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Rapid assay of dinitrophenyl derivative of taurine by high-performance liquid chromatography

B. Polanuer*, S. Ivanov, A. Sholin

Institute for Genetics of Industrial Microorganisms, 1 Dorozny pr. 1, 113545 Moscow, Russian Federation

Abstract

A rapid and simple method for the determination of taurine (2-aninoethanesulphonic acid) in complex samples is described. It is based on the HPLC separation of the dinitrophenyl (DNP) derivative of taurine. The reaction conditions are selected to allow complete derivatization of taurine within 15 min. DNP-taurine samples are stable for at least 3 days. DNP-taurine was separated by reversed-phase liquid chromatography within 12 s. The recovery of taurine was 102 ± 3% (S.D. = 2.5%, $n = 6$) and the detection limit was 10 pmol for taurine (signal-to-noise ratio of 10). The method was applied to the determination of taurine levels in different samples including marine products, infant formulas and fermentation media of different bacterial species.

1. Introduction

Taurine (2-aminoethanesulphonic acid) is found in different animal species and tissues. The role of this non-protein amino acid is not known, although it has been suggested that it may function as a neurotransmitter, membrane stabilizer and antioxidant [1]. Taurine is an essential component of milk powders for infants. For this purpose, taurine may be chemically synthesized or isolated from different marine species (fish, squid, etc.).

The determination of taurine in different biological samples has been carried out by techniques such as spectrophotometry [2,3], gas chromatography [4] and high-performance liquid chromatography (HPLC) [5–10]. The last technique is currently the most popular. Usually, methods developed for amino acid analysis are applied to the determination of taurine without any modification. The main disadvantage of these techniques is poor selectivity. Usually, simultaneous derivatization of all amino acids is carried out, which leads to the necessity to analyse a complex mixture. Ion-exchange chromatography with postcolumn ninhydrin detection, widely used for amino acid analysis, when applied to the determination of taurine [5] results in long analysis times (over 1 h). The analysis of the dansyl derivative of taurine in plasma was less time consuming (about 30 min), but required complex sample preparation [6].

Recently, an approach to the determination of taurine in feline plasma based on dansyl chloride derivatization with reversed-phase separation and fluorimetric or mass spectrometric detection of DNS-taurine has been described [7]. Although this method is very sensitive, both sample pretreatment and separation are time consuming (*ca.* 45 and 15 min, respectively). Using a pre-

* Corresponding author.

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liminary separation of taurine on a dual-bed ion-exchange column and derivatization with *o*-phthalaldehyde (OPA), it becomes possible to determine taurine in whole blood within 7 min [8]. Similar sample pretreatment on a dual ion-exchange column with OPA derivatization and reversed-phase separation has been used for the determination of taurine in rat and chick plasma [9]. OPA derivatization followed by HPLC separation within 2 min on a short column (45 × 4.6 mm I.D.) was used recently for the determination of taurine in the brain [10]. However, with complex amino acid mixtures containing components such as leucine, valine and tryptophan, the separation based on this technique takes much longer [10].

In our study of the preparation of taurine from marine species and screening of possible microbial taurine producers, a simple and convenient method for taurine assay in different samples was necessary. It must be mentioned that only taurine assay in the samples was of our interest, and the concentrations of other components were not considered. In an early study of the spectrophotometric determination of taurine in the form of the DNP derivative, it was found that DNP derivatives of most amino acids, nucleotides, etc., are easily extracted with chloroform [2]. The only DNP derivatives retained in the aqueous layer were those of taurine and cysteic acid. These results were used in our study during the development of an HPLC method for taurine determination.

2. Experimental

2.1. Materials

Taurine, cysteic acid, sodium 1-octanesulphonate and sodium 1-hexanesulphonate were purchased from Sigma (St. Louis, MO, USA), acetic acid, sodium hydroxide, orthophosphoric acid and triethylamine (ACS grade) from Aldrich (Milwaukee, WI, USA) and acetone, ethanol, 2-propanol and 2-butanone (all of analytical-reagent grade) from Soiuzkhimreaktiw (Moscow, Russian Federation). Methanol and water were distilled in glass. 2,4-Dinitrofluorbenzene (DNFB) was a kind gift from Prof. Yu. Belokon (Institute of Organo-Element Compounds, Russian Academy of Sciences) and was used as received. Solutions of 2,4-dinitrofluorbenzene (DNFB) in organic solvents were stored in dark glass bottles for up to 1 week.

2.2. Sample preparation

The meat of tuna or squid (0.5 kg) was extracted with 1 l of boiling water within 2 h. A 1-g sample of infant formula Nutrilon (Nutricia, Ireland) was diluted in 1 ml of distilled water. Different species of microorganisms (*Escherichia coli*, *Bacillus subtilis*, etc.) were cultivated in liquid media under appropriate conditions. A 1-ml aliquot with different taurine concentrations was centrifuged on an Eppendorf Model 5414S microcentrifuge, at 15 000 *g* for 3 min, and 100 μl were processed as described below.

Samples were prepared in disposable 1.5-ml plastic test-tubes (DIA-M, Moscow, Russian Federation) using micropipettes with disposable tips (Gilson, Villers le Bel, France). A 100-μl volume of 4% NaOH solution was added to 100 μl of sample and, after mixing, 20 μl of DNFB or 20 μl of DNFB solution in organic solvent were added. The samples were shaken vigorously. The reaction was terminated by addition of 100 μl of 10% (v/v) orthophosphoric acid. After the addition of 500 μl of chloroform, the samples were shaken and the phases were separated by centrifugation on an Eppendorf Model 5414S microcentrifuge at 15 000 *g* for 15 s. A 200-μl aliquot of the upper (aqueous) layer was transferred into another test-tube and extracted once more with 500 μl of chloroform. A 50-μl sample of the aqueous layer was taken for chromatographic separation. Quantification was effected by using an external standard.

2.3. Chromatographic separation

The chromatographic system consisted of a Model 2150 HPLC pump and a Model 2151

variable-wavelength monitor operated at 350 nm (LKB, Bromma, Sweden). Samples were injected using a Model 7410 injector with a 1-μl internal sample loop (Rheodyne, Cotati, CA, USA). The data were processed using an IBM PC/AT compatible data station with a Model 960 interface (PE Nelson, Cupertino, CA, USA) and Chrom&Spec software (Ampersend, Moscow, Russian Federation).

A stainless-steel column (50 × 4 mm I.D.) with 10-μm Silosorb C_{18} sorbent (Elsico, Moscow, Russian Federation) was used for all separations. The eluent was water–methanol (75:25, v/v). After filtration through GF/F and GF/C glass-fibre filters (Whatman, Maidstone, UK), 1% of glacial acetic acid and 0.1% of triethylamine were added and the eluent was degassed in an ultrasonic bath. The eluent for ion-pair chromatography consisted of a 0.01 *M* solution of ion-pair reagent in water–methanol (90:10, v/v) and was filtered as described earlier. The eluent was recycled for up to 300–400 analyses, after which it was replaced.

Amino acid analysis was carried out using a Model 3000 amino acid analyser (Biotronic, Maintal, Germany). Spectrophotometric analysis was carried out using a Model UV-160 scanning spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Recovery

To control the recovery of taurine, samples of fish extract were spiked with different concentrations of taurine, derivatized and analysed twice (before and after spiking).

3. Results and discussion

3.1. Selection of derivatization conditions

The reaction of amino acids with DNFB takes up to 16 h [2]. Therefore, faster reactions with dansyl chloride, *o*-phthalaldehyde, etc., are usually used nowadays for amino acid analysis. However, the possibility of extracting DNP derivatives of other interfering compounds and simplifying the chromatographic separation conditions makes this derivatization technique very attractive. Therefore, we considered different possibilities for decreasing the time of the reaction. Utilization of pure DNFB instead of its 3% ethanol solution allows the reaction time to be decreased to 30 min. However, DNFB is not soluble in water and intensive shaking is necessary during reaction. Therefore, we compared the derivatization properties of DNFB solutions in several organic solvents of different polarities that are easily miscible with water. The best results were obtained with acetone. We then determine the time necessary to obtain complete derivatization of taurine. It was found that using a 10% solution of DNFB in acetone it becomes possible to decrease the reaction time to 15 min.

In the original method [2], sodium hydrogencarbonate buffer was recommended for reaction. Subsequent acidification of the reaction mixture leads to a high yield of carbon dioxide. Therefore, we used sodium hydroxide instead of sodium hydrogencarbonate. It was found that 4% NaOH solution gave the best results.

Chloroform extraction of the acidified reaction mixture is an important step in sample pretreatment which leads to the removal of DNP derivatives of most low-molecular-mass compounds, including not only protein amino acids but also nucleotides, amino sugars, etc. Excess of the derivatizing reagent is also extracted from the reaction mixture. Attempts to inject the unextracted reaction mixture led to multiple peaks. Under selected analytical conditions DNP-taurine is eluted before DNP derivatives of other amino acids. However, the occurrence of other peaks makes it necessary to increase the analysis time and/or to use gradient elution. Therefore, chloroform extraction must be considered as an essential step of sample handling. It was found during this study that a one-step extraction is not sufficient for total removal of the reaction products. Better results were obtained with a two-step extraction. A third extraction seems to be unnecessary.

The DNP derivative of taurine thus obtained is stable in the reaction mixture at room temperature for at least 3 days. Therefore, preliminary derivatization and subsequent analysis of tens of

samples per day is possible if the chromatographic procedure is fast and simple enough.

3.2. Selection of chromatographic conditions

During the preliminary study, it was found that DNP-taurine and DNP-cysteic acid are the only DNP derivatives in the reaction mixture after chloroform extraction. This pair of DNP derivatives is easily separated and detected under simple isocratic conditions. The UV spectrum of DNP-taurine has two maxima (254 and 350 nm). The former is common to different aromatic compounds (nucleotides, vitamins, etc.). As some of them may interfere in the determination of taurine, we detected DNP-taurine at 350 nm. The time of analysis and solvent consumption were decreased by using a short column (50 × 4 mm I.D.). Nevertheless, the efficiency of this column was sufficient for baseline separation of DNP-taurine and DNP-cysteic acid. In order to decrease the retention, different concentrations of methanol in the eluent were tried. It was found that 25% of methanol in the eluent gave the best results. If the flow-rate was 2.0 ml/min, the pressure drop with 10-μm spherical particles was only about 60 bar. Hence it is possible to increase the flow-rate to 3–5 ml/min without any risk for the pump and without a drastic decrease in selectivity. It is then possible to separate DNP-taurine and DNP-cysteic acid within 1 min (Fig. 1).

An additional decrease in retention was obtained by using ion-pair reagents. As was demonstrated earlier [11], addition to the eluent of an ion-pair reagent with the same charge as the analyte compound leads to a drastic decreasing in retention. Variation of the ion-pair reagent concentration in the range 2–20 m*M* did not influence the solute retention [11]. In the present case, DNFB reacts only with the amino group of taurine. Therefore, the resulting DNP derivative has a sulpho group and possesses acidic properties. Consequently, a decrease in retention is possible by addition of an acidic ion-pair reagent. The addition of hexanesulphonic acid allows the retention of DNP-taurine to be decreased by a factor of *ca.* 2. Under these analytical conditions, the retention time of DNP-taurine was about 30 s. The use of octanesulphonate instead of hexanesulphonate leads to a further decrease in retention of DNP-taurine. It is possible to separate DNP-taurine and DNP-cysteic acid within 12 s with this ion-pair reagent, a short column and a fast flow-rate (Fig. 2).

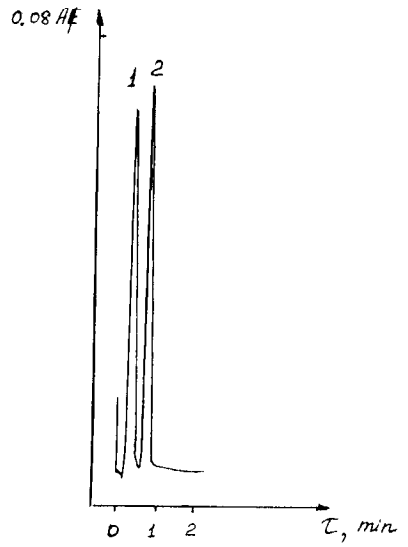

Fig. 1. Separation of DNP-taurine and DNP-cysteic acid. Eluent, 1% acetic acid and 0.1% triethylamine in 25% aqueous methanol; flow-rate, 5.0 ml/min; detection, 350 nm. Peaks: 1 = DNP-taurine; 2 = DNP-cysteic acid.

With the aim of obtaining quantitative results during the analysis of complex samples, we spiked aqueous extracts with different amounts of taurine. The results obtained clearly demonstrated the possibility of using the developed method for quantitative analysis (Table 1). Typi-

Table 1
Determination of taurine in fish extract ($n = 6$)

Concentration added (mg/l)	Concentration determined (mean ± S.D.) (mg/l)	Coefficient of variation (%)	Recovery (%)
0	1350 ± 5	4	–
200	1560 ± 16	5	105
500	1852 ± 24	0.5	100
700	2005 ± 18	0.3	101

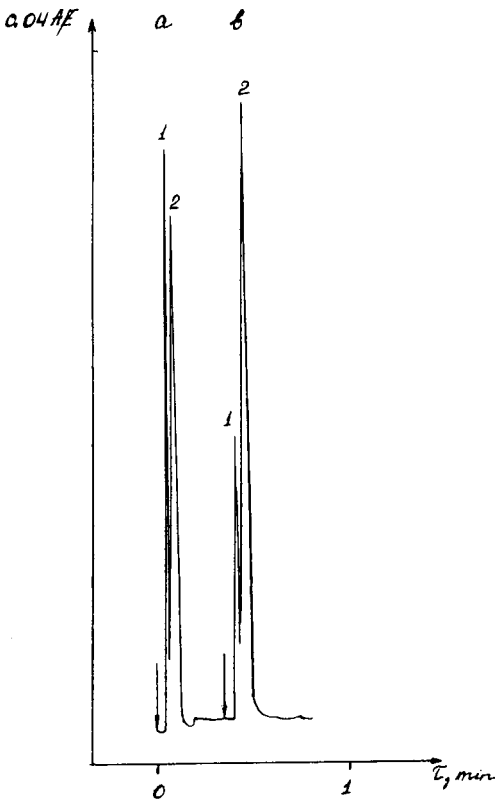

Fig. 2. Ion-pair reversed-phase separation of DNP-taurine and DNP-cysteic acid. (a) Standard mixture; (b) aqueous extract from tuna meat. Eluent, 0.01 *M* sodium 1-octanesulphonate in 10% aqueous methanol; flow-rate, 5.0 ml/min; detection, 350 nm. Peaks: 1 = DNP-taurine; 2 = DNP-cysteic acid.

cal recoveries were 100 ± 3% and the calibration graphs were linear and similar for both standard solutions and spiked extracts. The results obtained for taurine in the infant formula Nutrilon agreed with the manufacturer's data (35 and 36 mg per 100 g, respectively).

The results obtained with the developed technique were also confirmed using an alternative method, ion-exchange chromatography with ninhydrin detection (amino acid analyser). This approach was successfully applied to the analysis of fish aqueous extracts and technological solutions from different stages of taurine separation and purification. The results obtained were compared with those obtained for the same samples using the amino acid analyser. The two techniques gave similar results. However, the amino acid analyser is less sensitive and the analysis is more time consuming.

The described technique was also used for screening fermentation media of different bacterial strains with the aim of detecting potential taurine producers. However, the levels of taurine were found to be around 1 mg/1, which is not sufficient for industrial application.

The method described in this paper may be used for the assay of taurine in different samples of biological and technological origin. Its sensitivity may be increased, for example, by concentration of the derivitized sample, by evaporation and subsequent dissolution in a small volume, by increasing the volume injected or by using fluorimetric detection. However the sensitivity of 10 pmol of taurine (signal-to-noise ratio = 10) is sufficient for most purposes.

4. References

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