Determination of Taurine in Biological Samples by High-Performance Liquid Chromatography Using 4-Fluoro-7-Nitrobenzofurazan as a Derivatizing Agent

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Abstract

Objective   A highly sensitive and rapid high-performance liquid chromatography method with pre-column derivatization with 4-fluoro-7-nitrobenzofurazan was developed for determination of taurine in biological samples, including plasma, brain, and liver.

Methods   The optimum derivatization reaction temperature was 70 °C, and at this temperature the reaction was complete within 3 min. The derivatized taurine was separated using phosphate buffer (0.02 mol/L, pH 6.0):acetonitrile (84:16, v/v) as the mobile phase at a flow rate of 1.0 mL/min, and a column temperature of 25 °C. The taurine derivatives were separated within 20 min (tR:14.5 min) and fluorometrically detected at 530 nm with excitation at 470 nm.

Results   The intra- and the inter-day coefficients of variation for the method were 5.3% and 7.7%, respectively. The calibration curve was linear from 0.1 µmol/L to 30.0 µmol/L with a correlation coefficient of 0.9995.

Conclusion   This method can be used to determine the taurine contents in plasma, brain, and liver from normal rats and human plasma.

Key words: Taurine; HPLC; 4-fluoro-7-nitrobenzofurazan; Derivatization

INTRODUCTION

Taurine, α-aminoethanesulfonic acid, is a derivative of the sulfur-containing amino acid cysteine. It is one of the most abundant free amino acids in the human body and is widely distributed in biological fluids and tissues. Taurine has many physiological functions as an antioxidant, neurotransmitter and toxinicide, and in conjugation of bile acids, modulation of the levels of intracellular ions (e.g. calcium, potassium, sodium), membrane stabilization, and osmosis[1-7]. Taurine plays an important role in prenatal development, especially in neural development as an inhibitory and neuroprotective neurotransmitter and neuromodulator[8-10]. Taurine is essential in the diets of some animals such as felines and passerine birds[11].

Changes in the taurine levels in physiological fluids and tissues occur in various diseases or pathological conditions such as psychosis (depression, schizophrenia, epilepsy), inflammation, hepatic damage, sepsis, retinitis pigmentosa, and cancer[12-16]. The taurine concentrations in plasma are related to those in cerebrospinal fluid[17]. Taurine is a useful biomarker of some diseases and/or the conditions of pathological disorders[18].

To date, several methods have been developed for the determination of taurine in plasma. These methods include gas chromatography[19],

high-performance liquid chromatography (HPLC) with evaporative light scattering or fluorescence detection[20-21], HPLC with electrospray ionization/mass spectrometry[22-23], and capillary electrophoresis[24]. These methods differ in sensitivity, selectivity, specificity, and susceptibility to interferences. HPLC with mass spectrometry has good sensitivity, selectivity and specificity. However, mass spectrometers are expensive and complicated, which limits the routine application of this technique in clinical research, particularly in studies calling for the analysis of many samples. Several derivatives have been used for the determination of taurine, including o-phthalaldehyde, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride[25] and fluorescamine[26]. The o-phthalaldehyde derivative is unstable, which affects the accuracy and reproducibility of taurine analysis. 4-(5,6-Dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride and fluorescamine derivatives can be used for the determination of taurine, but the pre-treatment procedures are difficult and time-consuming (>20 min).

4-Fluoro-7-nitrobenzofurazan (NBD-F) is an extremely sensitive fluorescent derivatization reagent for the determination of amino acids (Figure 1) and some drugs[27-28]. Compared with other derivatization reagents, NBD-F has several advantages, including convenient excitation/emission wavelengths, few reagent related interfering peaks, and mild reaction conditions. In this paper, NBD-F was used for pre-column derivatization in a highly sensitive, simple HPLC method for the determination of taurine in plasma, brain and liver with fluorescence detection.
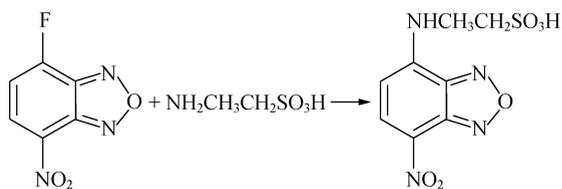

Figure 1. Derivatization of taurine with NBD-F.

MATERIALS AND METHODS

Chemicals

Taurine was purchased from Sigma-Aldrich (St. Louis, MO, USA). NBD-F was purchased from TCI (Tokyo, Japan). Human plasma samples were obtained from the Shandong Blood Center (Jinan, China), and kept frozen at –20 °C until analysis. HPLC grade acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA). Analytical grade potassium tetraborate and potassium dihydrogen phosphate were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Ultrapure water was prepared using a Millipore Milli-Q water system (Billerica, MA, USA).

Taurine Standard Solution

A sample of taurine was accurately weighed (31.3 mg) and quantitatively transferred into a 25 mL volumetric flask, and dissolved in water to produce a stock solution (10.0 mmol/L). The stock solution was further diluted with water to obtain a working standard solution.

Chromatography

A Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with two LC-10ATVP pumps, a 20 μL injection loop, HT-230A column heater, RF-10AXL fluorescence detector, and SCL-10Avp system controller was used for analysis. Chromatographic separations were performed on a Diamonsil C_{18} (250 mm× 4.6 mm, 5 μm) column (Dikma Technologies Inc., Lake Forest, CA). The column temperature was 25 °C. The mobile phase was acetonitrile:phosphate buffer (0.02 mol/L, pH 6.0) (16:84, v/v) with a flow rate of 1.0 mL/min. The mobile phase was filtered through a Millipore vacuum filtration system equipped with a 0.45 μm pore size filter, and degassed by ultrasonication. The fluorescence detector was set at 470 and 530 nm for the excitation and emission wavelengths, respectively.

Sample Collection, Preparation, and Derivatization

Blood samples from 36 healthy medical students (24 males and 12 females) and 20 female Wistar rats were analyzed. Informed consent was obtained from all human subjects after explaining the aims and risks of the study. Five milliliters of venous blood was collected from each subject by venipuncture into heparinized tubes. Twenty female Wistar rats was obtained from the Breeding Center for Experimental Animals of Shandong University (Jinan, China). Experiments were performed according to the guidelines of the University Committee on Animal Welfare.

Blood samples were centrifuged (1 500 rpm, 15 min, room temperature) immediately after collection to obtain the plasma. The plasma samples were

labeled and kept frozen at –20 °C until analysis.

Rat brain and liver samples were quickly excised and stored at –80 °C after measuring their wet weight. The tissues were homogenized in a 0.9% (mass fraction) NaCl solution, and the homogenates were centrifuged at 4 500 ×g for 5 min to obtain the supernatants.

The plasma and supernatant samples were mixed with twice their volume of acetonitrile in centrifuge tubes, vortexed for 1 min, and centrifuged at 12 000 rpm for 10 min. The supernatant from each sample was collected for derivatization after filtration through a 0.45 µm pore size Millipore filter. Each filtrate was diluted with borate buffer (pH 9.5) several fold and thoroughly mixed. For derivatization, the samples were mixed with an equal volume of NBD-F (1 mmol/L) and kept at 70 °C for 3 min in the dark. After cooling to room temperature, 10 µL of each sample was analyzed by HPLC.

RESULTS

Linearity and Sensitivity

A seven-point (0.1-30.0 µmol/L) taurine calibration curve was constructed using standard solutions prepared from the stock solution. Each solution was analyzed in triplicate. The regression equation of the calibration curve was $y=143\ 379\ x+25\ 503$, where x and y are the taurine concentration and detector signal, respectively, and had a correlation coefficient of 0.9995. The limit of detection determined using a signal-to-noise ratio of 3:1 was approximately 0.003 µmol/L, which is considerably lower than the values (0.05-688 µmol/L) established using other chromatographic methods. This confirmed the proposed method has high sensitivity. The regression curve was used to calculate the concentrations of taurine in the biological samples based on peak area.

Precision and Accuracy

The intraday precision and accuracy were evaluated by analyzing 6 replicates of taurine quality control samples at three different concentrations (0.50, 2.50, 10.0 µmol/L) over 1 day. The interday precision and accuracy were evaluated by analyzing 12 replicates of the taurine quality control samples over 3 days (Table 1). The precision is presented as the relative standard deviation, and accuracy is expressed as recovery of the analyte from samples

spiked with different concentrations of taurine, which covered the linear range of the method. The intra- and interday relative standard deviations were less than 5.3 % and 7.7 %, respectively. These results indicate that this method is reliable, reproducible and accurate.

Table 1. Intra- and Interday Precision and Accuracy for the Determination of Taurine

Added (µmol/L)		Measured (µmol/L, mean±SD)	RSD (%)	Recovery (%)
Intraday (n=6)	0.50	0.46±0.02	5.3	92.0
	2.50	2.59±0.10	3.8	103.6
	10.0	9.78±0.41	4.2	97.8
Interday (n=12)	0.50	0.46±0.04	7.7	92.0
	2.50	2.62±0.11	5.1	104.8
	10.0	9.80±0.30	3.1	98.0

Analysis of Biological Samples

The proposed method was successfully applied to the determination of taurine in the human and rat samples (Figure 2). The mean concentrations of taurine in the human and rat plasma were 37.6±5.1 and 263±24 µmol/L, respectively. The amounts of taurine in the rat brain and liver were 1.9±0.2 and 1.4±0.1 µmol/g of wet tissue, respectively.

DISCUSSION

Selection of the Mobile Phase

The chromatographic conditions were optimized to develop a sensitive, accurate, and reproducible method for detection of taurine in biological samples. Because the taurine derivative was easily retained on the column, acetonitrile was chosen as the organic solvent in the mobile phase for its elution strength. The retention time of NBD-F was relatively stable with different percentages of acetonitrile in the mobile phase, but the retention time of the taurine-NBD-F derivative was very sensitive to the percentage of acetonitrile. When the percentage of acetonitrile in the mobile phase was decreased from 25% to 15%, the retention time of the NBD-taurine derivative changed from 8 to 16 min, while that of NBD-F changed little. There were some interferences from plasma that were located before the NBD-F peak, and 16% acetonitrile was used in the mobile phase to separate these interferences from the taurine derivative.
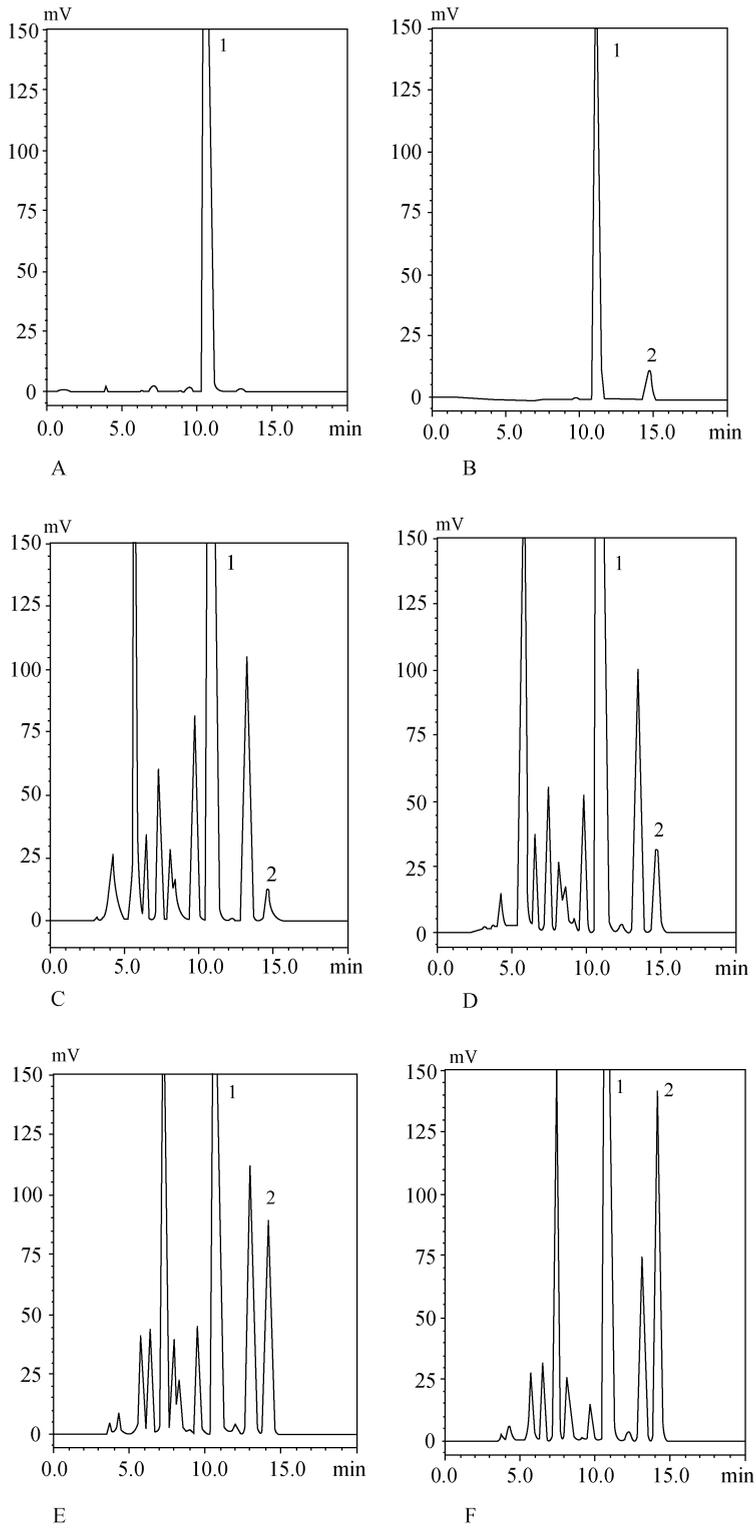
Figure 2. HPLC chromatograms (1. NBD-F, 2. NBD-Taurine) of (A) blank NBD-F; (B) standard solution of taurine (2.5 μmol/L); (C) human plasma sample; (D) human plasma sample spiked with taurine (2.5 μmol/L); (E) rat liver sample; and (F) rat liver sample spiked with taurine (10 μmol/L).

Selection of the Mobile Phase pH and the Column Temperature

The pH of the mobile phase and the column temperature affected the separation of NBD-F and the derivatives. At higher pH values, better separation from the interferences in the samples was achieved. When the column temperature was increased to 35 °C, taurine could not be separated from the interferences. The optimum separation conditions were acetonitrile:phosphate buffer (0.02 mol/L, pH 6.0) (16:84, v/v) at a flow rate of 1.0 mL/min and a column temperature of 25 °C. These conditions provide a good peak shape, satisfactory resolution, and a relatively short analysis time.

Derivatization Reaction Conditions

Derivatization of amino acids with NBD-F can occur at 20 °C, and higher reaction temperatures reduce the time required for completion of the reaction. The rates and the yields of the derivatization reaction of taurine with NBD-F were examined at different temperatures, and the results are shown in Figure 3. To achieve a high yield of the derivative and reduce the reaction time, the optimum reaction temperature was 70 °C. At this temperature, the reaction was complete within 3 min.
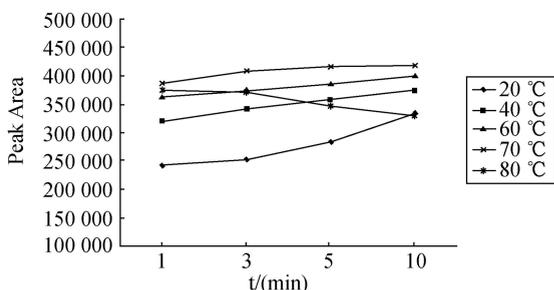

Figure 3. Effects of temperature and time on the derivatization reaction.

The derivatization reaction of amino acids with NBD-F is a nucleophilic reaction, and alkaline conditions are expected to favor this reaction. Both borate and phosphate buffers have been used for the reaction of NBD-F with amino acids. In this study, we compared the fluorescence intensity obtained with borate and phosphate buffers at different pH values. The NBD-taurine peak areas were calculated at pH values ranging from 7.5-10.0. The maximum fluorescence intensity was achieved with borate buffer at pH 9.5.

Stability of the Taurine NBD-F Derivative

The stability of the taurine NBD-derivative was analyzed by determining the peak areas of standard solutions of different concentrations that had been kept in the dark in room temperature for 1, 4, 8, 24, or 48 h after the derivatization reaction. The taurine peak areas for these samples were approximately the same as those for samples analyzed immediately after preparation. When the derivative was kept at 4 °C, there was no obvious decrease in the peak area even after 1 week.

We then evaluated the stability of taurine in samples using the difference in the taurine concentration before and after storage for 1 month at −20 °C. The relative variation (%) was calculated as [(concentration found − concentration added)× 100/concentration added]. The average relative variations (n=6) of three quality control samples at concentrations of 0.5, 2.5, and 10.0 µmol/L were −4.3%, 3.3 %, and −2.7%, respectively. These results indicate that taurine in the samples was stable for at least 1 month when stored at −20 °C.

In conclusion, an optimized HPLC-fluorescence method for determination of taurine in biological samples was developed and validated. Compared with previously reported HPLC methods, the proposed method is shorter, has improved reproducibility, and requires only a small amount of sample. The method is suitable for taurine analysis in clinical and biochemical research.

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