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Short communication

High-performance liquid chromatographic determination of hypotaurine and taurine after conversion to 4-dimethylaminoazobenzene-4′-sulfonyl derivatives and its application to the urine of cysteine-administered rats

Shinya Futani, Toshihiko Ubuka*, Tadashi Abe

Department of Biochemistry, Okayama University Medical School, 2-5-1 Shikatacho, Okayama 700, Japan

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Abstract

A method for the determination of urinary hypotaurine and taurine for the purpose of studying hypotaurine–taurine status in mammals is described. Hypotaurine and taurine were converted into 4-dimethylaminoazobenzene-4′-sulfonyl (dabsyl) derivatives under conditions minimizing hypotaurine oxidation. The dabsylhypotaurine and dabsyltaurine formed were determined by reversed-phase HPLC. Average excretions of taurine and hypotaurine in rat urine were 270.5 and 2.5 μmol/kg body mass per day, respectively. After intraperitoneal injection of 5 mmol of L-cysteine/kg body mass, the increased excretions of taurine and hypotaurine corresponded to 22.1 and 2.5%, respectively, of L-cysteine administered, indicating that hypotaurine production exceeded the capacity of hypotaurine oxidation in vivo.

1. Introduction

Taurine is one of the main final end-products of L-cysteine metabolism in mammals [1] and is excreted in urine. Taurine is formed through the cysteinesulfinate pathway [2] and cysteamine pathway [3], and hypotaurine is the immediate precursor of taurine biosynthesis in these pathways. It has been reported that hypotaurine is contained in the rat brain [4], accumulates in the liver of L-cysteine-administered rats [2] and in the regenerating rat liver [5] and is excreted in the urine of rats fed L-cystine [6]. Hypotaurine increased in the blood plasma of rats injected with L-cysteine [7]. Although the mechanism of the oxidation of hypotaurine to taurine is not fully understood [1,8], it has been proposed that the physiological significance of hypotaurine is its antioxidant action [9,10]. Therefore, the hypotaurine–taurine status in the animal body seems to be important for the elucidation of the physiological role(s) of hypotaurine and taurine.

The separation and determination of hypotaurine have been performed by means of paper chromatography [2], ion-exchange chromatography [11], amino acid analysis [5,7,12], radioactivity measurement [8], high-performance liquid chromatography (HPLC) [13,14] and gas chromatography [15]. In contrast to stable taurine, it has been shown that hypotaurine is oxidized to

* Corresponding author.

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taurine spontaneously [2,9] or by ultraviolet irradiation [16]. This instability of hypotaurine hindered the development of simple methods for the determination of hypotaurine. Therefore, we intended to establish a simple and rapid method for hypotaurine determination under conditions minimizing the oxidation of hypotaurine.

HPLC of 4-dimethylaminoazobenzene-4'-sulfonylamino acids (dabsylamino acids) has been reported to be a sensitive method for the determination of amino acids [17]. In this study, we applied this method to the determination of hypotaurine and taurine, and found that dabsylhypotaurine is, in contrast to free hypotaurine, very stable. We report here a method for the dabsylation of urinary hypotaurine and taurine and HPLC determination of these dabsylated amino acids. The method was applied to the urine of cysteine-administered rats as an example of the study of the hypotaurine–taurine status in mammals.

2. Experimental

2.1. Materials

Hypotaurine and taurine were obtained from Sigma (St. Louis, MO, USA). S-Carboxymethyl-L-cysteine (CMC) was synthesized as reported [18]; it may also be obtained from commercial sources. Other amino acids, acetonitrile (HPLC grade) and acetone (analytical-reagent grade) were obtained from Wako (Osaka, Japan). Dabsyl chloride was obtained from Dojin Laboratories (Kumamoto, Japan) and crystallized once from acetone. Male Wistar rats weighing 250–300 g were used and fed ad libitum a laboratory diet MF (Oriental Yeast, Tokyo, Japan).

2.2. Dabsylation of standard amino acids

Dabsylation of standard amino acids except for hypotaurine was performed according to Chang et al. [17].

Dabsylation of hypotaurine was performed in 65% acetone solution at 40°C as follows. Hypotaurine was dissolved in water at a concentration of 5.0 μmol/ml and 10 μl of the solution was mixed, in a test-tube (100 × 15 mm I.D.) with a Teflon-lined screw-cap, with 0.5 ml of 0.1 M sodium hydrogencarbonate buffer (pH 9.0) and 1.0 ml of 0.45 m*M* dabsyl chloride solution (3.25 mg in 5 ml of acetone). The mixture was incubated in a water-bath at 40°C for 30 min. After addition of equal volume of ethanol, the mixture was allowed to stand for about 30 min. The precipitate that formed (sodium hydrogencarbonate) was centrifuged off, the resulting supernatant was filtered through a 0.2-μm filter and an aliquot of the filtrate was applied to HPLC.

2.3. Reversed-phase (RP) HPLC

As the retention times of dabsyl derivatives of hypotaurine and taurine depend on column temperature, the HPLC separation was optimum at 16°C in this study.

An RP-HPLC system from Tosoh (Tokyo, Japan) was used. The system consisted of a CCPM pump, a UV-8010 detector, a Chromatocorder 12 integrator and a TSKgel ODS-80Ts column (150 × 4.6 mm I.D., particle size 5 μm) with a guard column (TSKguardgel ODS-80Ts, 15 × 3.2 mm, I.D., particle size 5 μm). Solvent A was 50 m*M* sodium acetate (pH 4.00) and solvent B was acetonitrile. The column was inserted in a plastic bag and immersed in a water bath regulated at 16 ± 0.5°C, and was preconditioned with solvent A–solvent B (72:28). The bottles of the solvents were also dipped in a water-bath regulated at 16 ± 0.5°C.

An aliquot (routinely 10 μl) of sample was applied to the system. Elution was performed with a gradient of solvent B as follows: 0–20 min, linear gradient from 28 to 31%; 20–40 min, linear gradient from 31 to 32%. The column was then washed with solvent A–Solvent B (5:95) for 20 min and reconditioned as above for 20 min. The effluent was monitored at 430 nm. All solvents and samples were filtered through a 0.2-μm filter before application to the column.

2.4. Preparation of calibration graphs

CMC was used as an internal standard. CMC was dissolved in water at a concentration of 5.0 m*M* by warming in a water-bath. The solution was not kept in a refrigerator, because CMC tended to crystallize at low temperature. A mixture of hypotaurine or taurine and CMC solutions was dabsylated as above and analysed by HPLC. Calibration graphs were obtained by plotting the ratio of the peak area of dabsylhypotaurine or dabsyltaurine to that of dabsyl-CMC obtained by HPLC against the corresponding molar ratio of these amino acids added to the dabsylation reaction mixture.

2.5. Oxidation of dabsylhypotaurine to dabsyltaurine with hydrogen peroxide

Hydrogen peroxide solution (3%) was added at a final concentration of 0.2% to 60% ethanol solution containing ca. 20 nmol/ml of dabsylhypotaurine. After incubation at 25 or 40°C for various times, aliquots were taken for the determination of hypotaurine and taurine.

2.6. Collection and dabsylation of urine samples

Each rat was housed in a metabolic cage and injected intraperitoneally with an aqueous L-cysteine solution (0.5 mmol/ml) at a dose of 5 mmol of L-cysteine per kg body mass. Urine (24 h) was collected in a light-protected bottle containing 1 ml of toluene and 5 ml of water. A 10-μl volume of the urine, 50 μl of a standard CMC solution (5.0 μmol/ml), 0.5 ml of 0.1 *M* sodium hydrogencarbonate buffer (pH 9.0), 50 μl of acetone and 1.0 ml of 0.45 m*M* dabsyl chloride solution in acetone were mixed in a test-tube and processed as described above for the dabsylation of hypotaurine. When the volume of the standard CMC solution was increased, acetone was added to give a 65% concentration.

When urine samples contain large amounts of amino acids other than taurine which may interfere with the HPLC determination of dabsyltaurine, taurine was determined after collection by means of a cation-exchange resin column as follows. A 1-ml volume of a 1:20 diluted urine sample was applied to a column of Dowex 50W (2.0 ml, 5.5×0.7 cm I.D., H^+ form) and the column was washed with 8 ml of water. All the effluent was collected and evaporated to dryness with a flash evaporator at 40°C. To the residue , an aliquot of the standard CMC solution was added and dabsylation was performed as above. Hypotaurine was not determined by this procedure because it was retained on the Dowex 50W column.

3. Results and discussion

The dabsylation reaction is routinely performed by heating amino acid with dabsyl chloride at pH 9 and 70°C for 10 min [17]. Under these conditions, part of hypotaurine was converted into taurine and dabsyltaurine was detected by HPLC. When 50 nmol of hypotaurine was dabsylated under the above conditions, the dabsyltaurine formed constituted 20–50% of the sum of the dabsylhypotaurine and dabsyltaurine. We found that dabsylhypotaurine, once formed, was very stable and it was not converted into dabsyltaurine on storage at room temperature for several months or on flash evaporation at 40°C. Therefore, the formation of dabsyltaurine seemed to be due to the oxidation of non-dabsylated hypotaurine during the dabsylation reaction. Therefore, in the present study, milder conditions of dabsylation reaction were investigated and it was found that the oxidation of hypotaurine was minimized when dabsylation was performed at 40°C in 65% acetone solution.

Fig. 1 shows the effect of the amount of dabsyl chloride on the formation of dabsylhypotaurine and dabsyl-CMC under the present conditions. The amounts of dabsylhypotaurine and dabsyl-CMC formed were proportional to the dabsyl chloride added and the ratio of dabsylhypotaurine to dabsyl-CMC was constant when the dabsyl chloride concentration was less than 0.3 m*M*. Fig. 1 also shows that an increase in dabsyl chloride above 0.3 m*M* resulted in an increase in

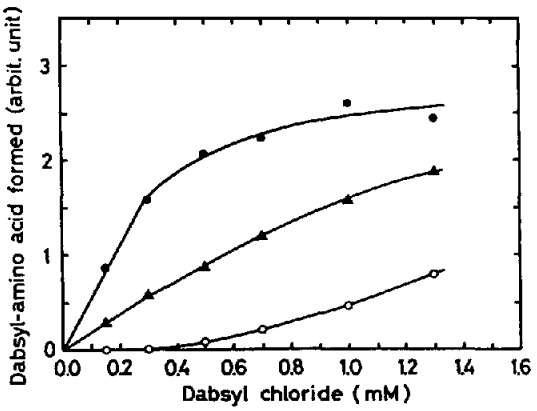

Fig. 1. Effect of dabsyl chloride concentration on the formation of (●) dabsylhypotaurine and (▲) dabsyl-CMC. Hypotaurine (50 nmol) or CMC was incubated in 3 ml of 65% acetone containing 33 m*M* sodium hydrogencarbonate buffer (pH 9) and various concentrations of dabsyl chloride at 40°C for 30 min. The reaction products were analysed by HPLC as described under Experimental. Dabsyltaurine (○) was formed in the presence of dabsyl chloride at concentrations >0.3 m*M*. Abbreviations: dabsyl, 4-dimethylaminoazobenzene-4'-sulfonyl; CMC, S-carboxymethyl-L-cysteine.

the formation of dabsyltaurine, i.e., hypotaurine was partly oxidized to taurine during the dabsylation reaction.

Calibration graphs for dabsylhypotaurine and dabsyltaurine were prepared under the present conditions. The peak-area ratios (x_H and x_T, respectively) of dabsylhypotaurine and dabsyltaurine to that of dabsyl-CMC were linearly proportional to the molar ratios (y_H and y_T, respectively) of the corresponding amino acids when the molar ratio was less than 3. The regression lines were $y_H = 0.317x_H + 0.023$ for dabsylhypotaurine and $y_T = 0.343x_T + 0.005$ for dabsyltaurine, and the correlation coefficients were both 1.000. As the intercepts are considered to be close to zero, the concentrations of hypotaurine (H) and taurine (T) (μmol/sample solution used) were calculated by the following equations: $H = 0.317x_H S$ and $T = 0.343x_T S$, where S is the amount of standard CMC (μmol) added.

The detection limit of both dabsylhypotaurine and dabsyltaurine under the present HPLC conditions was 4.0 pmol at a signal-to-noise ratio of 3. Therefore, the original sample solutions should contain more than ca. 50 pmol/ml of hypotaurine or taurine.

It is well known that hypotaurine is oxidized to taurine by hydrogen peroxide and this has often been used for the detection and determination of hypotaurine [2,5]. We attempted the quantitative oxidation of hypotaurine to taurine at various concentrations of hydrogen peroxide in order to determine hypotaurine. At 0.02% hydrogen peroxide, hypotaurine seemed to be completely oxidized, but the recovery of taurine was not quantitative. However, as shown in Fig. 2, we found that dabsylhypotaurine was, in contrast to free hypotaurine, quantitatively converted into dabsyltaurine with 0.2% hydrogen peroxide. The reaction proceeded at room temperature, as shown. When 20 nmol/ml of dabsylhypotaurine were incubated with 0.2% hydrogen peroxide in 60% ethanol, the dabsylhypotaurine was quantitatively converted into dabsyltaurine in 8 and 2 h at 25 and 40°C, respectively. Hence the stability of dabsylhypotaurine and its conversion into dabsyltaurine by hydrogen peroxide are useful for the identification and determination of small amounts of hypotaurine in the biological samples.

Fig. 3 is a typical chromatogram of dabsyl derivatives of authentic amino acids eluted near

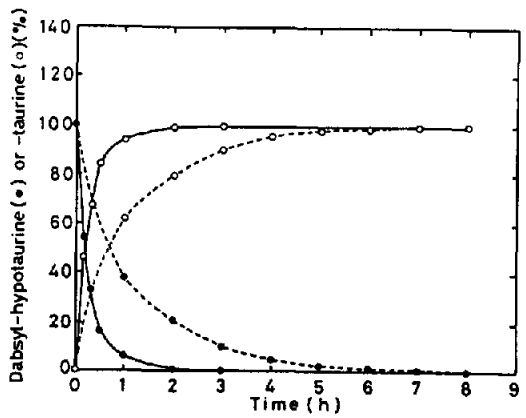

Fig. 2. Oxidation of (●) dabsylhypotaurine to (○) dabsyltaurine by 0.2% hydrogen peroxide at (solid lines) 40°C and (dashed lines) 25°C. Approximately 20 nmol/ml of dabsylhypotaurine in 60% ethanol containing 0.2% hydrogen peroxide was incubated at 25 or 40°C. After various time intervals, 10 μl of the reaction mixture were withdrawn and analysed by HPLC.

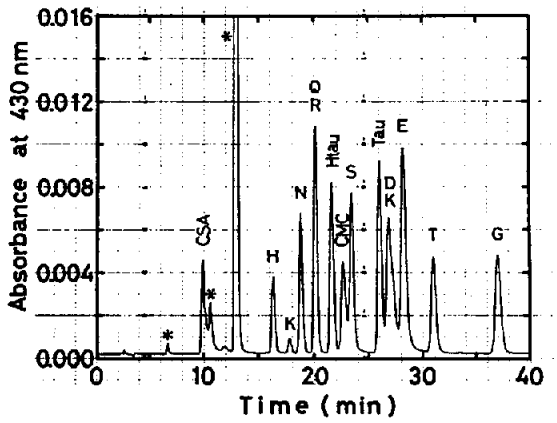

Fig. 3. Chromatogram of dabsyl derivatives of authentic amino acids. A 10-μl volume of a mixture of authentic dabsylamino acids (ca. 0.1–0.2 nmol each) was analysed by HPLC at 16°C. For detailed chromatographic conditions, see Experimental. Abbreviations: CSA, L-cysteinesulfinic acid; CMC, S-carboxymethyl-L-cysteine; Htau, hypotaurine; Tau, taurine; single letters, standard symbols for amino acids. Asterisks indicate peaks derived from excess reagent.

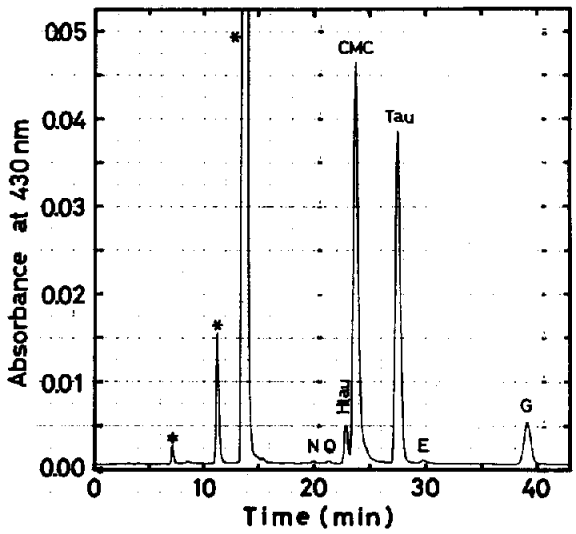

Fig. 4. Chromatogram of dabsylated urine of a rat injected with L-cysteine. A 10-μl volume of rat urine was dabsylated and analysed by HPLC under the conditions described under Experimental. For abbreviations, see Figs. 1 and 3.

dabsylhypotaurine and dabsyltaurine under the present HPLC conditions. The retention times of dabsylhypotaurine and dabsyltaurine were found to be very sensitive to column temperature. Therefore, chromatography of these compounds should be performed at a constant temperature. In this study, chromatography was performed at 16°C, at which the optimum separation was obtained. When chromatography was performed at 30°C, dabsylhypotaurine and dabsyltaurine

Table 1
Taurine and hypotaurine excretion in the urine of rats injected with L-cysteine

Rat No.	Body[a] mass (g)	Excretion[b] (μmol/kg body mass per day)					
		Day 1[c]		Day 2[c]		Day 3[c]	
		Tau	Htau	Tau	Htau	Tau	Htau
1	256	286.0	4.2	1690.2	149.2	544.3	2.2
2	256	268.3	3.2	1978.4	237.6	746.8	5.7
3	263	206.6	1.7	947.8	21.6	485.9	3.8
4	264	333.5	5.1	2375.9	268.7	809.8	9.7
5	284	239.0	0.1	1018.5	85.9	437.4	2.0
6	292	162.2	1.6	719.5	59.8	233.1	2.5
7	296	336.0	3.4	1016.0	85.4	351.5	4.2
8	296	332.1	0.3	1263.1	105.1	444.0	1.0
Mean ± S.D.		270.5 ±64.5	2.5 ±1.8	1376.2 ±578.9	126.7 ±86.5	506.6 ±192.4	3.9 ±2.8

[a] Body mass at the start of the experiment.
[b] Taurine (Tau) and hypotaurine (Htau) in 24-h urine were determined by HPLC after dabsylation. For details, see Experimental.
[c] Days 1 and 3, no injection; day 2, 5 mmol/kg body mass L-cysteine were injected intraperitoneally.

were eluted after dabsyl-CMC and dabsylaspartate, respectively.

Fig. 4 shows a chromatogram of dabsylated urine of rat injected with L-cysteine. The main amino acids present in the urine were taurine, hypotaurine and glycine. In normal rat urine, the contents of amino acids other than taurine and glycine were usually very low.

Table 1 shows the excretion of hypotaurine and taurine in the urine of rats injected with L-cysteine. The average excretion of taurine in rats before cysteine administration was 270.5 ± 60.3 μmol/kg body mass per day and that of hypotaurine was 2.5 ± 1.7 μmol/kg per day. After the intraperitoneal administration of 5 mmol/kg body mass of L-cysteine, the taurine excretion increased to 1376.2 ± 541.5 μmol/kg per day. Moreover, 126.7 ± 80.9 μmol/kg of hypotaurine per day were excreted. Therefore, the increased excretion of taurine after administration of cysteine was 1105.7 and that of hypotaurine was 124.2 μmol/kg per day. Hence, the increased excretion of taurine plus hypotaurine was 1229.9 μmol/kg per day, which corresponded to 24.6% of the L-cysteine administered. It is noteworthy that hypotaurine constituted 10.3% of the sum of the increased hypotaurine and taurine. Therefore, the present results seem to indicate that the formation of hypotaurine exceeded the capacity of hypotaurine oxidation to taurine in rat tissues in vivo, and that the present method is useful for the study of hypotaurine–taurine status in vivo.

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