Metabolism of Cystathionine, *N*-Monoacetylcystathionine, Perhydro-1,4-thiazepine-3,5-dicarboxylic Acid, and Cystathionine Ketimine in the Liver and Kidney of D,L-Propargylglycine-Treated Rats

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Experimental cystathioninuria was induced by injection of D,L-propargylglycine in rats. The novel cystathionine metabolites, *N*-monoacetylcystathionine (NAc-cysta), perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), and cystathionine ketimine (CK), were identified previously in the urine of patients with cystathioninuria and D,L-propargylglycine-treated rats. In this study, we identified these compounds in the liver and kidney of D,L-propargylglycine-treated rats using liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface system (LC/APCI-MS) and an amino acid analyzer. The metabolism of these compounds in the liver and kidney of D,L-propargylglycine-treated rats was also studied. PHTZDC, NAc-cysta, and CK were accumulated in the rat tissues in proportion to the content of cystathionine after D,L-propargylglycine administration. The concentrations of these compounds in the liver were higher than those in the kidney, and these compounds reached maxima earlier in the liver than in the kidney.

CYSTATHIONINURIA, described by Mudd et al,[1] is an autosomal recessive hereditary disorder. Phenotypical homozygotes persistently excrete large amounts of cystathionine in the urine due to cystathionine γ-lyase deficiency.[1] Secondary cystathioninuria is associated with various pathological conditions.[2-4] Pathologically increased urinary levels of cystathionine may reflect either an inherited enzyme defect, or transient impaired adaptation in premature infants, or a secondary phenomenon in neuroblastoma and certain liver disorders. Secondary cystathioninuria was found in biliary atresia, cytomegalovirus infection, neuroblastoma, vitamin D intoxication, and hyperglycinemia.[5] Isolation and identification of cystathionine and cystathionine metabolites were the basis for the determination of renal cystathionine excretion in healthy children. The investigation of the metabolism of cystathionine and cystathionine metabolites in various tissues of rats with experimental cystathioninuria may be important for the clarification of the physiological and pathological roles of these compounds in mammals.

We have identified several cystathionine metabolites in the urine of cystathioninuric patients such as *N*-monoacetylcystathionine (NAc-cysta), *S*-(carboxymethyl)homocysteine (CMHC), *S*-(3-hydroxy-3-carboxy-*n*-propyl)cysteine (HCPC), *S*-(2-hydroxy-2-carboxyethyl)homocysteine (HCEHC), *S*-(2-carboxyethyl)cysteine (β-CEC), perhydro-1,4-thiazepine-3,5-dicarboxylic acid (PHTZDC), *N*-acetyl-*S*-(3-hydroxy-3-carboxy-*n*-propyl)cysteine (NAc-HCPC), *N*-acetyl-*S*-(2-carboxyethyl)cysteine (NAc-β-CEC), cystathionine sulfoxide, *N*-acetylcystathionine sulfoxide, and cystathionine ketimine (CK).[6-11] Experimental cystathioninuria was induced in rats by administration of D,L-propargylglycine,[12] a suicide substrate of cystathionine γ-lyase (EC 4.4.1.1). This acetylenic substrate inactivates rat liver cystathioninase and, when administered to mice, leads to a rapid decrease in the hepatic activity of this enzyme.[12] It also inactivates pig heart alanine aminotransferase (EC 2.6.1.2),[13] rat liver aspartate aminotransferase (EC 2.6.1.1),[14] and hog kidney D-amino acid oxidase (EC 1.4.3.3).[15] We have previously determined the concentrations of D,L-propargylglycine and *N*-acetylpropargylglycine in urine samples and several tissues of D,L-propargylglycine-treated rats.[16] Among the cystathionine metabolites identified in the urine of patients with cystathioninuria, we have identified NAc-cysta, CMHC, β-CEC, HCEHC, HCPC, NAc-HCPC, NAc-β-CEC, PHTZDC, and CK in the urine of D,L-propargylglycine-treated rats.[17-19] In a recent study, we have suggested that in rats with cystathioninuria induced by injection of D,L-propargylglycine, cystathionine could be metabolized via three pathways, namely *N*-monoacetylation, sulfide oxidation, and monodeamination (Fig 1). The main route was from mono-oxo acids to PHTZDC through CK.[18]

The present study was undertaken to further test a prediction of this metabolism and to continue the studies of the effect of D,L-propargylglycine on cystathionine metabolism in rats. We present evidence in this report for identification of cystathionine, NAc-cysta, PHTZDC, and CK in the liver and kidney of D,L-propargylglycine-treated rats using liquid chromatography–mass spectrometry with an atmospheric pressure chemical ionization interface system (LC/APCI-MS), high-performance liquid chromatography (HPLC), and an amino acid analyzer. We also determined the time course of accumulation of cystathionine metabolites in the liver and kidney of D,L-propargylglycine-treated rats after administration of D,L-propargylglycine.

MATERIALS AND METHODS

Chemicals

CK was prepared according to the description of Ricci et al.[20] PHTZDC was kindly supplied by Dr S. Doprè, Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza" (Rome, Italy). Cystathionine and D,L-propargylglycine were purchased from Sigma Chemical (St Louis, MO). Phenylisothiocyanate (PITC) and acetonitrile (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). NAc-cysta was prepared from the urine sample of a patient with cystathioninuria according to the method of Kodama et al.[21] All other chemicals used were of analytical grade.

Preparation of Tissue Samples

Male Wistar rats (mean body weight, 200 g) were divided into two groups and transferred to individual metabolic cages. One group was intraperitoneally injected with 20 mg D,L-propargylglycine/200 g body

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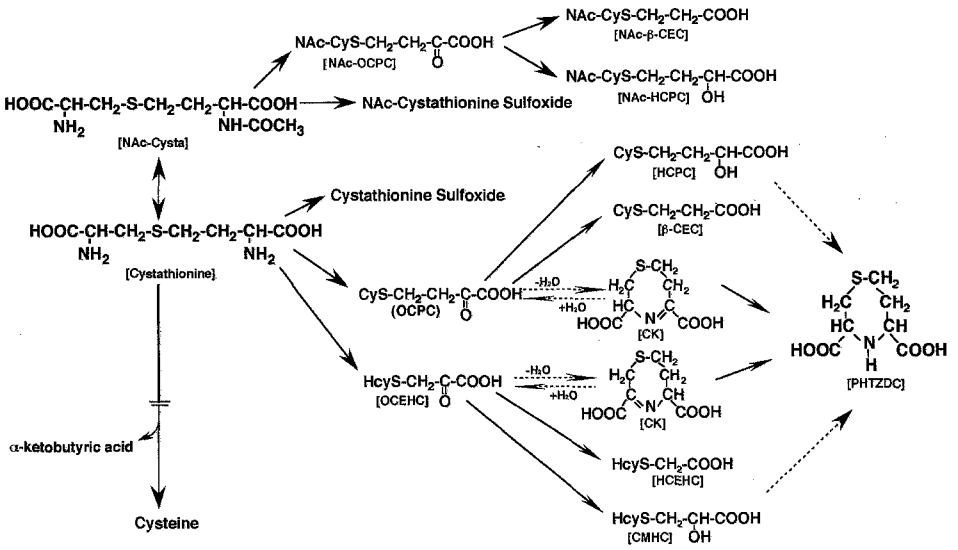

Fig 1. Unusual metabolism of cystathionine in cystathioninuric patients.

weight. Physiological saline solution (0.5 mL) was injected into control rats. Rats were killed by decapitation at various times after injection of D,L-propargylglycine. Each tissue (1 g) was homogenized in 3 vol 2% sulfosalicylic acid, and the homogenate was centrifuged at 2,000 × *g* for 15 minutes. The supernatant was applied to a column containing 10 mL Diaion SK-I (H-form of sulfonated cation exchanger, 1 × 10 cm, 100 mesh; Mitsubishi, Tokyo, Japan), washed with 150 mL water, and eluted with 30 mL 2-mol/L ammonia.

Preparation and Analysis of PHTZDC and CK Fractions

The water eluate was adjusted to pH 8.0 with 2 mol/L NH_4OH and evaporated to dryness under reduced pressure with a rotary evaporator at 40°C. The dried residue (sample 1) was dissolved in 0.5 mL water and analyzed for PHTZDC using LC/APCI-MS. Derivatization with PITC for the analysis of CK was performed as described by Pecci et al,[22] as follows.

Sample 1 was dissolved in 0.2 mL water, and 0.6 mL of the coupling buffer (acetonitrile:pyridine:triethylamine:water 10:5:2:3 vol/vol) and 40 μL PITC were added. After 30 minutes at room temperature, the solution was dried under reduced pressure at 50°C, the residue was dissolved in 200 μL 0.01-mol/L potassium phosphate buffer (pH 8.0), and 20-μL aliquots corresponding to 0.1 g of the original tissue were analyzed with a Hitachi L-6200 HPLC equipped with a 5-μm Inertsil ODS-2 column (150 × 4.6 mm ID) from Gasukuro Kogyo (Tokyo, Japan). The solvents used were (A) 0.05 mol/L ammonium acetate, (B) 0.05 mol/L ammonium acetate/acetonitrile (65:35 vol/vol), and (C) acetonitrile/water (70:30 vol/vol). After washing with solvent C for 10 minutes, the column was preconditioned with solvent A for 15 minutes before sample loading. Chromatography was performed with a linear gradient from 100% A to 100% B for 30 minutes at a flow rate of 1.0 mL/min at room temperature. The detection wavelength for PITC derivatives was 380 nm.

Preparation and Analysis of Cystathionine and NAc-cysta Fractions

The ammonia eluate thus obtained was evaporated under reduced pressure, and the residue was dissolved in 1 mL water and then analyzed by an automatic amino acid analyzer (Hitachi model 835 liquid chromatography).

Instrumentation

The apparatus used was a Hitachi L-6200 HPLC, equipped with a 5-μm Inertsil ODS-2 column (150 × 4.6 mm ID) from Gasukuro

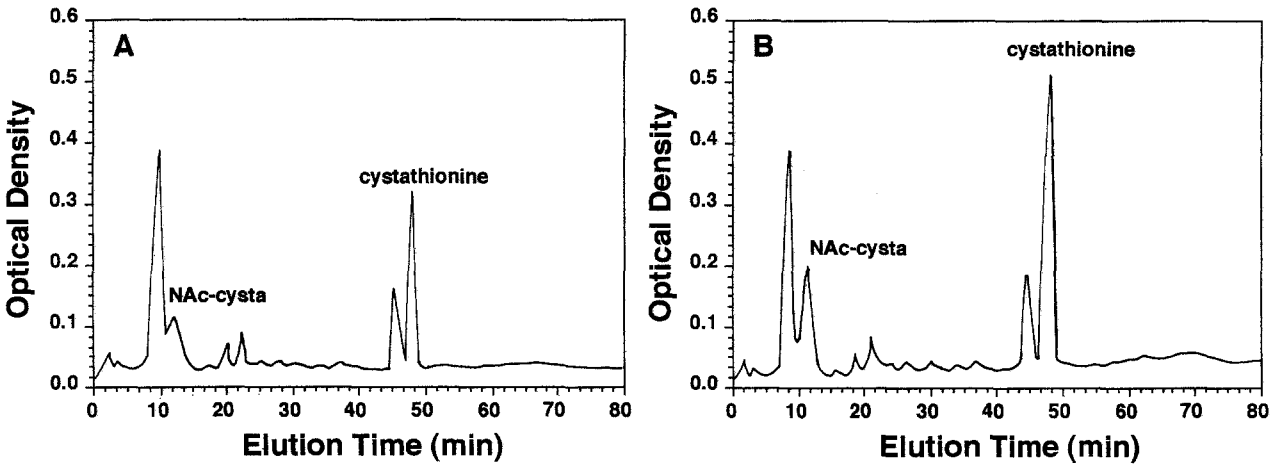

Fig 2. Amino acid chromatogram of (A) liver sample from rats treated with D,L-propargylglycine and (B) liver sample plus authentic cystathionine and NAc-cysta.

Kogyo, connected to a Hitachi M80B mass spectrometer/computer system through the APCI interface. The nebulizer and vaporizer temperatures were 260° and 390°C, respectively. Synthetic samples and tissue 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.[23]

RESULTS

Synthetic cystathionine, NAc-cysta, and tissue samples (liver and kidney) from D,L-propargylglycine-treated rats were analyzed by an amino acid analyzer. Results obtained from the liver samples are shown in Fig 2A and B. The retention time for cystathionine and NAc-cysta in the liver samples was almost the same as for synthetic cystathionine (48.2 minutes) and NAc-cysta (11.7 minutes). When authentic cystathionine and NAc-cysta were added to the liver samples, the peaks at 48.2 minutes and 11.7 minutes increased, respectively. The same results were obtained from the kidney samples of D,L-propargylglycine-treated rats. These results indicate the presence of cystathionine and NAc-cysta in the samples of liver and kidney from D,L-propargylglycine-treated rats and coincide well with those obtained by LC/APCI-MS.

Mass chromatograms and spectra of standard PHTZDC and water fractions of tissue samples from D,L-propargylglycine-treated rats are shown in Fig 3. In the LC/APCI-MS system, the quasi-molecular ion $[M+H]^+$ of PHTZDC was observed as a base peak at *m/z* 206 either with synthetic PHTZDC or in tissue samples. Additional ions, $[M\text{-}COCH_3]^+$ (*m/z* 160) and

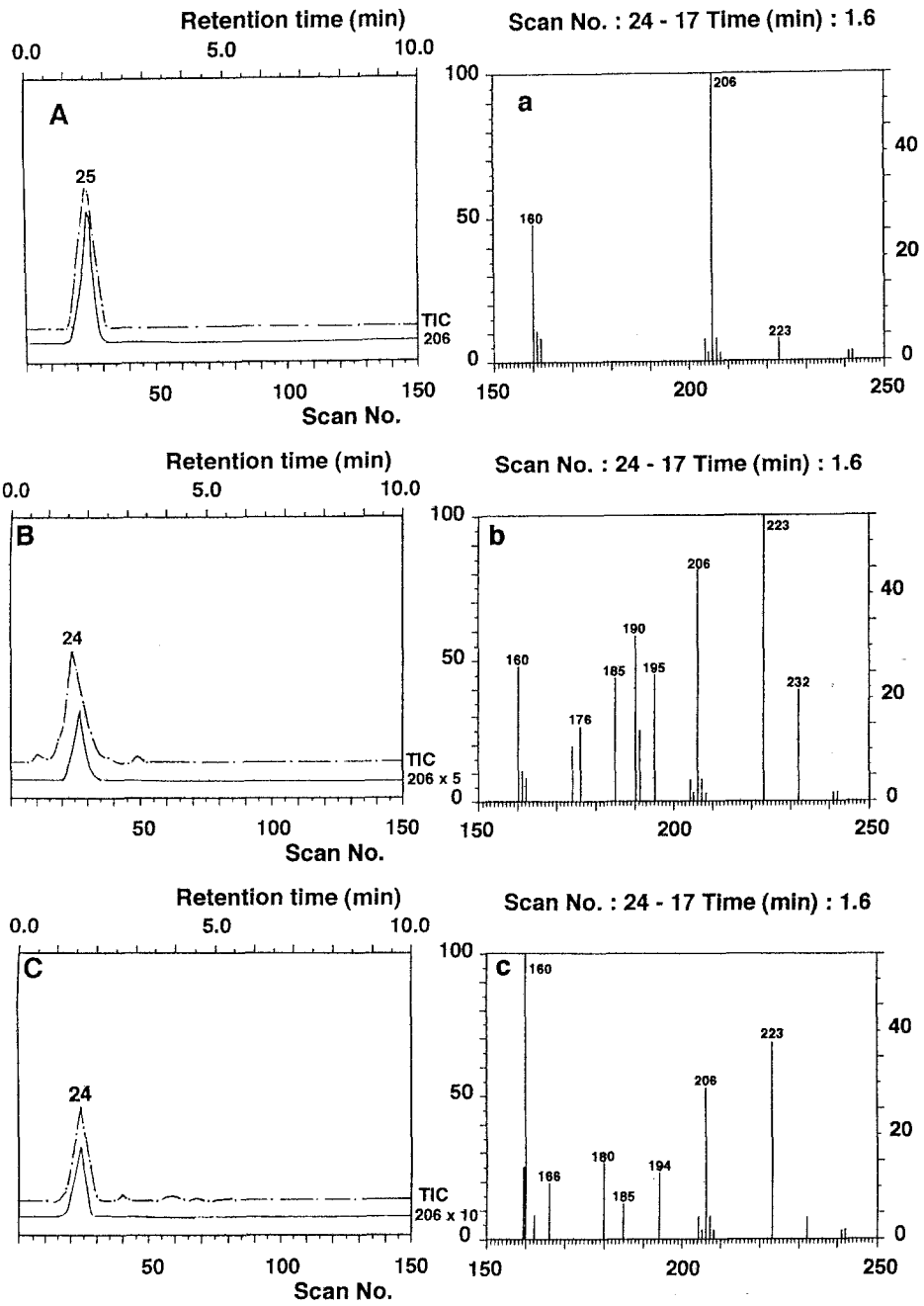

Fig 3. Mass chromatograms and spectra of synthetic PHTZDC (A,a) and water fractions from the liver (B,b) and kidney (C,c) of rats treated with D,L-propargylglycine.

$[M+NH_4]^+$ (*m/z* 223), were also detected with authentic PHTZDC and tissue samples.

HPLC elution profiles of the PITC derivatives of authentic CK and of liver samples from D,L-propargylglycine-treated rats are shown in Fig 4. The detection was performed at 380 nm, which is the characteristic absorption maximum of PITC derivatives of CK. The detection at 380 nm for tissue samples of D,L-propargylglycine-treated rats produced one peak (Fig 4B) at the same retention time as that of the authentic CK (Fig 4A). When the authentic CK was added to the tissue sample of D,L-propargylglycine-treated rats, the mixture also produced one peak (Fig 4C), the retention time of which corresponded to that of the authentic CK. The same results were observed in kidney samples of D,L-propargylglycine-treated rats (Fig 5). These results indicate that CK is present in the liver and kidney of D,L-propargylglycine-treated rats.

The amount of cystathionine, NAc-cysta, PHTZDC, and CK

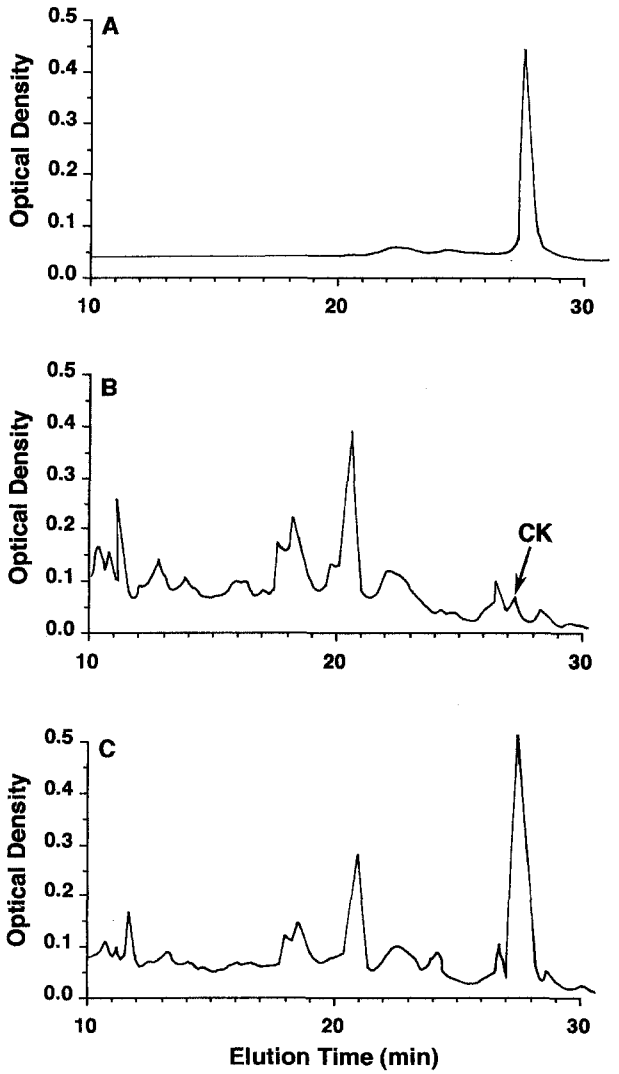

Fig 4. HPLC elution patterns of CK from synthetic compound and rat liver sample after reaction with PITC. (A) Chromatogram of authentic CK, (B) sample corresponding to 0.1 g original liver tissue from D,L-propargylglycine-treated rats, (C) sample A plus B.

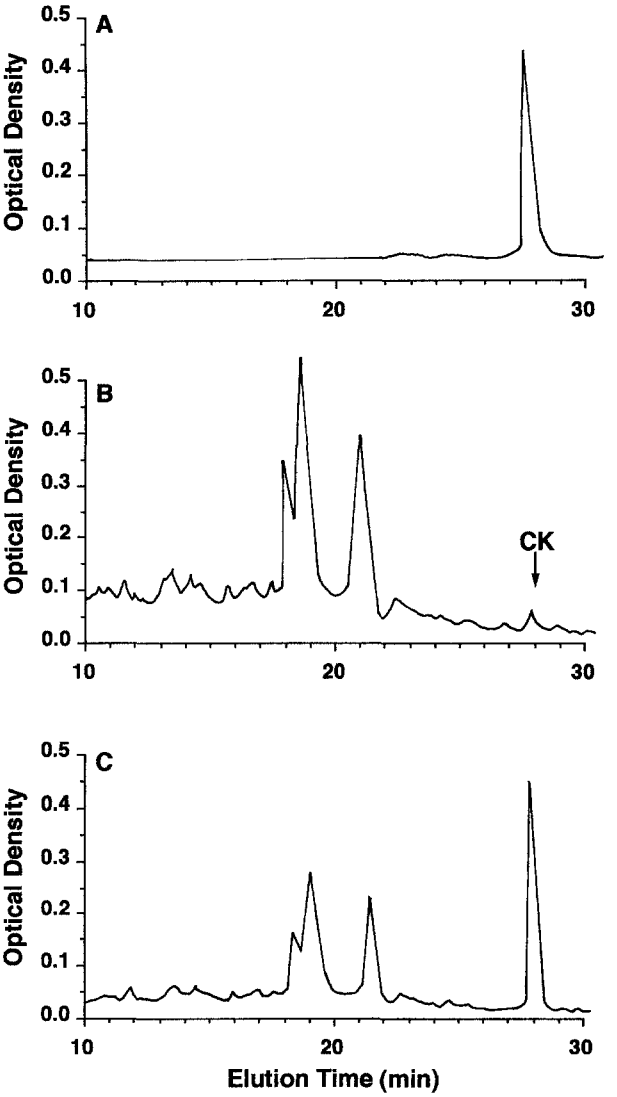

Fig 5. HPLC elution patterns of CK from synthetic compound and rat kidney sample after reaction with PITC. (A) Chromatogram of authentic CK, (B) sample corresponding to 0.1 g original kidney tissue from D,L-propargylglycine-treated rats, (C) sample A plus B.

in the liver and kidney of rats after a single injection of D,L-propargylglycine (20 mg/200 g body weight) was determined using LC/APCI-MS (for PHTZDC), an amino acid analyzer (for cystathionine and NAc-cysta), and HPLC (for CK). The results are shown in Figs 6 and 7. The content of these compounds increased rapidly after the injection. Cystathionine, NAc-cysta, and PHTZDC reached maximum levels at about 18 hours in the liver and about 24 hours in the kidney after the injection, and returned to the original levels at about 120 hours in both the liver and kidney. On the other hand, CK reached a maximum level at about 16 hours in the liver and about 20 hours in the kidney, and returned to the original level at about 72 hours in both the liver and kidney after D,L-propargylglycine injection. The levels of these compounds were higher in the liver than in the kidney. They reached the maximum levels later and maintained high concentrations longer in the kidney than in the

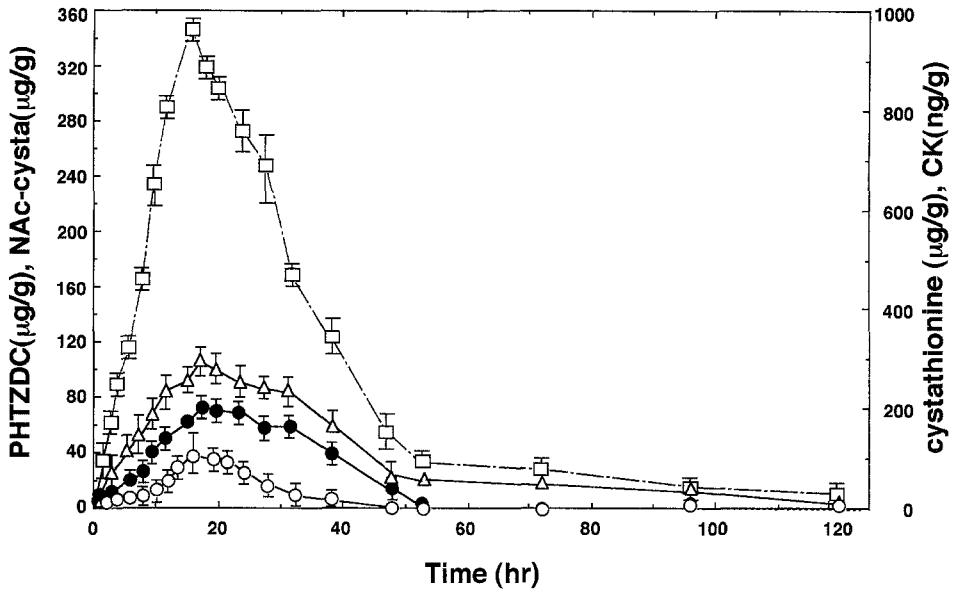

Fig 6. Cystathionine, NAc-cysta, PHTZDC, and CK levels in rat liver after a single injection of D,L-propargylglycine. PHTZDC was determined by LC/APCI-MS, cystathionine and NAc-cysta by an amino acid analyzer, and CK by HPLC. (□) Cystathionine, (△) PHTZDC, (○) NAc-cysta, (●) CK.

liver. PHTZDC was detected at a higher level than NAc-cysta in the liver and kidney of D,L-propargylglycine-treated rats. This result was the same as observed in the urine of cystathioninuric patients (318.56 mg PHTZDC/g creatinine and 76.29 mg NAc-cysta/g creatinine were found in a patient with cystathioninuria).[11]

DISCUSSION

The present results indicate the presence of cystathionine, NAc-cysta, PHTZDC, and CK in the liver and kidney of rats treated with D,L-propargylglycine, and describe additional metabolic routes of cystathionine in rats treated with D,L-propargylglycine. Normally, cystathionine is synthesized from serine and homocysteine by cystathionine β-synthase and is degraded to cysteine, 2-oxo-butyric acid, and ammonia by cystathionine γ-lyase in mammalia.[24] Injection of D,L-propargylglycine in rats led to a decrease in cystathionine γ-lyase activity in the liver and kidney. The activity decreased more markedly and more rapidly in the liver versus the kidney of rats after treatment with D,L-propargylglycine.[25] We have reported that administration of 1 mg propargylglycine/200 g body weight causes an almost complete inactivation of liver cystathionine γ-lyase in rats, but kidney cystathionine γ-lyase at a dose of 20 mg propargylglycine could produce an activity of about 20% of the control value.[25] We have also reported previously that cystathionine β-synthase was not affected by administration of D,L-propargylglycine.[25] The present results also demonstrate that the D,L-propargylglycine arrived first in the liver and then in the kidney. By an alternative metabolic pathway, cystathionine is monodeaminated either by L-amino acid oxidase[20] or by a transaminase exhibiting the properties of glutamine transaminase.[26] Monodeaminated cystathionine mono-oxo acids form CK through a nonenzymatic cyclization process,[20] and CK is reduced to PHTZDC by lactic dehydrogenase.[27] The PHTZDC level in the liver and kidney of rats treated with D,L-propargylglycine was higher than the other cystathionine metabolites (such as NAc-

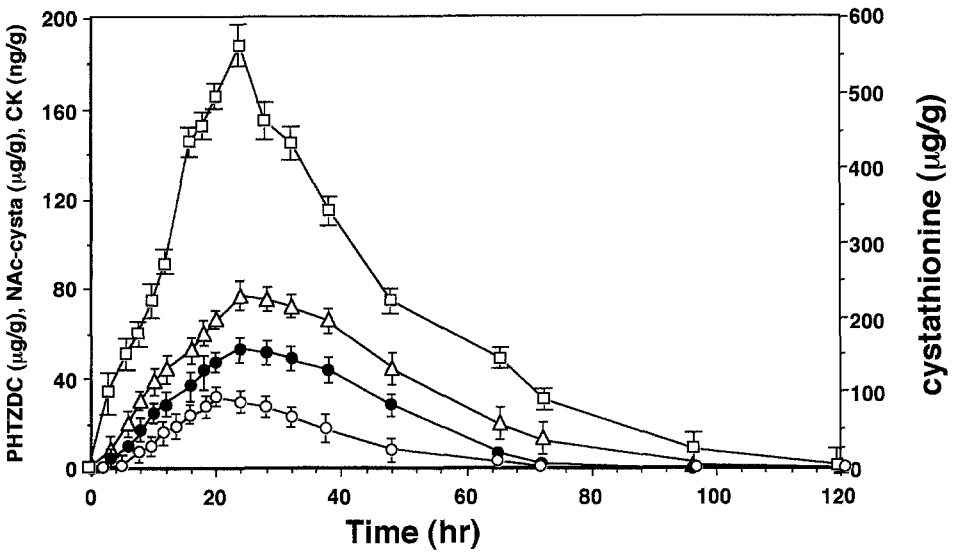

Fig 7. Cystathionine, NAc-cysta, PHTZDC, and CK in rat kidney after a single injection of D,L-propargylglycine. PHTZDC was determined by LC/APCI-MS, cystathionine and NAc-cysta an amino acid analyzer, and CK by HPLC. (□) Cystathionine, (△) PHTZDC, (○) NAc-cysta, (●) CK.

cysta). This result suggests that in rats treated with D,L-propargylglycine, the pathway from CK to PHTZDC through monodeamination of cystathionine is a main route of cystathionine metabolism. CK was found in the patient's urine at a level of 3.61 mg/g creatinine, 20 times higher than the ratio in healthy human urine.[11] CK was also detected in bovine brain cerebellum[28] and was found to bind to brain membranes with high affinity.[29] The sulfur-containing cyclic ketimines and their reduced products, such as PHTZDC and CK described herein, represent an interesting new class of compounds with similar properties whose biochemical significance is only now beginning to be understood. The increased levels of cystathionine, NAc-cysta, PHTZDC, and CK in the urinary samples have been described for hereditary cystathioninuric patients and D,L-propargylglycine-treated rats. The metabolism of these compounds in the liver and kidney of D,L-propargylglycine-treated rats was similar to that found in the urine of hereditary cystathioninuric patients and of D,L-propargylglycine-treated rats. These findings may make it possible to use D,L-propargylglycine-treated rats as an animal model of cystathioninuria for clarification of the mechanisms underlying the formation of various new metabolites from cystathionine and the physiological roles of cystathionine and its metabolites in mammals.

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