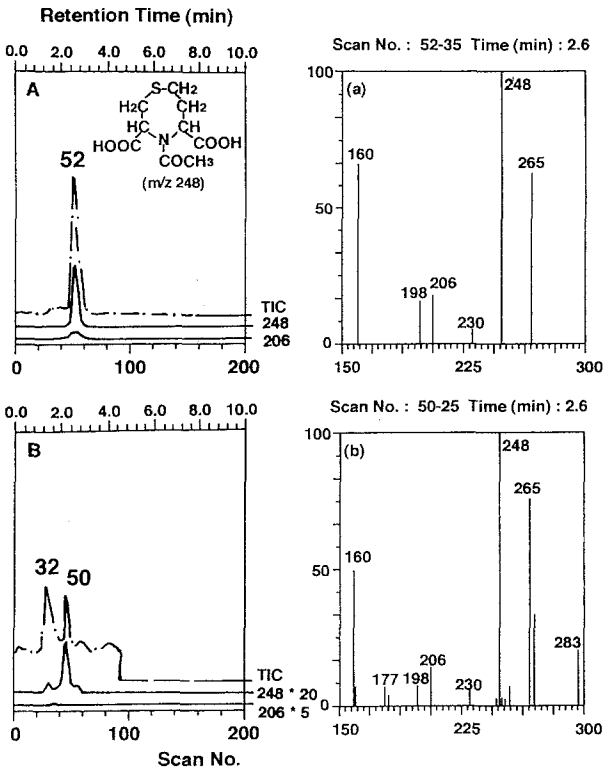


Fig 3. Mass chromatograms and spectra of synthetic NAc-cyclic cysta (A, a) and the 2-mol/L acetic acid fraction (0 to 20 mL) from the urine of a patient with cystathioninuria (B, b).

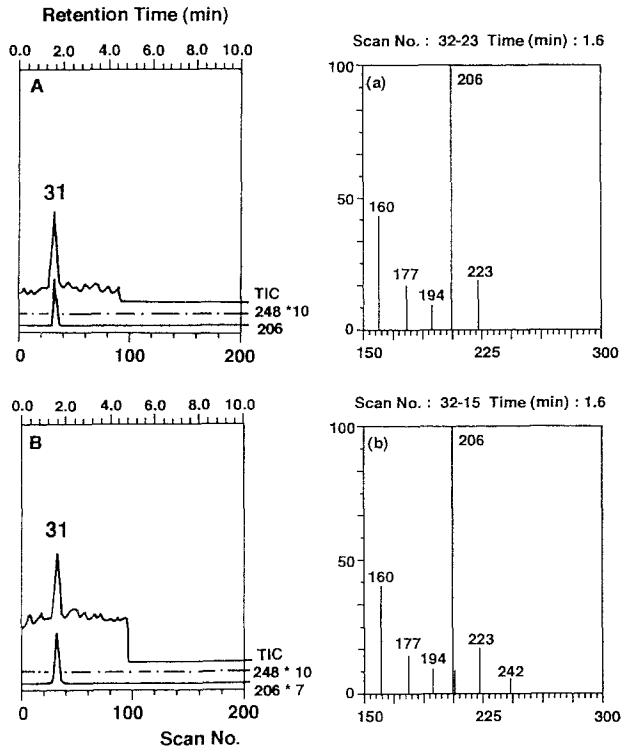


Fig 4. Mass chromatograms of the hydrolysates of synthetic NAc-cyclic cysta (A, a) and the fraction containing NAc-cyclic cysta from the urine of a patient with cystathioninuria (B, b).

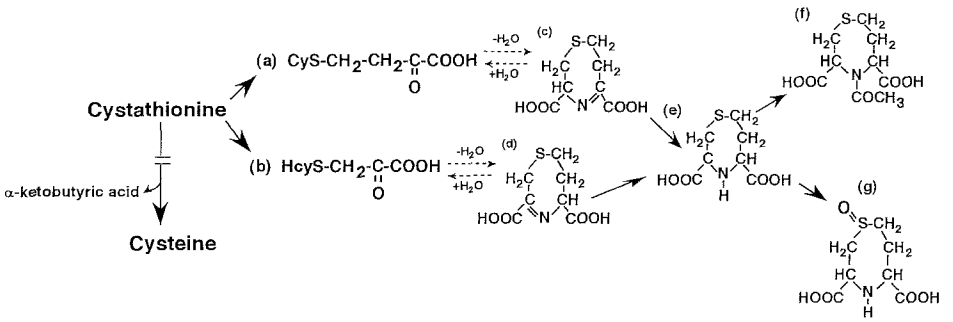
A mass chromatogram of a mixture of synthetic NAc-cyclic cysta (m/z 248) and cyclic cysta (m/z 206) was also analyzed by LC/APCI-MS. Both compounds were separated clearly and had different retention times.

Mass chromatograms of the hydrolysates of synthetic NAc-cyclic cysta and the fraction containing NAc-cyclic cysta from the patient's urine are shown in Fig 4. The peak of the molecular ion (m/z 248) corresponding to NAc-cyclic cysta disappeared completely after hydrolyzation of NAc-cyclic cysta, but the peak of the protonated molecular ion (m/z 206) of cyclic cysta from which the acetyl group (m/z 43) had been removed from NAc-cyclic cysta was observed newly as a base peak.

The result of paper chromatography of synthetic NAc-cyclic cysta, the hydrolysate of synthetic NAc-cyclic cysta, cyclic cysta, the urinary sample containing NAc-cyclic cysta from a patient with cystathioninuria, and the hydrolysate of the same urinary sample coincided well with the result of LC/APCI-MS. The spot of urinary sample containing NAc-cyclic cysta on the paper chromatogram had almost the same R_f value (0.402) as synthetic NAc-cyclic cysta (R_f 0.401). The spots corresponding to NAc-cyclic cysta disappeared after the hydrolyzations of the synthetic NAc-cyclic cysta and urinary sample, but the spot that had the same R_f value (0.241) as synthetic cyclic cysta (0.240) appeared newly. These results obtained by LC/APCI-MS and paper chromatography indicate that NAc-cyclic cysta was present in the urine of a patient with cystathioninuria. The content of NAc-cyclic cysta in the urine of a patient with cystathioninuria was 25.23 ± 0.83 mg/g creatinine.

It was impossible to detect cyclic cysta, cyclic cystaSO, and NAc-cyclic cysta using an amino acid analyzer, because these compounds had no free amino group in their structures. But these compounds could be determined easily using LC/APCI-MS.

We have suggested that cyclic cycta was excreted as the end product of cystathionine metabolites in the urine of patients with cystathioninuria, because this compound was contained at a higher level than other cystathionine metabolites in the urine of patients with cystathioninuria. But the presence of cyclic cystaSO and NAc-cyclic cysta in the urine of a patient with cystathioninuria suggests that cystathionine was mainly degraded to cyclic cysta, and part of the cyclic cysta was oxidized to cyclic cystaSO or acetylated to NAc-cyclic cysta in the patient's tissues and excreted into the urine (Fig 5). The relationship between these cystathionine metabolites identified in the urine and clinical symptoms (such as mental retardation and disorders of the central nervous system) of patients with cystathioninuria 'has not been studied until now. The physiological role of cystathionine and its metabolites remains a problem to be solved in a future study.



(a) S-(3-oxo-3-carboxy-n-propyl)cysteine
(b) S-(2-oxo-2-carboxyethyl)homocysteine
(c),(d) Cystathionine ketimine
(e) Perhydro-1,4-thiazepine-3,5-dicarboxylic acid
(f) NAc-cyclic-cystathionine
(g) Cyclic-cystathionine sulfoxide

Fig 5. Unusual metabolism of cystathionine in a patient with cystathioninuria.

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