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Note

Gas chromatographic determination of hypotaurine

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Hypotaurine (2-aminoethanesulphinic acid) has been reported to occur in rat brain [1], reproductive organs of the guinea pig [2, 3] and certain molluscs [4, 5], and also to be excreted in the urine of rats fed cysteine or cystine [6]. It is an important intermediate in the metabolism of sulphur-containing amino acids and is considered to be a precursor of taurine [7].

The determination of hypotaurine has been achieved by using a spectrophotometric method [8] and an amino acid analyser [1, 3, 5]. However, these methods lack the sensitivity required for determining hypotaurine contents in tissue samples and require time-consuming pre-treatment of the sample. Recently, high-performance liquid chromatographic [9, 10] and enzymatic [11] methods have been reported. Both methods appear to be sufficiently sensitive but their applicability to biological materials has not been well established.

Some years ago, we developed a specific and reliable method for the determination of taurine by gas chromatography (GC), in which taurine was analysed as its N-isobutoxycarbonyl (N-isoBOC) dibutylamide derivative, and we demonstrated that biological materials such as urine [12], blood [13] and tissues [14] could be analysed accurately and precisely without prior clean-up of the sample.

The aim of this paper is to describe the logical extension of this work to the analysis of hypotaurine.

EXPERIMENTAL

Reagents

All chemicals were of analytical-reagent grade. Taurine and hypotaurine were

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purchased from Nakarai (Kyoto, Japan) and Sigma (St. Louis, MO, U.S.A.), respectively. Isobutyl chloroformate (isoBCF) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Cetyldimethylbenzylammonium chloride (CDMBA-Cl) (Tokyo Kasei Kogyo) was dissolved in methanol at a concentration of 10%. Thionyl chloride and di-*n*-butylamine (DBA) were purchased from Nakarai and used after distillation. N-*n*-Butoxycarbonyl (N-*n*-BOC) hypotaurine, used as an internal standard (I.S.), was prepared as follows: 50 mg (0.46 mmol) of hypotaurine were dissolved in 5 ml of 0.2 *M* sodium hydroxide solution and to this solution was added 1 ml (7.3 mmol) of *n*-butyl chloroformate (Tokyo Kasei Kogyo). The mixture was extracted three times with 10 ml of diethyl ether in order to remove the excess of reagent, and the ethereal extracts were discarded. The aqueous layer was saturated with sodium chloride, acidified to pH 1 with 2 *M* hydrochloric acid and then extracted three times with 10 ml of diethyl ether. The combined ethereal extracts were evaporated at 50°C and the residue was reconstituted in water to prepare a 0.25 m*M* solution.

Derivatization procedure

Hypotaurine was converted into its volatile derivative, 2-(isobutoxycarbonylamino)-N,N-dibutylethanesulphonamide (N-isoBOC dibutylamide derivative of taurine), by the following procedure. An aliquot of the sample solution (containing 5—100 nmol of hypotaurine) was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.2 ml of 0.25 m*M* N-*n*-BOC hypotaurine, the solution was adjusted to pH 10 with 2 *M* sodium hydroxide solution and the total reaction volume was made up to 1.5 ml with distilled water if necessary. Immediately after addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 5 min at room temperature. The reaction mixture was washed with 3 ml of diethyl ether in order to remove the excess of reagent, and the ethereal extract was discarded. The aqueous layer was saturated with sodium chloride, acidified to pH 1 with 2 *M* hydrochloric acid and then extracted twice with 3 ml of diethyl ether. The combined ethereal extracts were washed with 1 ml of 0.05 *M* hydrochloric acid saturated with sodium chloride and then evaporated to dryness at 50°C. To the residue were added 0.5 ml of 0.1 *M* citrate buffer (pH 2) and 0.5 ml of 0.5 m*M* methylene blue. The mixture was irradiated with a 300-W tungsten lamp (Toshiba, Tokyo, Japan) for 5 min from a distance of 20 cm. The reaction mixture was washed twice with 3 ml of diethyl ether. Subsequently, 0.1 ml of 10% CDMBA-Cl solution and 2 ml of methylene chloride were added to the aqueous layer and the tube was shaken for 3 min at room temperature. After centrifugation at 2000 *g* for 1 min, the organic layer was transferred into another tube and the solvent was evaporated to dryness under a stream of nitrogen. The residue was treated as described previously [12]. The procedure mentioned above is summarized in Fig. 1.

Preparation of the reference compound

A reference sample of 2-(isobutoxycarbonylamino)-N,N-dibutylethanesulphonamide was prepared as described previously [12].

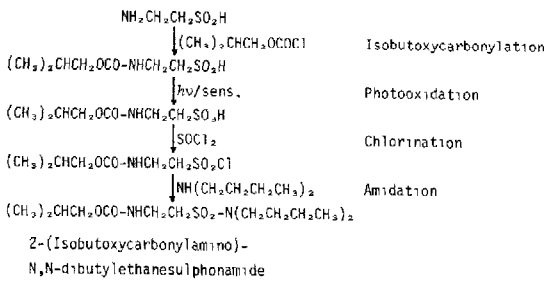

Fig. 1 Procedure for derivatization of hypotaurine.

Preparation of tissue samples

The following species were used in the experiment; three crabs* (*Portunus trituberculatus*), three clams* (*Meretrix lusoria*), six octopuses** (*Octopus vulgaris*), six mackerels* (*Scomber japonicus*), three carps** (*Cyprinus carpio*), nine frogs** (*Rana nigromaculata*), three tortoises** (*Geoclemys reevesii*), five chickens**, four Wistar rats** and two dogs**. Immediately after dissection each organ was removed and frozen. These frozen tissues were stored at −20°C in a deep-freezer until each assay. Each tissue was homogenized with 5—10 vols. of deproteinizing reagent (containing 0.033 *M* sulphuric acid and 1% of sodium tungstate) using a Model LK-21 ultra-disperser (Yamato Kagaku, Tokyo, Japan). After centrifugation at 2000 *g* for 5 min, the precipitate was re-extracted with the same volume of deproteinizing reagent. The supernatants were combined and made up to a constant volume with distilled water. A 1-ml portion of this solution was used for the analysis.

Gas chromatography

A Shimadzu 4 CM-PF gas chromatograph equipped with a hydrogen flame ionization detector and a 1.0 m × 3 mm I.D. glass column packed with 5% SE-54 on Uniport HP (100—120 mesh) was used. The column packing was prepared by the "solution coating" technique [15]. The column was conditioned with a nitrogen flow-rate of 30 ml/min at 290°C for 24 h. The operating conditions were as follows: column temperature, 230°C; injection and detector temperature, 285°C; nitrogen flow-rate, 45 ml/min. Peak heights for hypotaurine and the I.S. were measured and the peak-height ratios were calculated for the construction of calibration graph.

Mass spectrometry

A Shimadzu-LKB 9000 gas chromatograph—mass spectrometer with the same type of column as used for GC analysis was employed under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 250°C; separation temperature, 240°C; helium flow-rate, 38 ml/min.

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*Fresh marine products obtained from a fish market; for these species, there was some delay between dissection and death of the animal.

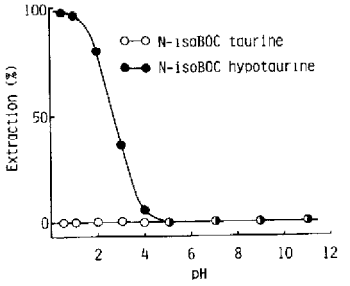
**Live species.

RESULTS AND DISCUSSION

Hypotaurine was analysed as the N-isoBOC dibutylamide derivative of taurine. In the first step of the derivatization, the amino function of hypotaurine was isobutoxycarbonylated with isoBCF in aqueous alkaline media, and the resulting N-isoBOC hypotaurine was extracted with diethyl ether. As shown in Fig. 2, N-isoBOC hypotaurine was quantitatively extracted with diethyl ether at pH 1, while N-isoBOC taurine remained completely in the water layer at this pH. This means that taurine, if present in the sample, can be removed at this stage of derivatization. The sulphinic acid function of the N-isoBOC hypotaurine was then oxidized to sulphonic acid by the photochemical procedure using methylene blue as a sensitizer. The photo-oxidation reaction proceeded rapidly and quantitatively under the conditions described under Experimental. The N-isoBOC taurine thus obtained was converted into the final derivative by essentially the same procedure as that used in the derivatization of taurine [12]. The mean derivatization yield throughout the procedure was determined to be 87% by comparison with the synthetic reference compound.

Fig. 3A shows a chromatogram obtained from a standard solution of hypotaurine. The peak represents about 0.5 nmol of hypotaurine. Taurine gave no peak when it was derivatized and analysed by this method (Fig. 3B). The calibration graph for hypotaurine in the range 5—100 nmol (the sample size injected into the GC system accordingly ranged from 0.1 to 2 nmol) was constructed by using N-*n*-BOC hypotaurine as the I.S. A linear relationship was obtained and the regression line was $y = 0.020x - 0.022$ ($r = 0.9998$, $n = 25$), where y = peak-height ratio and x = amount of hypotaurine.

The method developed was successfully applied to biological materials. The chromatogram in Fig. 3C shows the GC analysis of hypotaurine in octopus foot tissue. The recovery of hypotaurine added to tissue samples averaged 102% and the relative standard deviation was 0.2—4.7% ($n = 3$), indicating that this method is accurate and precise. The hypotaurine content of the various tissues examined is given in Table I.



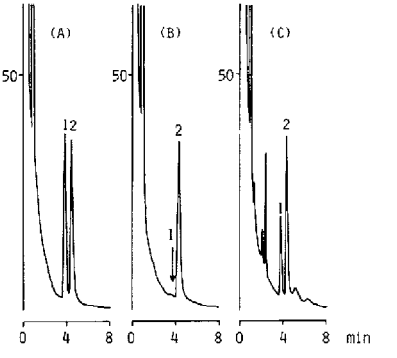


Fig. 2. Effect of pH on the diethyl ether extraction of N-isoBOC taurine (100 nmol) and N-isoBOC hypotaurine (100 nmol).

Fig. 3. Gas chromatograms obtained from (A) hypotaurine standard solution, (B) taurine standard solution and (C) octopus foot tissue GC conditions are given in Experimental. Peaks: 1 = hypotaurine; 2 = N-*n*-BOC hypotaurine (internal standard).

TABLE I

HYPOTAURINE CONTENT IN VARIOUS TISSUES

Each value represents an average of three experiments.

Tissue	Hypotaurine content (nmol/g wet weight)									
	Crab	Clam	Octopus	Mackerel	Carp	Frog	Tortoise	Chicken	Rat	Dog
Brain	—	—	—	55 2	84.2	97 7	—	25.4	42.8	42 7
Eye	—	—	7216	37 9	67.4	28 1	—	78.3	423.3	54 2
Heart	208.8	—	—	168 8	34.0	77 0	41 3	318.7	228 9	84 2
Lung	—	—	—	—	—	66 6	36 9	70.3	489.4	21 4
Gill	105 4	604 5	5018	39.2	34.4	—	—	—	—	—
Liver	273.3	—	7126	169 4	92 3	21 6	61.9	81 9	56.6	116.1
Spleen	—	—	—	339.9	41 3	43 9	139.7	550 1	286 1	22 3
Pancreas	—	—	2184	—	—	147.4	58.5	63.0	167 6	14 4
Kidney	—	—	—	120.1	152 0	43.6	61.3	—	227 0	79.7
Stomach	—	—	2005	113.4	—	—	—	79.7	115 6	16 6
Intestine	—	—	—	—	—	—	—	292 1	219 6	—
Caecum	—	—	4799	—	—	—	—	—	—	—
Ovary	235 7	—	28781	66.4	48 3	14 5	—	—	69 4	—
Testis	—	—	10583	—	—	—	—	—	50 0	—
Mantle	—	771 0	5187	—	—	—	—	—	—	—
Foot	425 3	1675 6	3961	—	—	—	—	—	—	—
Syphon	—	2243 3	—	—	—	—	—	—	—	—
Adductor muscle	—	1013 2	—	—	—	—	—	—	—	—
Skeletal muscle	—	—	—	41.2	47.3	28 5	188.6	298 4	72 9	—

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