Journal of Chromatography, 568 (1991) 212–218
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5917

Short Communication

High-performance liquid chromatographic determination of taurine in biological fluids by post-column fluorescence reaction with thiamine

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(First received January 16th, 1991; revised manuscript received March 22nd, 1991)

ABSTRACT

A high-performance liquid chromatographic method is described for the selective determination of taurine in biological fluids by post-column fluorescence reaction. Taurine was separated on an adsorption distribution type Shodex Ionpac KC-811 column. Then it was converted with hypochlorite into the corresponding N-chloramine, which was allowed to react with thiamine to give fluorescent thiochrome. As little as 6 ng per injection of taurine could be determined. The average recoveries of spiked taurine in serum and urine were 99.5 ± 2.7 and 101.8 ± 2.9%, respectively. The method could be applied to the assay of taurine in human serum and urine with simple pretreatment.

INTRODUCTION

Taurine is present in biological fluids and tissues as a non-protein free amino acid, which is one of the final products of cysteine metabolism. Taurine is known to conjugate with bile acids to form a key intermediate of their metabolism. Recently, taurine was reported to play a role as an inhibitory neurotransmitter in cerebellar stellate interneurons [1], and its blood level was observed to fluctuate in epilepsy [2] and depression [3].

Numerous methods have been examined for the assay of taurine in biological fluids and tissues, such as colorimetry [4], gas chromatography [5,6] and isotachophoresis [7]. Column liquid chromatography is a promising method for the separation of taurine from complex mixtures, such as biological fluids, which are highly hydrophilic. Perry *et al.* [8] reported the selective separation of taurine by ion-exchange chromatography. An amino acid analyser is also applicable for this

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purpose [9,10], but the detection of taurine should be devised to increase the sensitivity.

Recently, many workers have reported the pre-column derivatization of taurine with *o*-phthalaldehyde (OPA) [11,12], 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride) (Dns-Cl) [13] or 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) [14] and post-column derivatization with OPA [15]. These methods were based on the reaction of amino groups.

We have previously reported fluorometric assay methods for proteins in which the amide (peptide) bonds of proteins were chlorinated and the product was allowed to react with thiamine to give a fluorescent thiochrome [16,17]. We have found that taurine also undergoes this reaction to give intense fluorescence, whereas other amino acids give negative results [16]. This paper deals with the application of this reaction to the post-column detection of taurine in high-performance liquid chromatography (HPLC) of biological fluids.

EXPERIMENTAL

Chemicals

Taurine (2-aminoethanesulphonic acid), thiamine hydrochloride and analytical-reagent grade $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot H_2O$, H_3PO_4, $NaNO_2$, NaOH, $NaWO_4 \cdot H_2O$, H_2SO_4, 10% sodium hypochlorite solution (Antiformin®) and Brij-35 were obtained from Wako (Osaka, Japan). Ala-Phe and Ala-Ala-Ala were purchased from Sigma (St. Louis, MO, USA).

Mobile phase and derivatization reagents

The mobile phase for HPLC was 10 m*M* aqueous phosphoric acid solution. The solution was filtered through a 0.45-μm microfilter (Fuji Photo Film, Tokyo, Japan) and degassed prior to use.

The hypochlorite reagent was prepared by adding 60 ml of 1 *M* NaOH solution and 4 ml of 25% Brij-35 solution to 6 ml of Antiformin, and diluting the resulting mixture with 0.1 *M* phosphate buffer (pH 7.0) to make 1 l. The final pH and available chlorine concentration of this reagent should be pH 12.0 and 24 m*M*, respectively.

Thiamine reagent was prepared by dissolving 22.8 g of $NaNO_2$ and 200 mg of thiamine hydrochloride in 0.1 *M* phosphate buffer (pH 7.0), and adjusting the pH of the solution to 7.0 using 0.1 *M* Na_2HPO_4 solution and 0.1 *M* NaH_2PO_4 solution. The resulting mixture was diluted to 1 l with 0.1 *M* phosphate buffer (pH 7.0). This reagent was stable for at least 24 h at room temperature, and for one week in the refrigerator.

All reagent solutions were filtered before use to remove suspended material.

Stability of N-chlorides of taurine and peptides

A 2-ml volume of the hypochloride reagent was added to 2.5 ml of a 100 μg/ml

solution of taurine, Ala-Phe or Ala-Ala-Ala. The mixture was shaken well and allowed to stand for various times at room temperature. To the mixtures was added 1 ml of thiamine reagent, and the fluorescence intensity was measured after 30 min at excitation and emission wavelengths at 370 nm and 440 nm, respectively.

Sample pretreatment

Blood. A blood sample from an adult man was collected into a test-tube and centrifuged at 2000 *g* for 10 min. To 0.5 ml of the obtained serum were added 0.2 ml of distilled water, 0.2 ml of 0.167 *M* sulphuric acid, and 0.3 ml of 0.076 *M* sodium tungstate(VI) for deproteinization [18]. The mixture was mechanically shaken with an Iwaki KM shaker (Model VS, Iwaki, Tokyo, Japan) and allowed to stand for 10 min. After centrifugation at 2000 *g* for 10 min, 20 μl of the supernatant were submitted to the assay.

Urine. To 50 μl of 24-h urine from an adult man were added 0.2 ml of distilled water, 0.2 ml of 0.167 *M* sulphuric acid and 0.3 ml of 0.076 *M* sodium tungstate (VI). The mixture was mechanically shaken and allowed to stand for 10 min. After centrifugation, 20 μl of the supernatant were submitted to the assay.

Chromatographic system

Fig. 1 shows a schematic diagram of the HPLC system. Chromatographic separation was carried out on a 300 mm × 8.0 mm I.D. Shodex Ionpac KC-811 column (Showa Denko, Tokyo, Japan), equipped with a 50 mm × 8.0 mm I.D. Shodex Ionpac C-811P column as a guard column, operated at 45°C. The mobile phase and the two post-column derivatization reagents were pumped with a Shimadzu LC-6A HPLC solvent-delivery system.

The mobile phase was delivered at a flow-rate of 0.8 ml/min. The sample solution (20 μl) was injected using an HPLC auto-sampler KMT-60A (Kyowa-Seimitu, Tokyo, Japan). The eluate from the column was directly introduced into the fluorescence reactor system. The hypochlorite reagent was delivered into the eluate stream at a flow-rate of 0.3 ml/min, and the stream was allowed to flow

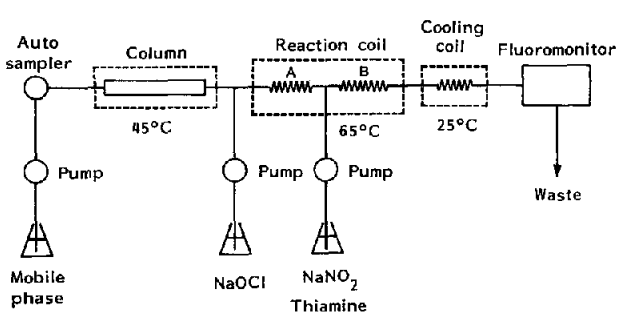

Fig. 1. Schematic diagram of the HPLC and fluorescence detection system.

through the PTFE reaction coil A (3 m × 0.5 mm I.D.), which was immersed in a water-bath at 65°C (Thermominder, Type Ace-80, Taiyo Service-center, Tokyo, Japan). The thiamine reagent was then delivered into the eluate stream at a flow-rate of 0.3 ml/min, and the mixture was passed through the PTFE reaction coil B (5 m × 0.5 mm I.D.), which was also placed in the 65°C water-bath. The effluent from the coil B was passed through the PTFE cooling coil (1 m × 0.5 mm I.D.).

The fluorescence intensity of the effluent was measured at excitation and emission wavelengths of 370 and 440 nm, respectively, using a Shimadzu RF-535 spectrofluorimeter equipped with a 20-μl flow-cell.

RESULTS AND DISCUSSION

The present method is based on the chlorination of taurine using hypochlorite to produce N-chlorotaurine, which reacts with thiamine to yield fluorescent thiochrome. The method is highly specific for taurine, because N-chlorotaurine is more stable than other N-chlorides of nitrogen-containing compounds, such as Ala-Phe and Ala-Ala-Ala as shown in Table I.

Reaction conditions were studied by the flow-injection method. The flow-injection system was essentially the same as the chromatographic system (Fig. 1), with the separation column omitted. Fig. 2 shows the fluorescence intensity plotted against the hypochlorite concentration. The fluorescence intensity increased with increasing chlorine concentration, but the net fluorescence intensity reached a maximum at the available chlorine concentration of 24 m*M* in the hypochlorite reagent, since the background fluorescence also increased when the concentration was above 32 m*M*. The optimum pH of the reaction was found to be 7.0 for both

TABLE I

STABILITY OF N-CHLORIDES OF TAURINE AND PEPTIDES

Reaction time[a]	Relative fluorescence intensity		
	Taurine	Ala-Phe	Ala-Ala-Ala
2 min	100.0	31.7	31.9
10 min	100.0	28.5	27.2
30 min	100.0	21.8	23.0
1 h	100.0	15.4	21.0
2 h	100.0	7.1	16.5
4 h	100.0	3.2	8.5
6 h	99.0	1.4	2.4
8 h	98.7	0.8	1.4

[a] Reaction times of N-chlorides with hypochlorite.

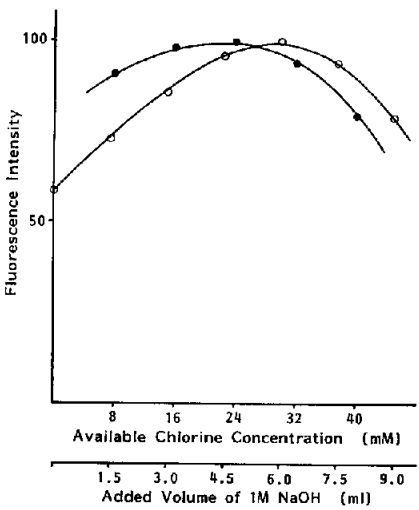

Fig. 2. Effect of available chlorine concentration (●) and added volume of 1 *M* NaOH (○) on fluorescence intensity. Amount injected, 0.2 μg of taurine. Other conditions of the post-column reaction as in Experimental.

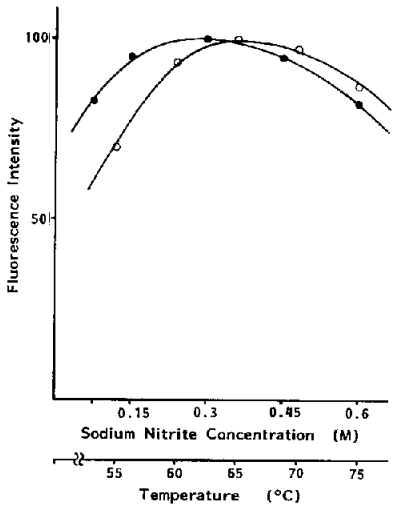

Fig. 3. Effect of nitrite concentration (●) and reaction temperature (○) on fluorescence intensity. Amount injected, 0.2 μg of taurine. Other conditions of the post-column reaction as in Experimental.

the chlorination and thiamine oxidation. The thiamine reagent was therefore adjusted to pH 7.0 with 0.1 *M* phosphate buffer. However, the pH of the hypochlorite reagent should be more alkaline because it is mixed with the acidic eluate from the column containing 10 m*M* phosphoric acid.

Fig. 2 also shows the fluorescence intensity in relation to the added volume of 1 *M* NaOH in the hypochlorite reagent. The maximum fluorescence intensity was observed when 6 ml of 1 *M* NaOH solution were added to 100 ml of the reagent. The concentration of nitrite to decompose chlorine in the thiamine reagent was adjusted to 0.3 *M*, and the temperature of the waterbath was set at 65°C because the maximum fluorescence intensity was observed under these conditions (Fig. 3). The flow-rates of the reagents, the thiamine concentration in the reagent, and the coil lengths were the same as those described in the previous paper [19].

We used the adsorption–distribution Ionpac KC-811 column (sulphonic acid-type polystyrene porous polymer), because it is shorter and offers a higher resolution and faster elution than that used by Hirai *et al.* [15] for taurine analysis.

A linear relationship was observed between the relative peak height and the amount of taurine in the range from 20 ng to 3 μg per injection, with a correlation coefficient of 0.998. The limit of detection for taurine was 6 ng per injection at a signal-to-noise ratio of 2.0.

Fig. 4B and C show typical chromatograms of human serum and urine, respectively. They show that other substances in the biological fluid do not interfere

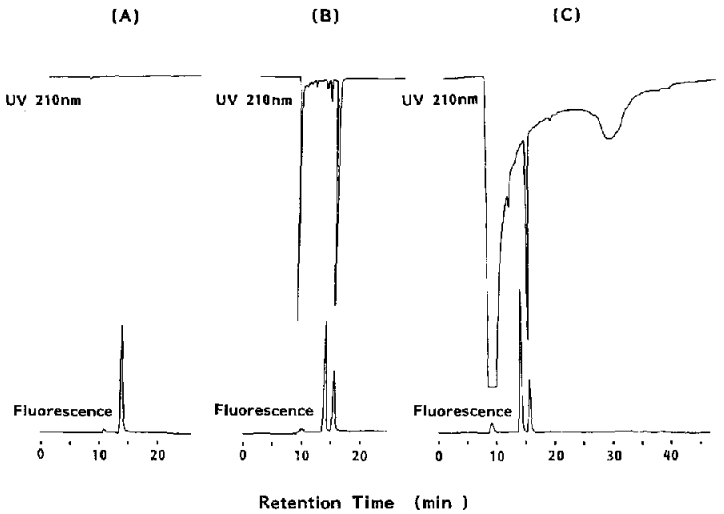

Fig. 4. Chromatograms of (A) taurine standard solution, (B) serum sample, and (C) urine sample, detected by UV absorbance at 210 nm (top) and by the present fluorimetric method (bottom). Conditions as in Experimental.

with the analysis of taurine. The peak at the retention time of 14 min was proved to be identical with that of the standard taurine, and the peak at 16 min was unknown.

On the other hand, taurine was not detected at 210 nm by a UV detector placed immediately after the column, though the peak at 16 min was detected.

Various UV-absorbing substances were found in large amounts in biological fluids, especially in urine. These substances were not detected by the present method, indicating its high selectivity towards taurine.

The recoveries of taurine added to serum in amounts of 20–120 nmol/ml were in the range 99.5 ± 2.7%, and those of taurine added to urine in amounts of 0.333–2.0 μmol/ml were in the range 101.8 ± 2.9%.

In conclusion, the present method facilitates rapid, sensitive and selective determination of taurine in biological fluids. Thus it is expected to be useful in biochemical and clinical fields.

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