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Determination of total cysteamine in urine and plasma samples by gas chromatography with flame photometric detection

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Abstract

A sensitive and selective method for the determination of total cysteamine in urine and plasma samples by gas chromatography (GC) has been developed. After reduction of the sample with sodium borohydride, the liberated cysteamine was converted into its N,S-diisobutoxycarbonyl derivative and measured by GC with flame photometric detection using a DB-210 capillary column. The calibration curve was linear in the range 0.2–5.0 nmol, and the detection limit, at a signal-to-noise ratio of 3, was *ca.* 0.5 pmol injected. Using this method, total cysteamine in urine and plasma samples could be accurately and precisely determined without any interference from coexisting substances. Analytical results for the determination of total cysteamine in urine and plasma samples from normal subjects are presented.

1. Introduction

Cysteamine (2-aminoethanethiol) is an important intermediate in the alternative pathway of taurine biosynthesis [1,2] and is formed in mammalian tissues by the enzymatic cleavage of panthetheine [3]. Cysteamine is present in the body as free form, as free oxidized forms (cystamine and mixed disulphides with low molecular mass thiols such as cysteine, homocysteine and glutathione) and as protein-bound form, and it has antioxidant activity [4,5] and gives protection against ionizing radiation [6–8] and hepatotoxicants [9–15]. In addition, cysteamine has been used therapeutically in the treatment of nephropathic cystinosis [16–18] and sickle cell anemia [19]. However, despite the physiological importance and clinical applications, only a few procedures have been described for the determination of cysteamine in biological fluids [20–23]. Ion-exchange column chromatography [20] and high-voltage electrophoresis [21] lack sensitivity and require a time-consuming preliminary clean-up of the samples. High-performance liquid chromatographic methods based on fluorometric [22] and electrochemical [23] detection are highly sensitive, but they lack specificity and require a time-consuming pretreatment of the samples. On the other hand, gas chromatographic analysis of cysteamine has been carried out by the preparation of neopentylidene [24] and trimethylsilyl [25] derivatives and with flame ionization detection. However, these methods lack sensitivity and specificity, and the preparation of the derivatives requires a lengthy procedure or anhydrous conditions.

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In our previous studies, it has been demonstrated that a number of amines [26–28] can be successfully analysed by GC as their N-alkoxycarbonyl derivatives, which are conveniently prepared by a simple procedure involving alkoxycarbonylation with alkyl chloroformate in an aqueous medium. In this paper, we describe a sensitive and selective method for the determination of total cysteamine in urine and plasma samples by gas chromatography with flame photometric detection (GC-FPD), in which cysteamine was converted into its N,S-diisobutoxycarbonyl derivative after reduction of the sample with sodium borohydride.

2. Experimental

2.1. Chemicals

Cysteamine and cystamine were purchased from Nacalai Tesque as their hydrochlorides (Kyoto, Japan). Each amine was dissolved in distilled water to make a stock solution with a concentration of 2 m*M*, and the stock solutions were stored at 4°C. The working standard solutions were made up freshly as required by dilution of the stock solution with distilled water. Thianthrene, the internal standard (I.S.), was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and was dissolved in ethyl acetate to make a 2 m*M* stock solution. Sodium borohydride (Nacalai Tesque) was used as a 100 mg/ml solution in 0.1 *M* sodium hydroxide. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo. All other chemicals were of analytical-reagent grade.

2.2. Preparation of samples

Early morning urine samples from healthy volunteers were collected and urinary creatinine concentrations were determined by the Jaffé reaction using Creatinine-test Wako (Wako Pure Chemical Industries, Osaka, Japan). Venous blood samples from healthy volunteers were collected in ethylenediamine tetraacetate (5 m*M*). After centrifugation at 2000 *g* for 5 min, the plasma layer was carefully collected. The collected urine and plasma samples were processed immediately or stored at −20°C until used. To obtain total cysteamine, urine and plasma samples were chemically reduced with sodium borohydride. To 0.1 ml of urine or plasma sample was added 0.2 ml of a 100 mg/ml sodium borohydride solution. The volume was made up to 1 ml with distilled water, and then the mixture was incubated at 100°C for 15 min. After cooling, the reaction mixture was used as the sample for derivatization.

2.3. Derivatization procedure

To the sodium borohydride reduced samples were added 0.05 ml of isoBCF and 0.05 ml of 2 *M* sodium hydroxide, and then the mixture was shaken at 300 rpm (up and down) for 5 min at room temperature. To the reaction mixture was added 0.05 ml of 10 μM thianthrene (I.S.) and then the mixture was extracted with 3 ml of *n*-pentane. The pentane extract was evaporated to dryness at 80°C under a stream of dry air. The residue was dissolved in 0.1 ml of ethyl acetate and then 1 μl of this solution was injected onto the gas chromatograph.

2.4. Gas chromatography

GC analysis was carried out with a Shimadzu 12A gas chromatograph equipped with a flame photometric detector (S-filter). A fused-silica capillary column (15 m × 0.53 mm I.D., 1.0 μm film thickness) of cross-linked DB-210 (J and W, Folsom, CA, USA) was used. The operating conditions were as follows: column temperature, programmed at 5°C/min from 170 to 250°C; injection and detector temperature, 260°C; nitrogen flow-rate, 8 ml/min.

3. Results and discussion

In order to determine the total concentration of cysteamine, disulphides in the sample must be reduced to the thiol and then derivatized with an appropriate reagent for GC analysis. Sodium

borohydride efficiently converts all cysteamine present as cystamine, mixed disulphides with other low molecular-mass thiols or protein-bound cysteamine to their thiol form [23]. In order to determine the reduction conditions for the disulphide forms of cysteamine, standard cystamine (2 nmol), urine and plasma samples were reduced with sodium borohydride. The reduction of these samples was accomplished within 15 min at 100°C by using 20 mg sodium borohydride. The sample treated with sodium borohydride could be directly derivatized after cooling. The N,S-diisobutoxycarbonylation of cysteamine with isoBCF proceeded rapidly and quantitatively in aqueous alkaline media. As shown in Fig. 1, the reaction was completed within 2 min at room temperature using 0.05 ml isoBCF. Subsequently, the isobutoxycarbonyl derivative of cysteamine was quantitatively extracted into *n*-pentane, and the excess reagent and solvent were removed by evaporation. The structure of N,S-diisobutoxycarbonyl cysteamine was confirmed by GC–mass spectrometry. A molecular-ion peak (M^+) with postulated *m/z* 277 and other prominent fragment ion peaks [$M^+ - OCH_2CH(CH_3)_2$, $M^+ - COOCH_2CH(CH_3)_2$, $M^+ - NHCOOCH_2CH(CH_3)_2$, and $M^+ - SCOOCH_2CH(CH_3)_2$] were useful for structure elucidation. The derivative was stable and no decomposition was observed.

As shown in Fig. 2A, cysteamine was eluted as a single and symmetrical peak at 7 min. The derivative provided an excellent FPD response and the minimum detectable amount of cysteamine at a signal-to-noise ratio of 3 under

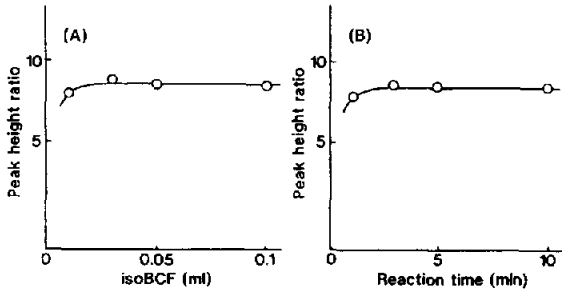

Fig. 1. Effects of (A) isobutyl chloroformate and (B) reaction time on the N,S-diisobutoxycarbonylation of cysteamine.

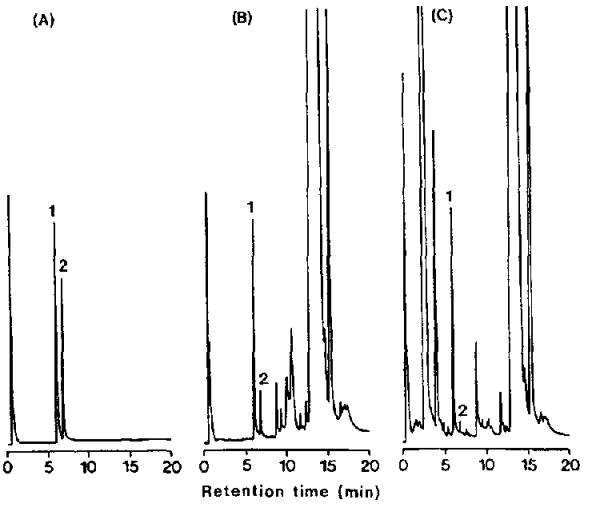

Fig. 2. Gas chromatograms obtained from the standard solution, urine and plasma samples. (A) Standard (containing 1 nmol of cysteamine), (B) urine (0.1 ml), (C) plasma (0.1 ml). GC conditions are given in Experimental. Peaks: 1 = thianthrene (I.S.), 2 = cysteamine.

our instrumental conditions was *ca.* 0.5 pmol injected (500 pmol/ml). By this method, other biological substances, such as cysteine, methionine and other amino acids and amines, were not detected at all. In order to test the linearity of the calibration curve, various amounts of cysteamine ranging from 0.2 to 5 nmol were derivatized, and aliquots representing 2–50 pmol were injected. A linear relationship was obtained from a double logarithmic plot, and the regression line was $\log y = 1.789 \log x - 0.165$ ($r = 0.9982$, $n = 15$), where y is the peak-height ratio and x is the amount (nmol) of cysteamine.

The developed method was successfully applied to urine and plasma samples without deproteinization. Fig. 2B and C show typical chromatograms obtained from 0.1 ml of urine and plasma samples, respectively. Although several unidentified peaks were observed, cysteamine in these samples could be detected without any interference from coexisting substances. The cysteamine peak obtained from each sample was confirmed by GC–MS. As shown in Table 1, the overall recoveries of cysteamine and cystamine added to urine and plasma samples were 91.0–102.2%, and the relative standard deviations

Table 1
Recoveries of cysteamine added to urine and plasma samples

Sample	Amine[a]	Concentration added (nmol/ml)	Concentration found[b] (nmol/ml)		Recovery (%)
			Non-addition	Addition	
Urine					
A	CYE	(5.00)	2.39 ± 0.22	7.30 ± 0.17	98.2
B	CYE	(5.00)	13.08 ± 0.02	18.18 ± 0.04	102.0
C	CYA	(2.00)	1.02 ± 0.08	2.85 ± 0.10	91.5
D	CYA	(2.00)	0.99 ± 0.07	2.81 ± 0.17	91.0
Plasma					
A	CYE	(5.00)	2.58 ± 0.09	7.69 ± 0.02	102.2
B	CYE	(5.00)	1.83 ± 0.09	6.66 ± 0.25	96.6
C	CYA	(2.00)	0.60 ± 0.09	2.49 ± 0.01	94.5
D	CYA	(2.00)	0.73 ± 0.03	2.60 ± 0.04	93.5

[a] CYE: cysteamine; CYA: cystamine.
[b] Mean ± S.D. ($n = 4$).

were 0.2–9.2% ($n = 4$). The quantitation limit of total cysteamine in these samples was 0.5 nmol/ml. Tables 2 and 3 show the urinary excretion and plasma concentration of total cysteamine in several healthy volunteers. Although physiological cysteamine concentrations have been reported in several tissues [29–31], cysteamine in biological fluids has not yet been detected. To our knowledge the results presented in this paper are the first reported for cysteamine concentrations in normal human urine and plasma samples.

In conclusion, a convenient and reliable method for the determination of total cysteamine in urine and plasma samples has been established. This method is sensitive and selective, and biological fluids can be directly analysed without deproteinization and without any interference from other coexisting substances. We believe that this method provides a useful tool in biochemical and pharmacological research.

Table 2
Urinary excretion of total cysteamine in normal subjects

Subject	Age	Sex[a]	Total cysteamine[b] (nmol/mg creatinine)
1	7	M	4.09 ± 0.01
2	23	M	1.53 ± 0.11
3	23	M	2.65 ± 0.02
4	24	M	1.00 ± 0.06
5	38	M	1.51 ± 0.01
6	5	F	2.88 ± 0.02
7	20	F	2.68 ± 0.02
8	21	F	0.59 ± 0.03
9	22	F	0.77 ± 0.03
10	38	F	1.93 ± 0.01

[a] M: male; F: female.
[b] Mean ± S.D. ($n = 4$).

Table 3
Plasma total cysteamine in normal subjects

Subject	Age	Sex[a]	Total cysteamine[b] (nmol/ml)
1	22	M	2.58 ± 0.09
2	23	M	1.88 ± 0.06
3	24	M	1.53 ± 0.07
4	37	M	1.83 ± 0.09
5	38	M	3.82 ± 0.01
6	21	F	1.12 ± 0.05
7	22	F	2.04 ± 0.09
8	22	F	1.20 ± 0.05

[a] M: male; F: female.
[b] Mean ± S.D. ($n = 4$).

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