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High-performance liquid chromatographic determination of taurine and hypotaurine using 3,5-dinitrobenzoyl chloride as derivatizing reagent

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Abstract

A method for the determination of taurine and hypotaurine in biological samples involving the preparation of their 3,5-dinitrobenzoyl derivatives followed by HPLC was established. Taurine and hypotaurine in aqueous media were reacted with 3,5-dinitrobenzoyl chloride in the presence of triethylamine to prepare 3,5-dinitrobenzoyl derivatives. These derivatives were separated on a C_{18} reversed-phase column and detected by recording the absorbance at 254 nm. Derivatives of taurine and hypotaurine were obtained in yields of 91.4 ± 3.3 and $85.6 \pm 2.6\%$, respectively. The calibration graphs for taurine and hypotaurine were linear between 2.5 and 500 μM with correlation coefficients of 0.999. The method was applied to the determination of taurine and hypotaurine in human and rat urine and blood and in rat liver and heart.

1. Introduction

Taurine and its precursor hypotaurine are metabolites of cysteine. It has been reported that taurine acts as a bile conjugator, a neuromodulator in the nervous system and an antioxidant, and that hypotaurine acts as a superoxide scavenger and a sperm mobility factor [1,2]. Many methods for the determination of taurine have been developed, including ion-exchange chromatography [3–5], gas chromatography [6,7], high-performance liquid chromatography (HPLC) [8–14] and electrophoresis [15,16]. However, some of these methods are time-consuming for the analysis of biological samples or technically difficult because of instability of the prepared derivatives. Moreover, most of these methods could not be applied to the simultaneous determination of taurine and hypotaurine. To study the physiological roles of these compounds, a more rapid and simpler method for the determination of taurine and hypotaurine in biological samples is necessary.

In this paper, we report an HPLC method for the determination of taurine and hypotaurine using 3,5-dinitrobenzoyl chloride as a derivatizing reagent.

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2. Experimental

2.1. Chemicals

Taurine and hypotaurine were purchased from Sigma (St. Louis, MO, USA). 3,5-Dinitrobenzoyl chloride and all other chemicals of analytical-reagent grade were purchased from Wako (Osaka, Japan).

2.2. Instruments and operating conditions

A Tosoh (Tokyo, Japan) HPLC system equipped with a UV detector was used. Separations were performed using a TSK gel ODS-80Ts C_{18} reversed-phase column (15 × 0.46 cm I.D.) (Tosoh).

The operating conditions for determination were as follows: the mobile phase was 16% acetonitrile in 100 m*M* ammonium acetate buffer (pH 3.7), the flow-rate was 0.8 ml min^{-1} and the UV absorbance at 254 nm was measured with a sensitivity of 0.01 AUFS.

2.3. Preparation of N-(3,5-dinitrobenzoyl)taurine

To 250 mg of taurine (2 mmol) in 15 ml of water, 2 ml of triethylamine and 2.3 g of 3,5-dinitrobenzoyl chloride (10 mmol) were added. The mixture was stirred at room temperature for 20 min, then 4 ml of 2 *M* hydrochloric acid were added and the reaction mixture was centrifuged at 1200 *g* for 10 min. The precipitate was washed twice with 5 ml of 0.01 *M* hydrochloric acid. The supernatant and washings were combined and the solvent was evaporated at 40°C under reduced pressure. The resultant residue was then dissolved in 3 ml of water and the suspension was filtered. The filtrate was applied to a Dowex 50W-X8 column (H^+ form, 35 × 2 cm I.D.) and eluted with water. The eluate between 30 and 40 ml was collected and neutralized with 2 *M* ammonia solution. After evaporation of the solvent, 470 mg of powder was obtained (ammonium salt, yield = 70%), m.p. 254–262°C (decomp.). Results of elemental analysis were as follows: calculated for $C_9H_{12}N_4O_8S \cdot 1/3H_2O$, C 31.58, H 3.70, N 16.37; found, C 31.85, H 3.64, N 15.93%. Spectral data were as follows: UV (H_2O), λ_{max} (ε, mM^{-1} cm^{-1} in parentheses) 207 (17.3), 235 (14.5) nm; IR (KBr disk), 1665 (CONH), 1550 (NO_2), 1350 (NO_2), 1220 (SO_3), 1030 (SO_3) cm^{-1}; fast atom bombardment mass spectrometry (FAB-MS, glycerol as a matrix), *m/z* (relative intensity, %) 110 ($C_5H_4NO_2$, 100), 131 (7), 149 (3), 195 ($C_7H_3N_2O_5$, 3), 202 (5), 303 (3), 304 (3), 320 (MH^+-NH_3, 3), 321 (4), 337 (MH^+, 10), 338 (5); high-resolution MS, calculated for $C_9H_{13}N_4O_8S$, M_r 337.0453; found, 337.0473.

2.4. Preparation of N-(3,5-dinitrobenzoyl)hypotaurine

Hypotaurine (2 mmol, 218 mg) was derivatized and the N-3,5-dinitrobenzoyl derivative was purified by the same procedure as described above. The eluate between 50 and 220 ml was collected and evaporated, yielding a crystalline solid (free acid, 383 mg, yield 63%), m.p. 128–132°C. UV (H_2O), λ_{max} (ε, mM^{-1} cm^{-1} in parentheses) 207 (19.2), 230 (15.4) nm; FAB-MS of the ammonium salt (glycerol) *m/z* (%) 110 (100), 115 (11), 304 ($MH^+ - NH_3$, 23), 305 (12), 321 (MH^+, 15), 322 (5).

2.5. Method for determination of taurine and hypotaurine

Derivatization procedure

A 1-ml volume of aqueous solution containing 2.5–500 nmol of taurine or hypotaurine was placed in a test-tube with a PTFE-lined screw-cap and 10 μl of triethylamine and 20 mg of freshly powdered 3,5-dinitrobenzoyl chloride were added. The mixture was shaken vigorously using a mechanical shaker for 10 min, then 0.1 ml of 2 *M* hydrochloric acid was added and the reaction mixture was centrifuged at 1200 *g* for 5 min. A 0.2-ml aliquot of the supernatant was diluted to 5 ml with water and this diluted solution (10–100 μl) was analysed by HPLC.

Oxidation of N-(3,5-dinitrobenzoyl)hypotaurine to N-(3,5-dinitrobenzoyl)taurine

To 1 ml of derivatized solution containing less than 20 nmol of N-(3,5-dinitrobenzoyl)hypotaurine, 0.1 ml of 5 m*M* *m*-chloroperbenzoic acid in water was added. After 30 min, the reaction mixture was analysed by HPLC.

Sample preparation

Urine from both humans and rats was adjusted to pH 7 by adding 2 *M* sodium hydroxide or hydrochloric acid, and then diluted 5 and 100 fold with water, respectively. The diluted urine was derivatized as described above.

Blood was deproteinized by the Folin–Wu method [17]. After centrifugation at 1200 *g* for 10 min, the supernatant was used for derivatization.

Male Wistar rats were killed by decapitation and the liver and heart were immediately removed. Tissues were homogenized with 7 volumes of water, 1 volume of 10% sodium tungstate and 1 volume of 0.33 *M* sulfuric acid in a Potter–Elvehjem homogenizer with a glass pestle, and centrifuged at 1200 *g* for 10 min. The supernatants from the liver and heart homogenates were diluted 2- and 5-fold with water, respectively, and these diluted solutions were used for derivatization.

3. Results and discussion

3,5-Dinitrobenzoyl chloride is a useful reagent for the derivatization of alcohols and amines, yielding strongly UV-absorbing products [18]. In general, the reaction of amino compounds with 3,5-dinitrobenzoyl chloride yields N-3,5-dinitrobenzoyl derivatives. The reaction (Schotten–Baumann reaction) is usually carried out by addition of 3,5-dinitrobenzoyl chloride in an organic solvent to amino compounds in aqueous alkaline solution [19,20]. However, for taurine in aqueous solution, the yield of the N-3,5-dinitrobenzoyl derivative obtained by this method was insufficient for accurate determination (about 10%). We therefore attempted to obtain a better yield in the following manner. An aqueous solution of 2.5–500 μM taurine was treated with freshly powdered 3,5-dinitrobenzoyl chloride in the presence of triethylamine and the amount of 3,5-dinitrobenzoyl derivative formed was determined by reversed-phase HPLC (Fig. 1A). N-(3,5-Dinitrobenzoyl)taurine was prepared in good yield by this method (91.4 ± 3.3%). For hypotaurine in aqueous solution, N-(3,5-dinitrobenzoyl)hypotaurine was prepared in a good yield of 85.6 ± 2.6% by the same procedure, and a small amount of by-product, N-(3,5-dinitrobenzoyl)taurine, was observed in a yield of 2.6 ± 1.1% (Fig. 1B). These N-3,5-dinitrobenzoyl derivatives were stable enough for the present analysis. About 5% of the hypotaurine derivative was oxidized by oxygen in the air to give N-(3,5-dinitrobenzoyl)taurine when the derivatized sample was left at room temperature for 2 days.

Calibration graphs for taurine and hypotaurine in aqueous solution obtained by the present method were linear between 2.5 and 500 μM with correlation coefficients of 0.999. The within-assay coefficients of variation determined at 2.5–500 μM taurine and hypotaurine were 2.8–4.8 and 1.8–4.7%, respectively. The detection limit for both compounds was 0.5 μM at a signal-to-noise ratio of 3 when 100 μl of derivatized solution were subjected to HPLC.

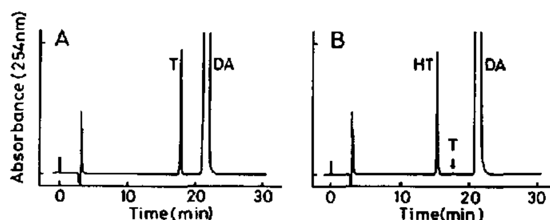

Fig. 1. Chromatograms for the determination of taurine and hypotaurine in aqueous solution. Taurine or hypotaurine (500 nmol ml^{-1}) in aqueous solution was converted into the N-3,5-dinitrobenzoyl derivative by reaction with 3,5-dinitrobenzoyl chloride in the presence of a triethylamine. Derivatized samples (10 μl) were separated on a C_{18} reversed-phase column and the absorbance at 254 nm was recorded at 0.01 AUFS. Details are given under Experimental. (A) Taurine; (B) hypotaurine. Peaks: HT = N-(3,5-dinitrobenzoyl)hypotaurine; T = N-(3,5-dinitrobenzoyl)taurine; DA = 3,5-dinitrobenzoic acid.

It should be noted that, as amino compounds react with 3,5-dinitrobenzoyl chloride, a mixture of amino acids and ammonia would be simultaneously analysed by the present method (Fig. 2). Peaks corresponding to several amino acids and ammonia were detected on the chromatograms, but these peaks were well separated from those of taurine and hypotaurine derivatives under the conditions used. As 5 m*M* *m*-chloroperbenzoic acid solution was added to the derivatized solution containing N-(3,5-dinitrobenzoyl)hypotaurine, the hypotaurine derivative was quantitatively oxidized to give N-(3,5-dinitrobenzoyl)-taurine. The peak corresponding to hypotaurine on the chromatograms was easily distinguished from other peaks. The 3,5-dinitrobenzoyl derivative of cysteinesulfinic acid was also converted into the cysteic acid derivative by this treatment (data not shown).

To check the accuracy of this method, we determined taurine in rat urine by both the present method and an ion-exchange chromatographic technique [3], consisting of separation with combined ion-exchange resin columns and ninhydrin reaction (Table 1). The values (x) obtained by the present method agreed well with those (y) obtained by the ion-exchange chromatographic technique, the regression line being

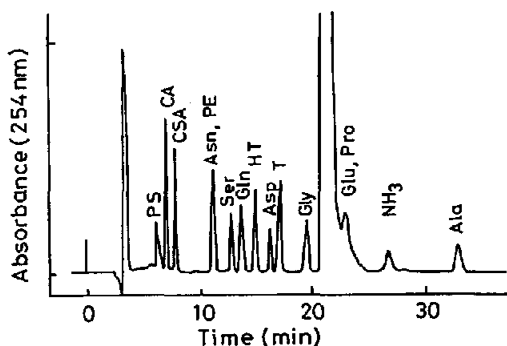

Fig. 2. Chromatogram of N-3,5-dinitrobenzoyl derivatives of amino acids and ammonia. A 1-ml aliquot of aqueous solution containing 200 nmol of amino acids and ammonia was analysed as described in Fig. 1. Abbreviations indicate the N-3,5-dinitrobenzoyl derivatives of the following compounds: PS = phosphoserine; CA = cysteic acid; CSA = cysteinesulfinic acid; PE = phosphorylethanolamine; Asn, Ser, Gln, Asp, Gly, Glu, Pro and Ala = amino acids; NH_3 = ammonia; HT = hypotaurine; T = taurine.

Table 1
Comparison between urinary taurine determined by HPLC and ion-exchange chromatographic methods[a]

Sample No.	Taurine determined (μmol ml^{-1} rat urine)	
	HPLC method[b] (x)	Ion-exchange chromatographic method[c] (y)
1	5.02 ± 0.20	5.23 ± 0.10
2	5.95 ± 0.30	6.16 ± 0.16
3	6.02 ± 0.30	6.35 ± 0.15
4	4.51 ± 0.13	4.71 ± 0.10
5	1.90 ± 0.08	2.00 ± 0.25
6	3.22 ± 0.10	3.19 ± 0.18

[a] Values are expressed as mean ± standard deviation of three determinations.
[b] Taurine was converted into N-(3,5-dinitrobenzoyl)taurine and the derivative was determined by HPLC.
[c] Taurine was separated by combined ion-exchange columns and determined by nihydrin reaction.

$y = 1.06x - 0.09$ and the correlation coefficient 0.999.

We applied the present method to the determination of taurine and hypotaurine in human and rat urine and blood and in rat liver and heart. Fig. 3 shows representative HPLC traces for human urine, blood and rat liver.

The recoveries of taurine (25–250 nmol) added to 1 ml of human urine and blood were 98.6 ± 1.1 and 102.0 ± 2.9%, respectively, and that of taurine (0.25–2.5 μmol) added to 1 g of rat liver was 99.8 ± 3.5%. The content of taurine in rat urine and blood was found to be 315.3 ± 128.6 μmol kg^{-1} body mass in 1 day ($n = 18$) and 395 ± 45 nmol ml^{-1} ($n = 3$), respectively. The taurine content in urine and blood from adult men was 0.92 ± 0.33 μmol mg^{-1} creatinine ($n = 8$) and 159 ± 19 nmol ml^{-1} ($n = 4$), respectively. The contents of taurine and hypotaurine in rat liver were 5.39 ± 4.67 and 0.18 ± 0.08 μmol g^{-1} wet mass (n = 4) and those in rat heart were 22.0 ± 2.2 and 0.23 ± 0.02 μmol g^{-1} (n = 4), respectively. These values are comparable to those reported previously [7–9,21].

Hence the proposed method, consisting of preparation of 3,5-dinitrobenzoyl derivatives and

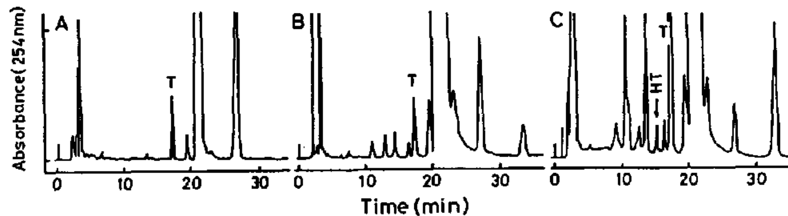

Fig. 3. Representative chromatograms for the determination of taurine and hypotaurine in human urine, blood and rat liver. Samples were deproteinized by the Folin–Wu method [17]. After centrifugation, the supernatants were analysed as described in Fig. 1. (A) Human urine; (B) = human blood; (C) rat liver. Peaks: HT = hypotaurine derivative; T = taurine derivative.

subsequent HPLC analysis, allows the simple and rapid determination of taurine and hypotaurine in biological samples.

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