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High performance liquid chromatography analysis of 2-mercaptoethylamine (cysteamine) in biological samples by derivatization with *N*-(1-pyrenyl) maleimide (NPM) using fluorescence detection

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Abstract

2-Mercaptoethylamine (cysteamine) is an aminothiol compound used as a drug for the treatment of cystinosis, an autosomal recessive lysosomal storage disorder. Because of cysteamine's important role in clinical settings, its analysis by sensitive techniques has become pivotal. Unfortunately, the available methods are either complex or labor intensive. Therefore, we have developed a new rapid, sensitive, and simple method for determining cysteamine in biological samples (brain, kidney, liver, and plasma), using *N*-(1-pyrenyl) maleimide (NPM) as the derivatizing agent and reversed-phase high performance liquid chromatography (HPLC) with a fluorescence detection method (λ_{ex} = 330 nm, λ_{em} = 376 nm). The mobile phase was acetonitrile and water (70:30) with acetic acid and *o*-phosphoric acid (1 mL/L). The calibration curve for cysteamine in serine borate buffer (SBB) was found to be linear over a range of 0–1200 nM (r^2 = 0.9993), and in plasma and liver matrix, the r^2 values were 0.9968 and 0.9965, respectively. The coefficients of the variation for the within-run and between-run precisions ranged from 0.68 to 9.90% and 0.63 to 4.17%, respectively. The percentage of relative recovery ranged from 94.1 to 98.6%.

Keywords: CSH; Cysteamine; NPM; Thiols; Cystinosis; Oxidative stress; HPLC; 2-Mercaptoethylamine

1. Introduction

2-Mercaptoethylamine (cysteamine) is an aminothiol compound used as a drug for the treatment of cystinosis [1]. The deficiency of a cystine carrier in the lysosomal membrane leads to cystine accumulation within the lysosomes, ultimately crystallizing in vital organs such as the liver, kidney, spleen, intestines, and cornea [2,3]. The kidney is most sensitive to cystine accumulation that causes renal tubular Fanconi syndrome to develop in children 6–12 months of age [4,5]. Cysteamine crosses the plasma and lysosomal membranes and reacts with the crystallized cystine within the lysosomes to form cysteine and cysteine-cysteamine mixed disulfides, which leave through the lysine porter [6]. The thiol functional group in cysteamine makes it a potential antioxidant in oxidative stress conditions such as after radiotherapy. Because of cysteamine's important role in clinical settings and its potential future applications as an antioxidant, it has become necessary to develop an analytical method for detecting cysteamine in biological samples. Various derivatizing reagents and procedures have been described in the literature for cysteamine analysis [7–9]. These procedures, however, are either complex or time-consuming, involving incubation of samples in the dark, deproteinisation, coupled enzyme reactions, and pre-treatment of the biological samples. We have, thus, developed a method that is simple, sensitive, and rapid for analyzing cysteamine in tissues from the brain, kidney, liver, and plasma. In this study, we have used *N*-(1-pyrenyl) maleimide (NPM), which has high affinity for free thiols as the derivatizing agent [10]. The derivatization procedure does not require previous extraction and takes place at room temperature with an incubation period of only 5 min. The adduct formed is stable for at least 4 weeks at 4 °C and reversed-phase high performance liquid chromatography (HPLC) is used for the quantitation. Various analytical methods have been cited in the literature for the deter-

Abbreviations: CSH, cysteamine; GSH, glutathione; CYS, cysteine; HCYS, homocysteine; HPLC, high performance liquid chromatography; NPM, *N*-(1-pyrenyl) maleimide; mBB, monobromobimane; NAC, *N*-acetyl-L-cysteine; SBB, serine borate buffer; BHT, butylated hydroxytoluene; DETAPAC, diethylenetriaminepentaacetic acid

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mination of cysteamine in biological fluids, such as ion exchange column chromatography [11], high voltage electrophoresis [12], electrochemical detection [13,14], and gas chromatography with flame photometric detection [15]. However, these methods lack sensitivity and have generally not been used with tissue samples to determine cysteamine. Because of this, we used reversed-phase HPLC with fluorescence detection since this method is very sensitive and specific for NPM-thiol adducts, in detecting and quantitating cysteamine in tissues of rats.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, acetic acid, and phosphoric acid (all HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA). NPM and diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Aldrich (Milwaukee, WI, USA). Cysteamine and Tris–HCl (Trizma hydrochloride), and all the other chemicals were obtained from Sigma (St Louis, MO, USA).

2.2. Animals

Adult Sprague Dawley rats, weighing 250–280 g each, were obtained from Charles River Laboratories (Wilmington, MA, USA). The rats were housed in a temperature-controlled (25 °C) room equipped to maintain a 12 h light-dark cycle. Standard rat chow (Purina rat chow) and water were given ad libitum. After over-night fasting, cysteamine was administered intraperitoneally at 300 mg/kg of body weight in 1 mL of saline solution. The animals were then anesthetized according to the University of Missouri Animal Care Regulations. Blood samples were collected after 30 min via intracardiac puncture into the sterile polystyrene tubes containing heparin as an anticoagulant. The animals were then sacrificed and liver, kidney, and brain samples were obtained and kept on ice for immediate derivatization and analysis. The remaining tissue samples were kept at −70 °C for later analysis. The blood was centrifuged for 5 min at 1000 × *g* to obtain plasma, which was immediately derivatized with NPM.

2.3. Preparation of solutions for calibration

Stock solutions of cysteamine were prepared by dissolving 1.2 mg of cysteamine in 10 mL of serine borate buffer (SBB) to make a 1 mM solution of cysteamine, which was further diluted with SBB to obtain 100 and 10 μM stock solutions. The stock solutions were used to prepare standards for the calibration curve. Appropriate volumes of cysteamine stock solutions were added to plasma and tissue samples in order to obtain final concentrations of 50, 100, 200, 400, 600, 800, 1000, and 1200 nM for the calibration standard. SBB was prepared by adding 15.74 g Tris–HCl, 0.618 g borate, 0.525 g serine, and 0.393 g diethylenetriaminepentaacetic acid in 1 L of HPLC grade water (pH 7.0). NPM (1 mM) solution was prepared by dissolving 0.003 g of NPM in 10 mL acetonitrile. Antioxidant buffer was prepared by dissolving 1.2 g disodium phosphate, 0.32 g sodiumdihydrogen phosphate, 100 μL butylated hydroxytoluene (BHT) solution (0.1102 g BHT in 1 mL 100% ethanol), 0.841 g aminotriazole, 0.039 g DETAPAC, and 0.065 g sodium azide in 1 L HPLC-grade water.

2.4. HPLC system

The HPLC system (Thermo Electron Corporation) consisted of a Finnigan™ Spectra SYSTEM SCM1000 Vacuum Membrane Degasser, Finnigan™ Spectra SYSTEM P2000 Gradient Pump, Finnigan™ Spectra SYSTEM AS3000 Autosampler, and Finnigan™ Spectra SYSTEM FL3000 Fluorescence Detector (λ_{ex} = 330 nm and λ_{em} = 376 nm). The HPLC column was a Reliasil ODS-1 C_{18} column (5 μm silica packing material) with 250 mm × 4.6 mm (Column Engineering, Ontario, CA, USA). The mobile phase was 70% acetonitrile and 30% HPLC water and was adjusted to a pH of about 2 through the addition of 1 mL of both acetic acid and *o*-phosphoric acid. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 mL/min. The chromatographic column temperature was ambient. There were very minor fluctuations of retention times which could be neglected.

2.5. Assay procedures

2.5.1. Derivatization of cysteamine

The tissue samples from the livers, kidneys, and brains of adult Sprague Dawley rats were homogenized (0.15 g/mL) in antioxidant buffer (prepared as described above under solution preparation) to avoid oxidation. The plasma samples were diluted (1/5) before derivatization. Ten microliters of tissue homogenates or diluted plasma samples were mixed with 240 μL of SBB and then derivatized at room temperature with 750 μL of NPM to form fluorescent derivatives. Standard solutions of cysteamine were prepared by taking appropriate volumes of stock solutions to obtain concentrations of 0, 50, 100, 200, 400, 600, 800, 1000, and 1200 nM in the tissue matrix. The resulting mixtures were derivatized with 750 μL of 1.0 mM NPM solution in acetonitrile and left to stand for 5 min at room temperature. At the end of the reaction time, 10 μL of 2 N HCl solution were added to stop the reaction and stabilize the adducts. The final pH of the solution was maintained at about 2 which is ideal for the stability of the NPM-cysteamine adducts. The derivatized samples were filtered through a 0.45 μm acrodisc filter, and then injected into the column in a reversed-phase HPLC system.

2.5.2. Protein assay

The protein contents of different tissue samples were determined by using the Bradford method [16] in order to compare the concentration levels of CSH obtained from the tissue samples. The homogenized samples were diluted to appropriate concentrations prior to determination of protein levels. Concentrated coomasie blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water; then 2.5 mL of the diluted dye were added to 50 μL of the homogenized sample. The mixture was incubated at room temperature for 5 min, then the absorbance was measured at 595 nm by a spectrophotometer. The concentrations of protein present

Table 1
Between-run and within-run precisions, accuracy, and relative recovery of replicate samples spiked with CSH (50, 100, 600, and 1000 nM) in plasma and tissue sample matrices, and standards

Sample matrix	Brain	Kidney	Liver	Plasma	Standard
Between-run precision ($n = 6$)	0.73–3.19%	0.63–1.83%	1.42–3.32%	1.15–4.17%	1.38–1.99%
Within-run precision ($n = 6$)	1.82–9.20%	1.55–4.04%	0.78–5.82%	2.91–4.49%	0.68–9.90%
Accuracy ($n = 6$)	0.03–6.90%	3.05–5.01%	4.90–4.98%	3.95–5.88%	1.50–3.60%
Relative recovery ($n = 3$)	94.1 ± 7.0%	94.9 ± 6.9%	94.7 ± 2.8%	98.6 ± 5.0%	N/A

Percentage relative recovery is reported as the average relative recovery ± standard deviation. N/A: not applicable.

in the homogenized samples were obtained by comparing the absorbance values of the samples against the standard curve. The standard curve was constructed using the bovine serum albumin (BSA), 0.25–1 mg/mL.

3. Method validation

3.1. Calibration curves

The calibration curves of CSH were plotted by using integrated peak areas as the *y*-axis versus standard CSH concentrations (0, 50, 100, 200, 400, 600, 800, 1000, and 1200 nM) as the *x*-axis. Linearity for the standards (without the tissue matrix) was obtained over a full range of 0–1200 nM with the calibration curve: $y = 876.35x - 5224.7$ and a correlation coefficient: $r^2 = 0.9993$. Linearity for standards (with the tissue matrix) was also obtained over a full range of 0–1200 nM, with the calibration curves in the plasma and liver tissue matrix: $y = 890.33x - 12871$ and $y = 856.62x - 14886$, respectively. The correlation coefficients were $r^2 = 0.9968$ in the plasma matrix and $r^2 = 0.9965$ in the liver tissue matrix.

3.2. Accuracy, precision, and recovery

Six replicates of plasma and tissue samples were prepared, spiked with 50, 100, 600, and 1000 nM of CSH, and then analyzed in order to determine accuracy. The concentration points (50, 100, 600, and 1000 nM) were used as the true values in the calculation of the deviations between the true values and the measured values. The calculated deviations were then expressed as percentage to yield a relative deviation (R.D.), which was used as a measure of accuracy. Within-run precision was determined by analyzing six replicates of CSH-spiked control plasma and tissue samples, at concentrations ranging from 50 to 1000 nM in one analytical run, and comparing the CSH concentrations calculated from the peak areas of the six replicates in the matrix. Between-run precision was determined by derivatizing six replicates of CSH-spiked control plasma and tissue samples, at concentrations ranging from 50 to 1000 nM in three different analytical runs, and comparing the CSH concentrations calculated from the peak areas of the six replicates in each matrix. The coefficients of variation were calculated in each matrix and used as a measure of precision. Relative recovery was determined by spiking the brain, kidney, liver, and plasma samples with 50, 100, 600, and 1000 nM CSH in three replicates. The recoveries were calculated by comparing the analytical results for the spiked samples with the unspiked pure standards at the four mentioned concentrations that represented 100% recovery. The coefficients of variation (CV) for between-run and within-run precision, accuracy, and relative recovery of the samples spiked with CSH (50, 100, 600, 1000 nM) in the tissue matrix and standards are reported in Table 1. The coefficients of variation for within-run precision and between-run precision ranged from 0.68 to 9.90% and 0.63 to 4.17%, respectively. The within-run precision of 9.90% was obtained at 50 nM. The percentages of relative recovery ranged from 94.1 to 98.6%.

3.3. Sensitivity and stability

The lower limit of quantitation (LLOQ) was the concentration of CSH when its peak area was 10 times that of the peak area of the blank (signal-to-noise = 10). The LLOQ of CSH was 50 nM (0.05 nmol/mL), and the detection limit was 10 nM (0.01 nmol/mL) (signal-to-noise = 3) with 5 μL injected sample volume. The autosampler stability was measured by determining the six replicates of derivatized CSH spiked plasma and tissue samples at three concentrations (100, 600, and 1000 nM). These were kept in HPLC autosampler vials and stored at room temperature following 0, 6, 12, 24, and 48 h of sample derivatization, or stored at 4 °C for 1, 3, 7, and 14 days after sample derivatization. The relative standard deviation was found to be less than 8% when the derivatized samples were stored at room temperature, and less than 15% for samples stored at 4 °C.

3.4. Investigating interference from other thiols

A standard mixture of 600 nM of cysteamine (CSH), *N*-acetyl-L-cysteine (NAC), glutathione (GSH), cysteine (CYS), and homocysteine (HCYS), in a plasma matrix was derivatized with NPM and analyzed by reversed-phase HPLC, as shown in the chromatogram in Fig. 2. All of the biological thiols mentioned above elute before CSH and do not interfere with its detection.

4. Results

In this investigation, CSH was derivatized with NPM and analyzed using the reversed-phase HPLC with fluorescence detection method in isocratic mode. The tissue samples (brain, kidney, liver) from treated adult Sprague Dawley rats were homogenized, derivatized with NPM, and analyzed. The plasmas were also analyzed. Tissues of control animals were spiked with

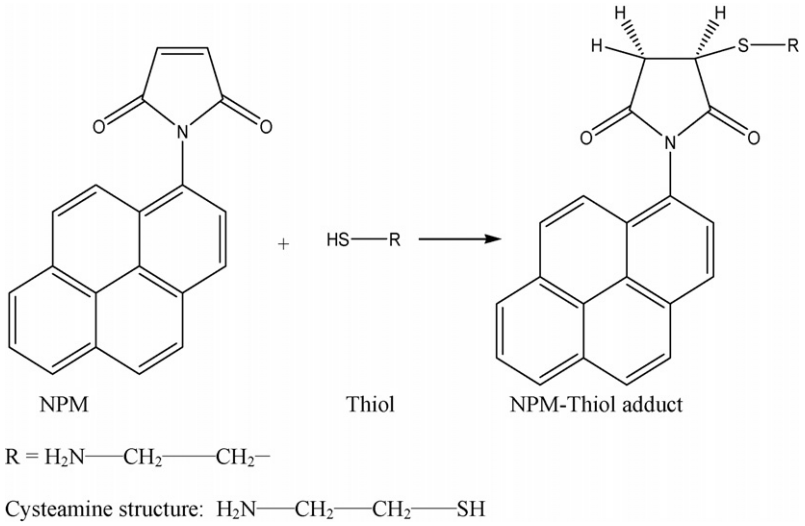

Fig. 1. Reaction of NPM with thiols to form fluorescent adducts.

varying concentrations of CSH and analyzed. Fig. 1 shows the derivatization reaction in which the thiols reacted with NPM to form fluorescent adducts. Fig. 2 shows the chromatogram of derivatized standard mixed thiols (600 nM): NAC, GSH, CYS, HCYS, and CSH in a plasma matrix. The biological thiols (CYS, GSH, NAC, and HCYS) do not interfere with the detection of CSH since their peaks come out before the CSH peak. Fig. 3 is the chromatogram of a kidney sample obtained from an animal administered 300 mg/kg body weight of CSH and sacrificed 30 min later. Fig. 4(a) shows the chromatogram of the control liver sample from a Sprague Dawley rat given phosphate buffered saline solution only. There is no CSH peak in this chromatogram. Fig. 4(b) shows the chromatogram of the liver sample obtained from a rat that was administered 300 mg/kg body weight of CSH and sacrificed 30 min later. The CSH peak was observed at 17 min. The concentrations of CSH obtained in the plasma and tissues are reported in Table 2. The levels

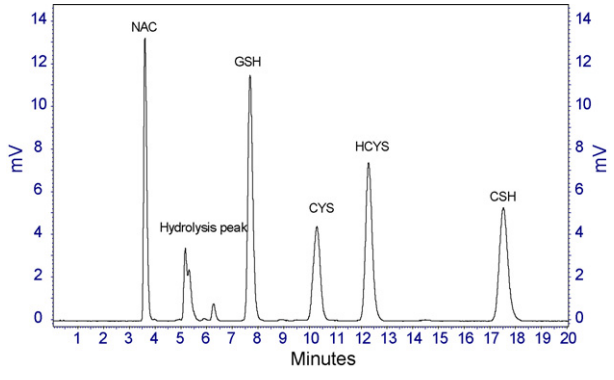

Fig. 2. Chromatogram of derivatized standard mixed thiols (600 nM): NAC, GSH, CYS, HCYS, and CSH in a plasma matrix. Separation conditions: an ODS-1 C_{18} Column (5 μm packing material) with 250 mm × 4.6 mm (i.d.) was used for the separation. The NPM derivatives were measured by a fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 376 nm). Flow rate was 1 mL/min. The mobile phase was 70% acetonitrile and 30% HPLC water and was adjusted to a pH of about 2 through the addition of 1 mL of both acetic acid and *o*-phosphoric acid in 1 L of mobile phase.

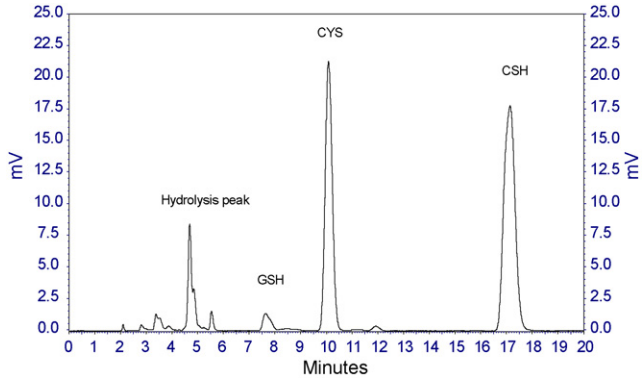

Fig. 3. Chromatogram of a kidney sample obtained from an animal sacrificed 30 min after the administration of 300 mg/kg body weight of CSH. The concentrations of GSH, CYS, and CSH were 3.3 ± 1.2, 34.5 ± 6.3, and 24.5 ± 1.2 nmol/mg protein, respectively. The separation conditions are the same as mentioned under Fig. 2.

of other important biological thiols (such as GSH and CYS) in the tissue samples and plasma were calculated using the thiol concentrations (nM) of homogenized tissue solutions, divided by the protein content in the tissue and expressed as nmol/mg

Table 2
Thiol levels in biological samples 30 min after oral administration of 300 mg/kg body weight

Samples (n = 3)		CSH	GSH	CYS
Brain	Control	ND	13.6 ± 1.1	1.6 ± 0.3
	CSH-treated	21.8 ± 1.7	15.8 ± 2.4	1.9 ± 0.5
Kidney	Control	ND	2.1 ± 0.5	28.2 ± 3.1
	CSH-treated	24.5 ± 1.2	3.3 ± 1.2	34.5 ± 6.3
Liver	Control	ND	19.2 ± 3.7	3.4 ± 0.2
	CSH-treated	9.5 ± 1.6	21.3 ± 4.2	2.7 ± 0.4
Plasma	Control	ND	6.2 ± 1.1	15.6 ± 1.8
	CSH-treated	22.4 ± 2.8	7.3 ± 1.4	18.4 ± 2.9

Plasma sample units are in μM, units for tissue samples are in nmol/mg protein. The values are mean ± standard deviation. ND: not detectable.

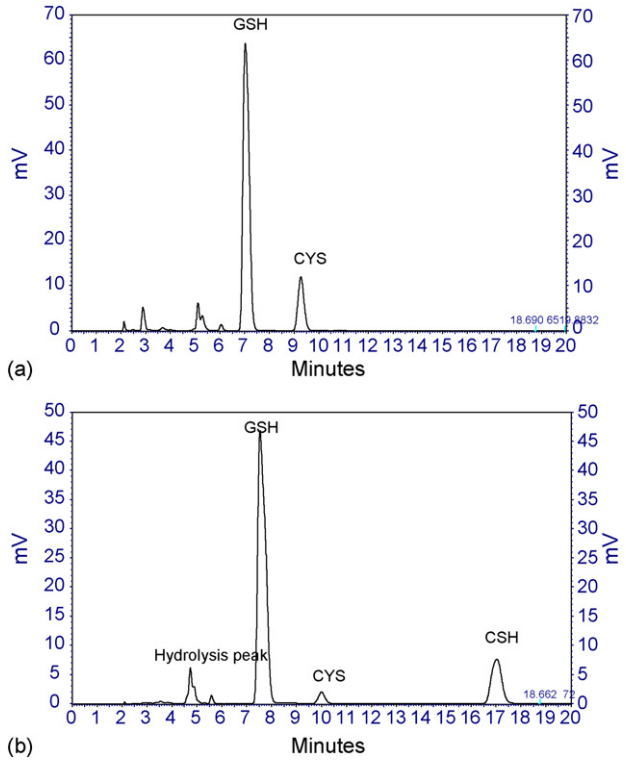

Fig. 4. (a) Chromatogram of a control liver sample obtained from an animal administered phosphate buffered saline solution only. There is no CSH peak. (b) Chromatogram of a liver sample obtained from an animal sacrificed 30 min after the administration of 300 mg/kg body weight of CSH. The separation conditions are the same as mentioned under Fig. 2.

protein. This data is reported in Table 2. The hydrolysis peak is the peak of NPM-water adduct, which results from the reaction of excess NPM with water.

5. Discussion

In spite of the attempts that have been made to analyze cysteamine in biological samples, to the best of our knowledge, no detailed study has been reported in the recent past on determination of cysteamine in biological samples. Ricci et al. [7] published an analytical method for determining cysteamine in biological samples but the derivatization procedure used is complex, involving coupled enzyme reactions along with detailed pre-treatment of the samples. Furthermore, no clear detection method has been shown. Stachowicz et al. [8] have reported determination of cysteamine in human serum. However, their work did not include the determination of cysteamine in other tissue samples such as the brain, kidney, and liver. Moreover, the derivatizing agent they used was mono bromobimane (mBBr) as a fluorescent probe, which can measure many biologically important thiols since it is both specific and sensitive to thiols. However, the sample preparation and derivatization are complex and time consuming, and involve the incubation of samples in the dark. In our study, we used *N*-(1-pyrenyl) maleimide (NPM) as the derivatizing agent [17]. NPM is very specific for thiols. The derivatization procedure using NPM is very simple (not requiring any special conditions), and the reactions take place at room temperature under ordinary laboratory conditions. NPM has the added advantage of increased sensitivity and rapid analysis, compared to the mBBr method. Although NPM has hydrolysis peaks, these come up well before the cysteamine peak; no interference has been observed. The methods of detection reported in the literature, such as high performance liquid chromatography with ultraviolet detection [18], ion exchange column chromatography, and capillary electrophoresis [19–21], are well documented as lacking sensitivity [22]. Therefore, we focused on the use of reversed-phase HPLC with fluorescence detection for determining cysteamine in biological samples. This is a very sensitive method with high reproducibility and precision. The LLOQ of CSH in biological samples by our method was 0.05 nmol/mL, and the detection limit was 0.01 nmol/mL (signal-to-noise = 3), which is better than the LLOQ of 0.1 nmol/mL of plasma reported by Kusmierek et al. [18] using HPLC with ultraviolet detection after precolumn derivatization, and the LOD of 0.061 μmol/L reported by Lochman et al. [9] using high-throughput capillary electrophoretic method for determination of total aminothiols in plasma and urine. A plasma cysteamine concentration of 5 μM in humans 8 h after an oral dose of 1200 mg is reported by Stachowicz et al. [8]. It is important to note that cysteamine easily undergoes oxidation at room temperature to form mixed disulfides, therefore, cysteamine should be dissolved in serine borate buffer, in which it remains stable for 7 days at 4 °C. In this study, we have demonstrated that HPLC with fluorescence detection, using NPM as the derivatizing agent, is a very suitable method for analyzing and detecting cysteamine in biological samples. The method is rapid, simple, and sensitive, and could be used in health institutions for pharmacokinetic studies.

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