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Abstract

A new volatile derivative of taurine, N-isobutoxycarbonyltaurine methyl ester (methyl 2-(N-isobutoxycarbonylamino)ethanesulfonate), was prepared by a three-step procedure for the gas chromatographic determination of taurine in urine. First, taurine was converted to its silver salt by reaction with silver oxide; next the silver salt was reacted with isobutyl chloroformate to form the N-isobutoxycarbonyl derivative, and finally the derivative was reacted with methyl iodide to form N-isobutoxycarbonyltaurine methyl ester. The volatile derivative was analyzed by gas chromatography using a column of 3% OV-101 on Chromosorb W. When methyl 3-(N-isobutoxycarbonylamino) propanesulfonate was used as an internal standard, the calibration curve was linear between 0.5 and 5.0 μmol of taurine/ml and showed a good reproducibility. This method was applied to the determination of taurine in human urine. Recovery was 98.6 $\pm$ 5.2%, when 1.25 to 5.0 μmol /ml of taurine was added to human urine.

KEYWORDS: taurine, gas chromatography, taurine determination, methyl 2-(N-isobutoxycarbonylamino) ethanesulfoate, taurine excretion

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Preparation of a Volatile Derivative of Taurine and Application to Gas Chromatographic Determination of Urinary Taurine

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A new volatile derivative of taurine, N-isobutoxycarbonyltaurine methyl ester (methyl 2-(N-isobutoxycarbonylamino)ethanesulfonate), was prepared by a three-step procedure for the gas chromatographic determination of taurine in urine. First, taurine was converted to its silver salt by reaction with silver oxide; next the silver salt was reacted with isobutyl chloroformate to form the N-isobutoxycarbonyl derivative, and finally the derivative was reacted with methyl iodide to form N-isobutoxycarbonyltaurine methyl ester. The volatile derivative was analyzed by gas chromatography using a column of 3% OV-101 on Chromosorb W. When methyl 3-(N-isobutoxycarbonylamino)propanesulfonate was used as an internal standard, the calibration curve was linear between 0.5 and 5.0 μmol of taurine/ml and showed a good reproducibility. This method was applied to the determination of taurine in human urine. Recovery was $98.6 \pm 5.2\%$, when 1.25 to 5.0 $\mu\text{mol/ml}$ of taurine was added to human urine.

Key words : taurine, gas chromatography, taurine determination, methyl 2-(N-isobutoxycarbonylamino)ethanesulfonate, taurine excretion

Taurine (2-aminoethanesulfonic acid) is one of the end products of cysteine metabolism in mammals and excreted in the urine. It has been suggested that taurine plays important physiological roles in higher animals (1). Many methods have been developed for the determination of taurine, including colorimetric (2), fluorometric (3) and high performance liquid chromatographic (4) methods, and methods employing an amino acid analyzer (5).

Gas chromatographic methods have also been reported, despite the fact that it is difficult to convert taurine to a volatile compound. Shahrokhi and Gehrke reported a method to prepare trimethylsilyl derivatives for the analysis of sulfur-containing amino acids including taurine (6). However, the method has not been used for the analysis of biological materials because the trimethylsilyl derivative of taurine is unstable in water and the method results in the production of many volatile derivatives. Recently, Kataoka *et al.* reported the gas chromatographic analysis of N-isobutoxycarbonyl

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dibutylamide derivative of taurine for estimating a small amount of taurine (7). In our previous paper (8), we reported a procedure for the preparation of a dimethyl ester of sulfate, which could be applied to the gas chromatographic determination of sulfate. Based on this procedure, we developed a new method to form a volatile derivative of taurine for analysis by gas chromatography. The derivative, N-isobutoxycarbonyltaurine methyl ester, is formed in two derivatization reactions: reaction of taurine silver salt with isobutyl chloroformate and esterification with methyl iodide.

Materials and Methods

Reagents. Taurine, sodium 3-aminopropane-sulfonate and cysteic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals used were of analytical grade and were obtained from Wako Pure Chemical Ind., Ltd., Osaka, Japan.

Instruments and operating conditions. A Shimadzu gas chromatograph (4CM, Shimadzu Seisakusyo, Ltd., Kyoto, Japan) equipped with a hydrogen flame ionization detector was used. A column of 3% OV-101 on Chromosorb W (80-100 mesh) packed in a silanized glass tube (0.3 mm i.d. $\times$ 2 m) was used. The column was conditioned at 280°C for 24 h with nitrogen gas at a flow rate of 30 ml/min. The operating conditions were as follows: oven temperature, 195°C; injection temperature, 220°C; flow rate of nitrogen gas as the carrier gas, 40 ml/min.

A Shimadzu gas chromatograph-mass spectrometer (GC-MS) (Shimadzu 9020-FD, Shimadzu Seisakusyo) was used for the identification of derivatized compounds. The operating conditions of the gas chromatograph were the same as those described above except that helium gas was used as the carrier gas at a flow rate of 30 ml/min. Electron impact (EI) mass spectra were taken under the following conditions: trap current, 60 μ A; ionizing voltage, 70 eV; accelerating voltage, 3.0 kV; ion source temperature, 250°C. Chemical ionization (CI) mass spectra were obtained under following conditions: reacting gas, isobutane;

emission current, 200 μ A; ionizing voltage, 150 eV; accelerating voltage, 3.0 kV; ion source temperature, 250°C.

NMR spectra and infrared (IR) spectra were recorded with a Hitachi R-22 FTS FT-NMR spectrometer (Hitachi Ltd., Tokyo, Japan) and JASCO IRA-102 infrared spectrometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan), respectively.

Preparation of volatile derivatives.

Preparation of N-isobutoxycarbonyltaurine methyl ester from taurine. The scheme for the formation of the N-isobutoxycarbonyl methyl ester derivative of taurine is shown in Fig. 1. To 100 ml of an aqueous solution containing 6.12 mmol of taurine, 8 g of silver oxide was added, and the suspension was shaken for 1.5 h. Then, 40 ml of isobutyl chloroformate was added, and the suspension was stirred for 5 min. The reaction mixture was immediately washed twice with 100 ml of ethyl ether. The water layer was centrifuged at $1200 \times g$ for 10 min, and the resulting supernatant was separated. The precipitate was washed once with a small amount of water. The supernatant and washing were combined and evaporated under a reduced pressure at 40°C. To the residue, 50 ml of methyl iodide was added and refluxed for 2 h. The reaction mixture was cooled and filtered. The filtrate was evaporated under a reduced pressure at 40°C. The residue was applied to a silica gel column (Wakogel C-200, 2×13 cm) and eluted once with 100 ml of chloroform and again with 200 ml of chloroform containing 1% methanol. The eluate between 40 and 240 ml was collected and evaporated. The yield of crude N-isobutoxycarbonyltaurine methyl ester was 97% (5.93 mmol). It was recrystallized from *n*-hexane, mp 33.0-34.0°C. Elementary analyses were as follows: Calculated for $C_8H_{17}O_5SN$: C, 40.16; H, 7.16; N, 5.85. Found: C, 40.25; H, 7.36; N, 5.68. Spectral data were as follows. IR (Neat): 3380 (NH), 1710 (CONH) cm^{-1} . NMR ($CDCl_3$): δ 0.92 (d, $J=7$ Hz, 6H, 2CH₃), 1.93 (1H, CH), 3.35 (2H, CH₂SO₃), 3.67 (2H, CH₂N), 3.88 (d, $J=7$ Hz, 2H, CH₂O), 3.93 (s, 3H, OCH₃), 5.27 (bs, 1H, NH). EI: m/e (%) 184 (14), 166 (90), 143 (11), 140 (100), 134 (28), 108 (15). CI: m/e (%) 240 (MH⁺, 100), 184 (64).

Preparation of methyl 3-(N-isobutoxycarbonyl-amino)propanesulfonate from sodium 3-aminopropanesulfonate. The scheme for forming the N-

isobutoxycarbonyl methyl ester derivative of 3-aminopropanesulfonic acid is shown in Fig. 1. An aqueous solution (6 ml) of 5.90 mmol of 3-aminopropanesulfonate sodium salt was applied to a Dowex 50 W $\times$ 8 column (1.5 $\times$ 10 cm, H⁺ form) and eluted with water to remove sodium ion. The eluate between 10 and 50 ml was collected, and diluted to 100 ml with water. The solution was subjected to derivatization and purification by exactly the same procedure as described for taurine. The eluate between 40 and 250 ml from the silica gel column was collected and evaporated. The yield of crude methyl 3-(N-isobutoxycarbonylamino)propanesulfonate was 97% (5.72 mmol). It was recrystallized from *n*-hexane, mp 34.0–35.0°C. Elementary analyses were as follows: Calculated for C₉H₁₉O₅SN: C, 42.67; H, 7.56; N, 5.53. Found: C, 42.51; H, 7.66; N, 5.55. Spectral data were as follows. IR (Neat): 3350(NH), 1705(OCNH)cm⁻¹. NMR(CDCl₃): δ 0.92(d, J=7Hz, 6H, 2CH₃), 1.9(1H, CH), 2.1(2H, CH₂), 3.20(2H, CH₂SO₃), 3.36(2H, CH₂N), 3.87(d, J=7Hz, 2H, CH₂O), 3.92(s, 3H, OCH₃), 4.92(bs, 1H, NH). EI: m/e(%) 253(M⁺, 8), 198(6), 197(7), 180(48), 154(35), 152(42), 148(92), 137(52), 130(100), 125(32). CI: m/e(%) 254(MH⁺, 100), 198(38).

Taurine determination by the present method. When less than 10 μ mol of taurine was derivatized, the yield of N-isobutoxytaurine methyl ester was less than 90%. We found, however, that the yield improved when small amounts of dimethyl sulfate and silver oxide were added in the reaction mixture of N-isobutoxycarbonyltaurine silver salt with methyl iodide as described below. To determine the exact concentration of small amounts of taurine by gas chromatography, 0.5 ml of 5 mM methyl 3-(N-isobutoxycarbonylamino)propanesulfonate solution was added at the step before gas chromatographic analysis as the internal standard.

Taurine in aqueous solution. One ml of a sample solution containing 0.5–5.0 μ mol of taurine was applied to a Dowex 50 W $\times$ 8 column (0.7 $\times$ 8 cm, H⁺ form) and eluted with water. The initial 10 ml of effluent was collected. To 5 ml of the effluent, 0.05 g of silver oxide was added, and the mixture was shaken for 90 min. Then 30 μ l of isobutyl chloroformate was added, and the mixture was shaken again for 5 min. Immediately after this reaction, the mixture was extracted

twice with 5 ml of ethyl ether to remove excess isobutyl chloroformate. The aqueous layer was centrifuged at 1200 $\times g$ for 5 min to remove precipitated silver salts. The supernatant was separated and 3.5 ml of the solution was evaporated at 40°C under a reduced pressure. To the residue, 0.5 ml of methyl iodide, 20 μ l of dimethyl sulfate, 10 mg of silver oxide and 0.1 g of sea sand were added and shaken vigorously for 1 min. Then the mixture was incubated at 55°C for 30 min. The mixture was shaken again for 1 min and incubated at 55°C for additional 30 min. After cooling, 0.5 ml of freshly prepared 5 mM methyl 3-(N-isobutoxycarbonylamino)propanesulfonate solution in chloroform was added. Two μ l of the reaction mixture was analyzed by gas chromatography under the conditions described above (Fig. 3A). The uniformity of each peak was confirmed by GC-MS. The amount of taurine was determined by measuring the peak areas of the chromatogram.

Taurine in urine. Urinary taurine was determined as above except that the length of the Dowex 50 W $\times$ 8 column and the amount of silver oxide were changed. A longer column (0.7 $\times$ 20 cm) was used in order to eliminate hippuric acid, which is contained in urine, and was eluted after the initial 10 ml of effluent as shown in Fig. 2. The amount of silver oxide was increased to 0.2 g in order to convert other anions present in urine to their silver salts. A typical gas chromatogram obtained with human urine is shown in Fig. 3B. Peaks corresponding to the derivatives of taurine and the internal standard showed no peak-tailing and good separation. The identity of each peak was confirmed by comparing with the mass spectra of the authentic derivative (data not shown).

Determination of urinary taurine by ion-exchange chromatography and ninhydrin reaction. Urinary taurine was also determined by ninhydrin reaction after the separation of the taurine fraction with a combination of anion-exchange and cation-exchange columns (9, 10).

Creatinine concentration in the urine. The creatinine concentration was determined by the Jaffe reaction (11).

Results and Discussion

In the present study, taurine was con-

verted to N-isobutoxycarbonyltaurine, and the silver salt of the derivative was esterified to form N-isobutoxycarbonyltaurine methyl ester in the same manner developed previously to form a dimethyl ester of sulfuric acid (8). In general, N-isobutoxycarbonyl derivatization of an amino group with isobutyl chloroformate is conducted in the presence of sodium hydroxide (7, 12, 15). However, we found that silver oxide could be used for the N-isobutoxycarbonyl derivatization reaction instead of sodium hydroxide. In the next step, silver salt of N-isobutoxycarbonyltaurine was reacted with methyl iodide to

form N-isobutoxycarbonyltaurine methyl ester. The yield of the derivative was good in the preparative scale, but it was low, when smaller amounts of taurine were derivatized. We found, however, that the addition of dimethyl sulfate and silver oxide to the reaction mixture improved the yield as shown in Table 1. Thus, N-isobutoxycarbonyltaurine methyl ester could be prepared from taurine with a good yield by a simpler procedure as shown in Fig. 1.

The structure of the derivative prepared in the present study was examined by elementary analyses, spectral analysis and GC-MS. Results of elementary analyses and spectral data agreed well with theoretical values. A peak which appeared at 4.7 min on the gas chromatogram (see Fig. 3) was subjected to EI and CI mass spectrometry. Although no molecular ion peak (m/e 239, M^+) in the EI mass spectra was observed, the prominent fragment peaks supported the structure. The CI mass spectra showed a base ion peak of m/e 240, which was consistent with a structure of MH^+ . When aqueous taurine solution was analyzed by the procedure described above, only a peak which appeared at 4.7 min was detected. All these data indicate that taurine was converted to N-isobutoxycarbonyltaurine methyl ester, and the peak of the derivative appeared at 4.7

Table 1 Effect of dimethyl sulfate on the recovery of taurine^a

Dimethyl sulfate added ^b (μ l/ml)	Taurine added (μ mol/ml)	Taurine determined ^c (μ mol/ml)	Recovery ^c (%)
0.0	5.00	4.00 ± 0.13	80.0 ± 2.6
2.0	5.00	4.73 ± 0.14	94.6 ± 2.8
5.0	5.00	4.70 ± 0.12	94.0 ± 2.4
10.0	5.00	4.88 ± 0.10	97.6 ± 2.0
20.0	5.00	4.85 ± 0.05	97.0 ± 1.0

a: Taurine was determined after derivatization to N-isobutoxycarbonyltaurine methyl ester. Details are described under Materials and Methods.

b: Dimethyl sulfate and 10 mg of silver oxide were added to the reaction mixture of N-isobutoxycarbonyltaurine silver salt with methyl iodide.

c: Values are expressed as mean $\pm$ SD of 3 determinations.

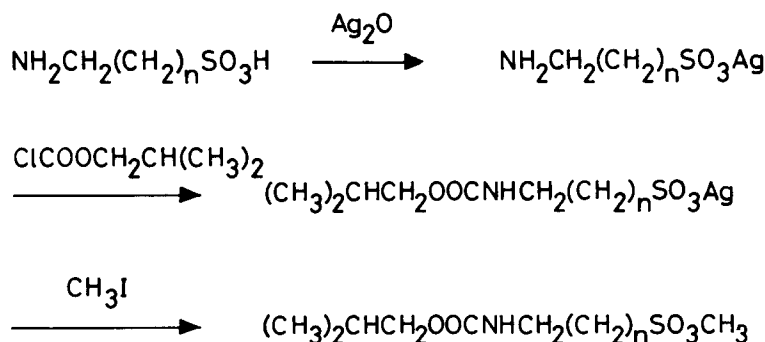


Fig. 1 Scheme of the conversion of taurine ($n=1$) and 3-aminopropanesulfonic acid ($n=2$) to N-isobutoxycarbonyl methyl ester derivatives.

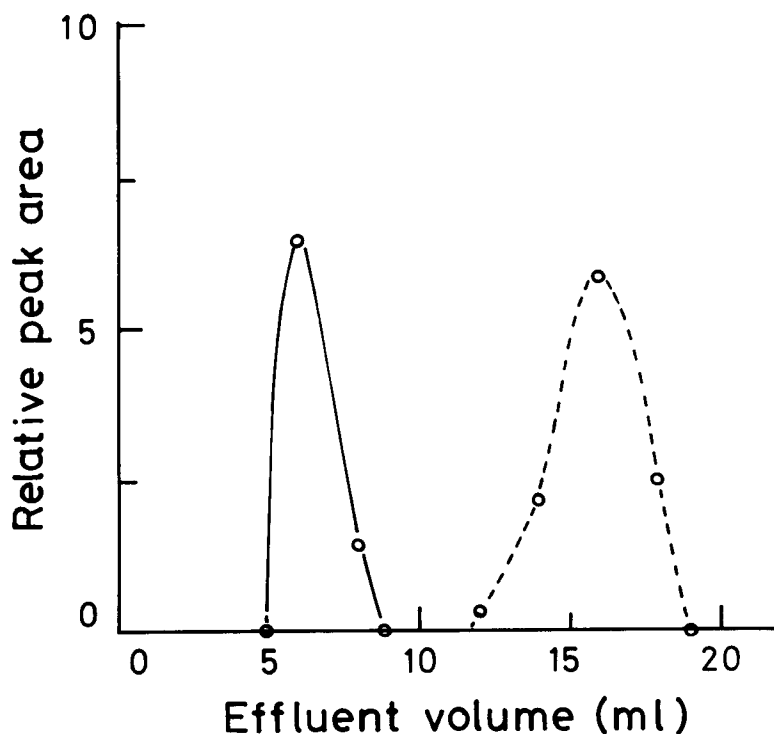


Fig. 2 Separation of hippuric acid from taurine by a Dowex 50W column. Five μmol each of taurine and hippuric acid were applied to a Dowex 50W $\times$ 8 column (0.7 $\times$ 20 cm, H^+ form) and eluted with water. Each 2 ml fraction was analyzed by the gas chromatographic procedure described under Materials and Methods. Taurine, —; hippuric acid,

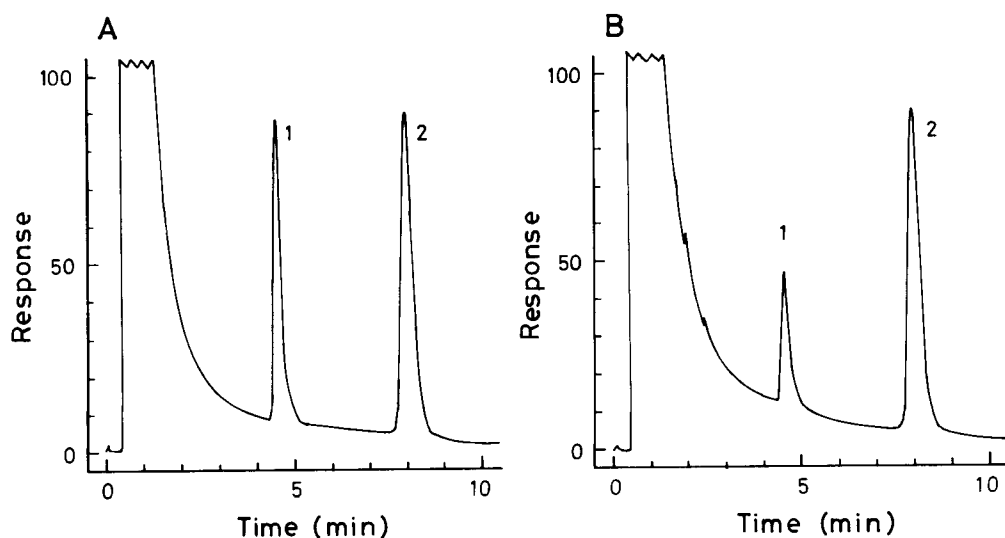


Fig. 3 Gas chromatograms of N-isobutoxycarbonyltaurine methyl ester derivative prepared from taurine and methyl 3-(N-isobutoxycarbonylamino)propanesulfonate (internal standard). Chromatogram A was obtained with a solution containing 5 $\mu\text{mol}/\text{ml}$ of taurine, and B with human urine. Peaks: 1, the derivative of taurine; 2, the internal standard.

min on the gas chromatogram.

Methyl 3-(N-isobutoxycarbonylamino)propanesulfonate, a homologue of the taurine derivative, was used as an internal standard in order to determine the exact taurine concentration by gas chromatography. The derivative was prepared from 3-aminopropane-sulfonic acid with as good a yield as that for taurine. Its structure was identified by elementary analyses and spectral data. The peak appeared at 8.0 min on the gas chromatogram (see Fig. 3).

Quantitative analysis of taurine by the present gas chromatographic method was performed using aqueous solutions containing 0.5–5.0 $\mu\text{mol/ml}$ of taurine. As shown in Fig. 3A, peaks corresponding to the derivative of taurine and the internal standard showed no peak-tailing and were separated well. The recovery of taurine was $100.7 \pm 3.7\%$ and the calibration curve obtained using aqueous taurine solution was linear between 0.5 and 5.0 $\mu\text{mol/ml}$. These results show that the present method can be used for the determination of taurine.

The present method was applied to the determination of taurine in urine. The interference by chloride ion in urine was eliminated by the addition of an excess amount of silver oxide as reported previously (8). When urine samples were analyzed, the peak at 4.7 min on the gas chromatogram corresponding to the taurine derivative was analyzed by GC-MS. It was found that the peak consisted of two compounds. One was the taurine derivative, and the other was identified to be methyl hippurate by the mass spectra. These two compounds could be separated into two peaks by gas chromatography when a column (0.3 mm i.d. $\times$ 2 m) of 2% OV-105 in Uniport HP (80–100 mesh) was used, but the separation was incomplete. Therefore, hippuric acid in urine must be removed before the derivatization. As shown in Fig. 2, hippuric acid could be separated

from taurine with a Dowex 50W $\times$ 8 column.

Possible interference by cysteic acid, which might be present in a small amount in urine, was examined. Cysteic acid was derivatized using the present procedure. The peak of the derivative formed appeared at 9.4 min on the gas chromatogram. Therefore, the taurine determination is not affected by the presence of cysteic acid. No other interfering material was detected as judged by mass spectra of each peak on the gas chromatogram. The recovery was $98.6 \pm 5.2\%$, when 1.25 to 5.0 $\mu\text{mol/ml}$ of taurine were added to the urine. Values (y) obtained by the present method were compared with those (x) obtained by a method using combined ion-exchange resin columns and ninhydrin reaction (9, 10) as shown in Table 2, both results agreed well. The regression line was $y = 0.93x + 0.19$ and the correlation coefficient r was 0.991. Thus, the present method was shown to be applicable to the

Table 2 Comparison of urinary taurine determined by gas chromatography and ion-exchange chromatographic method^a

Sample number	Taurine determined ($\mu\text{mol/ml}$ of rat urine)	
	Gas chromatography ^b (y)	Ion-exchange chromatographic method ^c (x)
1	8.13 ± 0.15	8.95 ± 0.08
2	5.63 ± 0.10	6.25 ± 0.16
3	6.58 ± 0.14	6.58 ± 0.15
4	4.19 ± 0.20	4.20 ± 0.05
5	3.02 ± 0.06	3.09 ± 0.03
6	7.22 ± 0.13	7.31 ± 0.14
7	9.11 ± 0.27	9.28 ± 0.11
8	5.47 ± 0.21	5.65 ± 0.15

a : Values are expressed as mean $\pm$ SD of 4 determinations. The regression line and correlation coefficient of both methods were $y = 0.93x + 0.19$ and $r = 0.991$.

b : Taurine was converted to N-isobutoxycarbonyltaurine methyl ester. The derivative was analyzed by gas chromatography.

c : Taurine was separated by ion-exchange chromatography and determined by ninhydrin reaction.

Table 3 Taurine excreted in human urine^a

Sample number	Taurine ^b ($\mu\text{mol}/\text{mg}$ of creatinine)
1	0.46 ± 0.01
2	0.75 ± 0.03
3	1.02 ± 0.03
4	1.49 ± 0.10
5	0.96 ± 0.03

a: Taurine was determined by gas chromatography. Details are described under Materials and Methods.

b: Values are expressed as mean $\pm$ SD of 3 determinations.

determination of taurine in urine.

Taurine concentrations in the urine of five healthy male Japanese ranging from 27 to 55 years of age were determined by present method. A typical gas chromatogram is shown in Fig. 3B. The amounts of taurine determined are shown in Table 3. These values are comparable to those of other reports (13, 14).

Compared to the method of taurine determination using the N-isobutoxycarbonyl dibutylamide derivative (7), the present method has some advantages: 1. The derivatization procedure is simpler. 2. Taurine and cysteic acid may be analyzed at the same time. 3. The volatile N-isobutoxycarbonyl methyl ester derivative has a smaller molecular weight, making the calculation of isotope-ratios of mass fragments easier. Therefore, this method seems to be more applicable to the isotopic analysis of taurine by GC-MS.

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