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Determination of cysteamine and cystamine by gas chromatography with flame photometric detection

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Abstract: A gas chromatographic method for the determination of cysteamine and its disulphide cystamine is described. Cysteamine and cystamine are converted into N,S-diisobutoxycarbonyl and N,N-diisobutoxycarbonyl derivatives, respectively. The derivatives are analysed by gas chromatography with flame photometric detection, using a DB-210 capillary column. The calibration curves for cysteamine and cystamine in the range of 0.2–5.0 nmol are linear and sufficiently reproducible for quantitative analysis, and the detection limit is about 0.5 pmol injected. Cysteamine in mouse tissues is found in the free reduced, free oxidized and protein-bound forms. Free oxidized and protein-bound forms are reduced to free cysteamine by the use of sodium borohydride, and then derivatized. Cysteamine and cystamine in mouse tissues can be measured without any interference from coexisting substances by this method. The recoveries of cysteamine and cystamine added to the tissue samples are 91–106%, and their reproducibilities are found to be satisfactory. Analytical results for the determination of various forms of cysteamine in mouse tissues are presented.

Keywords: *Cysteamine; cystamine; redox status; isobutoxycarbonylation; gas chromatography; flame photometric detection.*

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 pantetheine [3]. Cysteamine and its disulphide, cystamine, have various pharmacological effects, and have been used experimentally and therapeutically in the treatment of nephropathic cystinosis [4–6] and sickle cell anaemia [7], as a depleting agent of immunoreactive somatostatin levels [8–12], as an inhibitor of the secretion of several pituitary hormones [13, 14], as a potent duodenal ulcerogen [15], as a radioprotective agent [16–18], as an antioxidant [19, 20], and as a post-toxicant therapeutic agent for hepatic necrosis caused by several hepatic toxicants such as acetaminophen [21–25], phalloidin [26], galactosamine [27], carbon tetrachloride, bromobenzene, thioacetamide, and dimethyl nitrosamine [28]. However, despite the metabolic importance and clinical applications of cysteamine and cystamine, the mechanisms of their actions have not been elucidated.

The determination of cysteamine and cystamine in biological samples has been carried out by ion-exchange column chromatography [29–32], high-voltage electrophoresis [33], enzymatic assay [34, 35] and high-performance liquid chromatography (HPLC) [35–41]. However, ion-exchange column chromatography and electrophoresis lack sensitivity and require a time-consuming preliminary clean-up of the samples. Enzymatic methods are specific for cysteamine but lack sensitivity and the reaction product is unstable. HPLC methods based on the spectrophotometric [35], fluorimetric [36–39] and electrochemical [39–41] detection are highly sensitive, but some of these methods lack specificity and require a time-consuming preliminary clean-up of the sample by ion-exchange column chromatography to remove the interfering substances. On the other hand, gas chromagraphic methods which are based on the preparation of neopentylidene [42] and trimethylsilyl [43] derivatives lack specificity and sensitivity, and are not applied to biological samples.

The *in vivo* facile oxidation of cysteamine results in a variety of disulphide forms [1, 44], including cystamine and mixed disulphides with low molecular weight thiols (such as cysteine, homocysteine and glutathione) and proteins. Therefore, to elucidate the function

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of cysteamine in biochemical and pharmacological processes, it is necessary to determine the *in vivo* redox status of cysteamine. In this paper, a selective and sensitive method for the determination of cysteamine and cystamine by gas chromatography with flame photometric detection (FPD–GC) is described, in which these amines are analysed as N,S-diisobutoxycarbonyl and N,N-diisobutoxycarbonyl derivatives, respectively. By using this method, the *in vivo* redox status of cystamine in mouse tissues was investigated.

Experimental

Reagents

Cysteamine and cystamine were purchased from Nacalai Tesque (Kyoto, Japan). Each amine was dissolved in distilled water to make a stock solution at a concentration of 2 mM, and stored at 4°C. The working standard solutions were made up freshly as required by dilution of the stock solution with distilled water. *p*-Toluenesulphonylanilide (*p*-TSA), an internal standard (I.S.), was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and was dissolved in ethyl acetate to make a stock solution with a concentration of 2 mM. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo. Sodium borohydride ($NaBH_4$) (Nacalai Tesque) was used as a 100 mg ml^{-1} solution in 0.1 M NaOH. All other chemicals were of analytical grade.

Principles for the determination of the different forms of cysteamine in tissue samples

The outline of the method for the determination of the different forms of cysteamine in tissue samples is shown in Fig. 1. The principles of the different procedures described below involve fractionation into free and protein-bound fractions, reduction of the various disulphides to cysteamine by $NaBH_4$ and subsequent derivatization with isoBCF. Free cysteamine and cystamine contents were measured after direct derivatization of the free fraction. The oxidized cysteamine content in the free fraction was measured by subtraction of the free cysteamine content from the total free cysteamine content. Protein-bound cysteamine was measured after $NaBH_4$ reduction of protein-bound fraction.

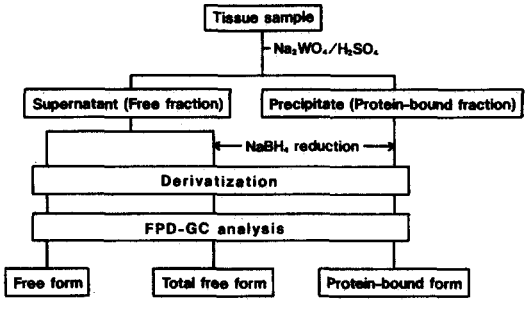

Figure 1
Outline of method for the determination of different forms of cysteamine in tissue samples.

Preparation of samples

Five male ddY mice 6 weeks old (28.2 ± 0.5 g) were used in the experiments. Immediately after dissection, each organ was removed and stored at −20°C before assay. Each pooled tissue was chopped up, and an aliquot (0.2–0.4 g) was homogenized with 2.4 ml of 0.05 M H_2SO_4 and 1.2 ml of 2.5% Na_2WO_4 with a Model LK-21 ultra-disperser (Yamato Kagaku, Tokyo, Japan). After centrifugation at 2000*g* for 5 min, the precipitate was re-extracted with same volumes of 0.05 M H_2SO_4 and 2.5% Na_2WO_4. The supernatants were combined and used to determine the free amines. The precipitate was dissolved in 8 ml of 0.1 M NaOH and used in the determination of the protein-bound fraction.

For the free cysteamine and cystamine analysis, 0.8 ml of the free fraction was directly derivatized.

For the total free cysteamine (cysteamine + cystamine + mixed disulphides of cysteamine with other low molecular weight thiols), to 0.5 ml of free fraction was added 0.2 ml of 100 mg ml^{-1} $NaBH_4$ solution, and the mixture was incubated at 100°C for 10 min. After cooling, the reaction mixture was used as the sample for derivatization.

For the protein-bound cysteamine, to 0.5 ml of protein-bound fraction was added 0.2 ml of 100 mg ml^{-1} $NaBH_4$ solution, and the mixture was incubated at 100°C for 10 min. After cooling, the reaction mixture was used as the sample for derivatization.

Derivatization procedure

To the free fraction sample and the $NaBH_4$ reduction samples of free and protein-bound fractions, which were obtained by the above method, were added 0.05 ml of isoBCF and 0.2 ml of 0.5 M NaOH and the mixture was shaken with a shaker set at 300 rpm (up and down) for 5 min at room temperature. To the

reaction mixture was added 0.1 ml of 10 μM *p*-TSA (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 into the gas chromatograph.

Gas chromatography

GC analysis was carried out with a Shimadzu 12A gas chromatograph equipped with a flame photometric detector (S-filter). Fused-silica capillary column of cross-linked DB-210 (J & W, Folsom, CA, 15 m × 0.53 mm i.d., 1.0 μm film thickness) was used. The stationary phase of this column is 50% trifluoropropyl–50% methyl polysiloxane and corresponds to OV-210. The operating conditions were as follows: column temperature, programmed at 5°C min^{-1} from 170 to 250°C; injection and detector temperatures, 260°C; nitrogen flow rate, 10 ml min^{-1}; hydrogen flow rate, 20 ml min^{-1}; air flow rate, 35 ml min^{-1}. The peak heights of cysteamine, cystamine and the I.S. were measured, and the peak height ratios relative to the I.S. were calculated before constructing a calibration curve.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett–Packard 5890A gas chromatograph was operated in conjunction with a VG Analytical 70-SE mass spectrometer and a VG 11-250J mass data system. The GC column was of the same type as used for GC analysis. The instrumental setting gave an ionizing voltage of 40 eV, an ion-source temperature of 250°C and a helium flow rate of 8 ml min^{-1}.

Results and Discussion

GC analyses of cysteamine and cystamine have been carried out by the preparation of neopentylidene [42] and trimethylsilyl [43] derivatives and with flame ionization detection. However, these methods lack sensitivity and the preparation of the derivatives requires a lengthy procedure or anhydrous conditions. We investigated a simple and rapid derivatization method involving aqueous media and using isoBCF as derivatizing reagent. The N- and S-isobutoxycarbonylation of cysteamine and cystamine with isoBCF proceeded rapidly and quantitatively in aqueous alkaline media. This reaction was completed within 2 min at room temperature. Subsequently, the isobutoxycarbonyl derivatives of cysteamine and cystamine were quantitatively extracted into *n*-pentane, and the excess of reagent and solvent were removed by evaporation. The derivative preparation could be performed within 10 min, and several samples could be treated simultaneously.

The structures of the derivatives were confirmed by GC–MS analysis. As shown in Fig. 2(A) and (C), a molecular ion peak (M^+) was observed for each of the derivatives and other common ion peaks which were useful for structure elucidation were $M^+ - 73$ $[OCH_2CH(CH_3)_2]$, *m/z* 176 $[SCH_2CH_2NHCOOCH_2CH(CH_3)_2]$, *m/z* 144 $[CH_2CH_2NHCOOCH_2CH(CH_3)_2]$, *m/z* 130 $[CH_2NHCOOCH_2CH(CH_3)_2]$ and *m/z* 57 $[CH_2CH(CH_3)_2]$. The derivatives were stable and no decomposition was observed even after standing in ethyl acetate for 2 weeks at room temperature.

As shown in Fig. 3(A), cysteamine and cystamine were eluted as separate symmetrical peaks and chromatographic run was completed within 16 min. The derivatives provided an excellent FPD response and minimum detectable amounts of cysteamine and cystamine to give a signal three times as high as the noise under our instrumental conditions were about 0.5 pmol injected. By this method, other biological substances such as amino acids were not detected at all. In order to test the linearity of the calibration curve, various amounts of cysteamine and cystamine ranging from 0.2 to 5 nmol were derivatized in a mixture, and aliquots representing 2–50 pmol of each compound were injected. In each case, a linear relationship was obtained from both logarithmic plots, and the regression lines of cysteamine and cystamine were $\log y = 1.884 \log x + 0.562$ ($r = 0.9995$, $n = 15$) and $\log y = 1.856 \log x + 0.355$ ($r = 0.9990$, $n = 15$), respectively, where y is the peak height ratio and x is the amount (nmol) of each amine.

In order to determine the concentrations of the different forms of cysteamine in tissue samples, the conditions for the fractionation of tissue sample and the reduction of disulphides were investigated. As shown in Fig. 1, the sample was separated into free and protein-bound fractions by precipitation with Na_2WO_4–H_2SO_4. Free cysteamine and cystamine were not observed in the protein-bound

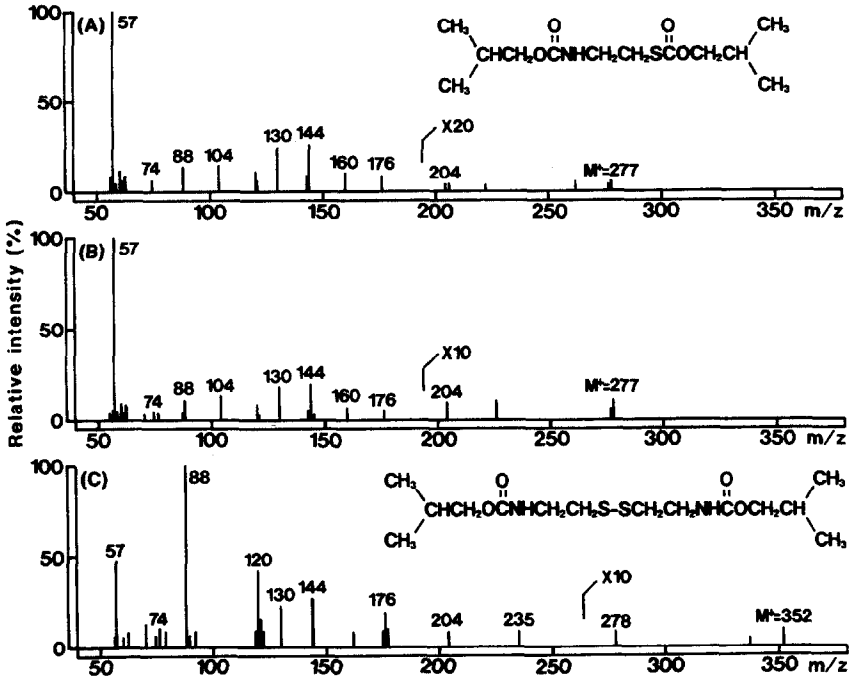

Figure 2
GC–MS spectra obtained from the N,S- and N,N-diisobutoxycarbonyl derivatives of authentic cysteamine and cystamine and from the peak identified with cysteamine in free fraction of mouse liver. (A) Authentic cysteamine; (B) liver cysteamine; (C) authentic cystamine.

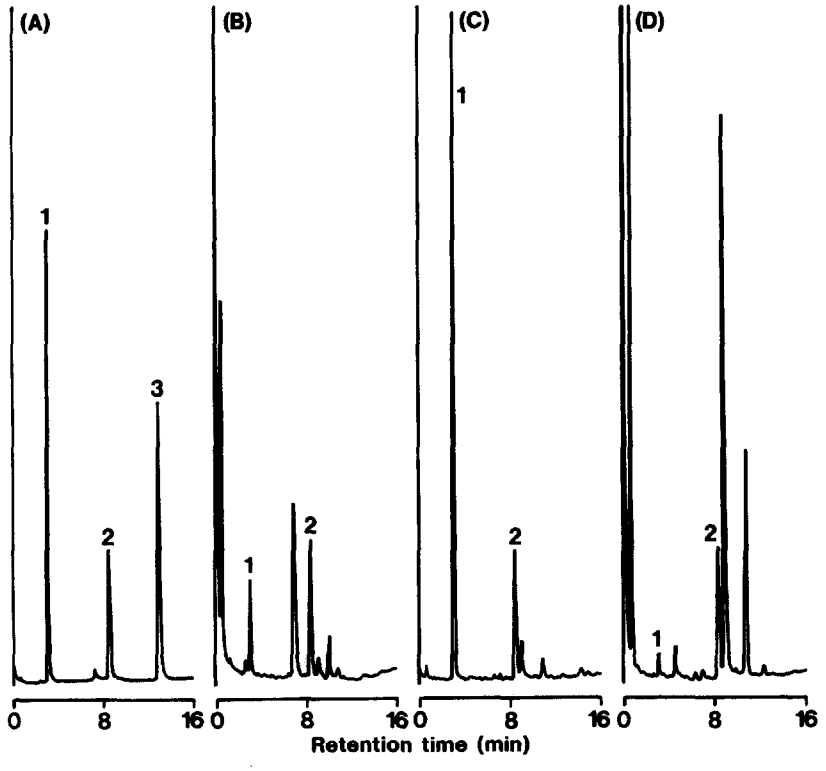

Figure 3
Gas chromatograms obtained from a standard solution and free and protein-bound fractions of mouse liver. (A) Standard containing 1 nmol of each compound; (B) free fraction (−reduction); (C) free fraction (+reduction); (D) protein-bound fraction (+reduction). GC conditions: see Experimental section. Peaks 1, cysteamine; 2, *p*-toluenesulphonylanilide (I.S.); 3, cystamine.

fraction. In order to determine the reduction conditions for disulphide forms of cysteamine, standard cystamine (2 nmol) and the free fraction of mouse liver containing added standard cystamine (2 nmol) were reduced with $NaBH_4$. As shown in Fig. 4, the reduction of standard cystamine was accomplished within 10 min at 100°C by using 20 mg $NaBH_4$ in the absence and presence of tissue sample. The sample treated with $NaBH_4$ could be directly derivatized after cooling.

As shown in Fig. 3(B)–(D), cysteamine in the free and protein-bound fractions could be detected without any interference from co-existing substances, although unidentified peaks were detected before and after the I.S. peak. Moreover, there was no peak which could cause interference with that of the I.S., when the I.S. was not added to each fraction. The identity of the cysteamine peak obtained from mouse liver extracts was confirmed by GC–MS. As shown in Fig. 2(B), the GC–MS spectrum of cysteamine originating from a liver sample agreed with that of authentic derivative. As shown in Table 1, the recovery rates of cysteamine and cystamine added to free and protein-bound fractions of mouse tissues were 91.6–105.7%, and their relative standard deviations were 1.1–5.8% (n = 4).

Table 2 shows the results obtained from the analysis of various forms of cysteamine in mouse tissues by the above methods developed. Free cysteamine was widely distributed in mouse tissues except for the eye. Kidney

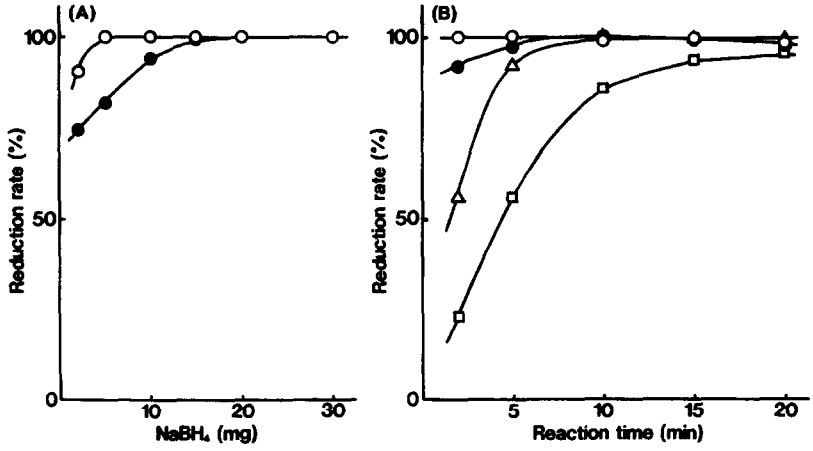

Figure 4
Effects of (A) $NaBH_4$ and (B) reaction time on the reduction of cystamine (2 nmol) in water and free fraction of mouse liver. Key: ○, in water (100°C); ●, in mouse liver (100°C); △, in water (80°C); □, in water (60°C).

Table 1
Recoveries of cysteamine and cystamine added to free and protein-bound fractions of mouse tissues

Fraction	Amine added (nmol g^{-1})		Amount found (nmol g^{-1})* Non-addition	Addition	Recovery (%)
Liver, free†	Cysteamine	(12.0)	17.0 ± 0.2	28.3 ± 0.3	94.2
	Cystamine	(12.0)	ND§	11.9 ± 0.3	99.2
Liver, free‡	Cysteamine	(38.6)	51.0 ± 0.7	91.8 ± 1.3	105.7
	Cystamine	(38.6)	25.5 ± 0.3‖	64.6 ± 1.4‖	101.3
Liver, bound‡	Cysteamine	(40.5)	8.7 ± 0.1	48.6 ± 1.2	98.5
	Cystamine	(40.5)	4.4 ± 0.05‖	41.5 ± 0.9‖	91.6
Brain, free†	Cysteamine	(10.5)	5.0 ± 0.3	14.8 ± 0.4	99.3
	Cystamine	(10.5)	ND	10.1 ± 0.4	96.2
Brain, free‡	Cysteamine	(33.7)	23.0 ± 0.4	55.4 ± 1.2	96.1
	Cystamine	(33.7)	11.5 ± 0.2‖	44.4 ± 0.7‖	97.6
Brain, bound‡	Cysteamine	(40.2)	37.7 ± 1.9	75.6 ± 4.4	94.3
	Cystamine	(40.2)	18.9 ± 1.0‖	60.4 ± 1.6‖	103.2

*Mean ± SD (n = 4).
†Derivatized without reduction.
‡Derivatized after reduction.
§Not detectable.
‖Calculated as cystamine amount after measurement of cysteamine produced by reduction with $NaBH_4$.

Table 2
Contents of free forms of cysteamine, cystamine and total cysteamine, and protein-bound form of cysteamine in mouse tissues

Tissue	Content (nmol g^{-1} wet weight)*			
	Free cysteamine	Free cystamine	Free total cysteamine	Protein-bound cysteamine
Eye	ND†	5.3 ± 0.1	27.1 ± 0.8	19.2 ± 0.5
Brain	2.1 ± 0.1	ND	20.5 ± 0.3	33.9 ± 1.7
Heart	2.5 ± 0.1	ND	47.1 ± 0.6	19.5 ± 1.3
Lung	2.2 ± 0.05	ND	38.7 ± 1.3	40.9 ± 1.6
Liver	9.7 ± 0.1	ND	46.3 ± 2.5	7.5 ± 0.3
Spleen	1.9 ± 0.05	ND	6.51 ± 0.2	4.2 ± 0.3
Pancreas	7.0 ± 0.5	ND	12.5 ± 0.4	4.6 ± 0.3
Kidney	70.3 ± 1.5	ND	100.0 ± 3.6	6.7 ± 0.3
Stomach	3.5 ± 0.1	ND	12.9 ± 0.7	5.6 ± 0.2
Intestine	7.0 ± 0.2	ND	20.7 ± 1.0	12.6 ± 0.3
Testis	4.6 ± 0.1	ND	12.7 ± 0.4	3.3 ± 0.2
Muscle	1.6 ± 0.04	ND	6.1 ± 0.4	3.5 ± 0.2

*Mean ± SD (n = 3).
†Not detectable.

Table 3
Total cysteamine contents and percentages of the different forms of cysteamine in mouse tissues

Tissue	Total cysteamine (nmol g^{-1})	Cysteamine (%)*		
		Reduced	Free oxidized	Protein-bound
Eye	46.3	0	58.5	41.5
Brain	54.4	3.9	33.8	62.3
Heart	66.6	3.8	67.0	29.3
Lung	79.6	2.8	45.9	51.4
Liver	53.8	18.0	68.0	13.9
Spleen	10.7	17.8	43.0	39.3
Pancreas	17.1	40.9	32.2	26.9
Kidney	106.7	65.9	27.8	6.3
Stomach	18.5	18.9	50.8	30.3
Intestine	33.3	21.0	41.1	37.8
Testis	16.0	28.8	50.6	20.6
Muscle	9.6	16.7	46.9	36.5

*The cumulative values of the reduced cysteamine, free oxidized cysteamine (cystamine and mixed disulphides with low molecular weight thiols) and protein-bound cysteamine were taken as 100% (total cysteamine).

contained high concentrations of free cysteamine. In contrast, free cystamine was only detected in eye and not detected at all in any of the other organs investigated in this study. Total free cysteamine and protein-bound cysteamine were found in all tissue samples examined. Kidney, heart, liver and lung contained high concentration of total free cysteamine. On the other hand, only lung and brain contained high concentration of protein-bound cysteamine. Table 3 shows the total cysteamine content calculated as the sum of the total free cysteamine and protein-bound cysteamine contents (Table 2) and the percentile distribution of the different forms of cysteamine against total cysteamine content in mouse tissues. Oxidized cysteamine (cystamine and mixed disulphides with low molecular weight thiols) was measured by subtracting the free reduced cysteamine content from the total free cysteamine content. Total cysteamine contents in kidney, lung, heart, brain, liver and eye were high, but those in muscle and spleen were very low. It clearly demonstrates that the predominant form of cysteamine in brain, heart, lung, spleen, stomach and muscle is the free oxidized or protein-bound form. Notably, the level of the reduced form of cysteamine in eye, brain, heart and lung was extremely low or not detected. In contrast, cysteamine in kidney is unique among mouse tissues as it predominantly occurs in the reduced form. Although the reason for such differences among tissues is not known at present, the wide distribution

and occasionally high content of cysteamine as various forms suggest that cysteamine plays an important role in the respective organs.

In conclusion, the present method is selective and sensitive, and applicable to the determination of reduced, oxidized and protein-bound forms of cysteamine in tissue samples. Therefore, this method is considered to be a useful tool for biochemical and pharmacological research requiring the measurement of cysteamine and cystamine concentrations.

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