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Quantitative determination of taurine in real samples by high-performance anion-exchange chromatography with integrated pulsed amperometric detection

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Abstract

As taurine is a very important compound involved in a large number of metabolic processes, it is naturally present in the mammal tissues and is often deliberately added in some foods as a fortifying component. A detailed knowledge of taurine metabolic roles in biological systems can be obtained only if a sensitive, reliable and rapid analytical method is available. This article describes the successful application of high-performance anion-exchange chromatography coupled with integrated pulsed amperometric detection (HPAEC-IPAD) for taurine determination in egg white and yolk samples, as well extracts of human serum and urine. Applications are shown for determination of taurine in soft drinks and pharmaceutical preparations where the taurine content was evaluated by standard additions. These results were achieved without prior derivatization of taurine.

Keywords: Taurine; Ion chromatography; Integrated pulsed amperometric detection; Egg; Biological samples; Beverages

1. Introduction

Commonly referred to as an amino acid, taurine contains a sulfonic acid group, rather than the carboxylic acid moiety present in other amino acids. This is probably one of the reasons why taurine is not incorporated into proteins, even though it is one of the most abundant free amino acids in many tissues, including skeletal and cardiac muscle, and brain. In the body, taurine is synthesised from the essential amino acid methionine and its related non-essential amino acid cysteine. Taurine is known to play an important role in numerous physiological [1] and pharmacological functions [2]. Among the physiological roles attributed to taurine are membrane stabilisation [3] antioxidation [4], and neuromodulation [5]. While conjugation of bile acids is perhaps its best-known function, this accounts for only a small proportion of the total body pool of taurine in humans. Other metabolic actions of taurine include: detoxification, osmoregulation, and modulation of cellular calcium levels. Taurine has been used, *inter alia*, in the treatment of a wide variety of clinical conditions, including cardiovascular diseases, epilepsy and other seizure disorders, macular degeneration, Alzheimer's disease, hepatic disorders, and cystic fibrosis [6]. Interestingly, intracellular taurine concentration is stringently controlled [7] and plasma levels are affected during trauma [8], sepsi [9] and cancer [10]. Therefore, as the role of taurine in various diseases has become more widely recognised, the need for a simple, rapid assay for routine estimation of this compound in plasma, urine and foodstuffs has increased.

Recently, much effort has been devoted to determination of taurine in medicinal granule, nutrient capsule and human urine by ion chromatography with electrochemical detection, which does not require any derivatization process [11]. More recently, the experimental conditions in high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD) were optimised and successfully applied to the analysis of milk samples [12]. Such a method has been demonstrated to be suitable for assaying taurine content at low micromolar levels. Pulsed amperometry is based on the application of a multistep potential waveform with a repeating sequence (detection,

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cleaning and conditioning pulses) and current measurement during a short sampling interval [13,14]. Thus, reaction products that would cause electrode fouling are removed upon application of an appropriate sequence of positive and negative potential pulses. The long-term stability and reproducibility of pulsed amperometric detection makes it one of the most widely used detectors in liquid chromatography [15]. Here, we illustrate how such a method of analysis can be used to provide a quantitative evaluation of taurine in a comprehensive set of samples, including urine and serum; the method is suitable for enzymatic and clinical studies as well. Eggs were also chosen as real samples because most determinations of taurine have been focused on biological fluids as sample matrices.

2. Materials and methods

2.1. Chemicals

Taurine (2-aminoethanesulfonic acid) (99%), sodium acetate (99%), carbonate-free sodium hydroxide (50%, w/w), barium acetate (99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Stock solutions were prepared with pure water supplied by Milli-Q RG unit from Millipore (Bedford, MA, USA). All the reagents used in this study were of the highest purity available. The sodium hydroxide solutions used as eluents were prepared by diluting a concentrate NaOH solution in water that was previously filtered with a 0.45 μm membrane and degassed with nitrogen.

2.2. Instrumentation

All chromatographic analysis were performed on a Dionex system (Sunnyvale, CA, USA) equipped with a metal-free isocratic pump (model IP 20), a Rheodyne injection valve (model RH9125, Cotati, CA, USA) with a 50 μL loop, and a pulsed amperometric detector (model ED40). The flow-through detection cell is made from a 1.0 mm diameter gold working electrode and a standard combination pH-Ag|AgCl reference electrode using the half-cell Ag|AgCl as the reference electrode; the counter electrode consisted of a titanium cell body across the 25 μm channel formed by a gasket. Separations were accomplished on a Dionex column, CarboPac PA1 (250 mm × 4 mm i.d.) coupled with a guard column (50 mm × 4 mm i.d.) of the same filling. Some experimental work was also carried out using an a CarboPac PA10 (250 mm × 4 mm i.d.) column coupled with a guard column (50 mm × 4 mm i.d.) of the same filling. The column temperature was regulated by using a home-made water jacket coupled with a circulating water bath model WK4DS from Colora (Colora, Messtechnik GmbH, Germany). The mobile phase solution consisted of 100 mM NaOH and 1 mM barium acetate, isocratically eluted at a flow rate of 1.0 mL/min. The plastic reservoir bottles (DX 500 2-L bottles, Dionex) were closed and pressurised with pure nitrogen to 0.8 MPa. As previously described [12], a six-potential waveform was employed for taurine detection. The system was interfaced, via proprietary network chromatographic software (PeakNet^{TM}), to a personal computer for instrumentation control, data acquisition and processing (Dionex). A centrifuge model ALC Refrigerated Centrifuge PK 120 R (ALC International, Milan, Italy) was used for the sample preparation.

2.3. Eluent preparation

The alkaline eluents were prepared by following the procedure developed in this laboratory. Pure water for the eluent preparation was degassed before use by sparging nitrogen for about 20 min. Barium acetate was dissolved and, upon addition of the proper amount of labelled carbonate-free 50% (w/w) NaOH, the eluent solution was kept in plastic bottles and a Dionex eluent organiser (EO1) was used to blanket it with N_2 gas to minimise CO_2 adsorption. The analytical column was periodically regenerated (usually every 2–3 weeks) by pumping 5 mM HCl, water, 200 mM NaOH, and then water in that order over a period of 20 min at a flow rate of 1.0 mL/min.

2.4. Samples preparation and assay procedure

In order to prevent the fouling of the analytical columns, we prepared egg samples in the following way: 0.5 g of lyophilised sample were placed in polypropylene containers, suspended in 5 mL of deionised water and shaken for 15 min in an ultrasonic bath. Next, the mixture was centrifuged at 4100 rpm for 10 min. Finally, the sample solutions were filtered through 0.2 μm nylon membranes (Whatman International Ltd., Maidstone, UK) and then injected.

Eight human serum samples, supplied by anonymous healthy donors, were analysed. The removal of proteins and fats was accomplished using Carrez clearing reagents. Five millilitres of serum was mixed with 0.87 mL of a 7.2% (w/w) $K_4Fe(CN)_6 \times 3H_2O$ water solution and 0.87 mL of a 14.4% (w/w) $ZnSO_4 \times 7H_2O$ solution. After stirring for 15 min, 3.3 mL of a phosphate buffer (pH 7.5) containing 0.1% (w/w) $MgSO_4 \times 7H_2O$ were added. Then, the mixture was centrifuged at 4100 rpm for 20 min at 4 °C; the sample solutions were filtered just prior to injection with 0.2 μm nylon membranes and diluted with deionised water (1:4).

Eight human urine samples, kindly provided by healthy volunteers from our group, were analysed. These samples were collected at 7:30 a.m. and stored at −20 °C until used. The sample cleanup was carried out using classical Sep-Pak C_{18} cartridge (Waters SpA, Milan, Italy), preconditioned by sequential treatment with methanol (2 × 5 mL) and water (2 × 5 mL). An aliquot of 5 mL of each sample, previously diluted with water (1:5), was passed trough the preconditioned cartridges. Hence, the final eluate (2 mL) was collected, properly diluted with deionised water and injected.

A soft drink sample produced by Red Bull S.r.l. and a pharmaceutical preparation called O-DUE by Teofarma S.r.l. (Pavia, Italy) were also analysed. These samples were prepared by simple dilution with water (i.e. up to 10,000-fold) and filtration on 0.2 μm nylon membranes. A stock solution of taurine in water was prepared and stored in a refrigerator (4 °C) at a concentration of approximately 1 mM. The content of taurine in all analysed real samples was obtained by standard additions.

3. Results and discussion

3.1. Assay of taurine in foodstuffs

Details of the analytical method to determine taurine by ion-chromatography with pulsed electrochemical detection have been described elsewhere [12]. To demonstrate the feasibility of the method, five different egg samples were analysed and taurine content was evaluated by standard additions. Before illustrating the results obtained with real samples, data relevant to the calibration parameters were evaluated. Standard solutions for taurine were between 0.3 and 130 μM and the curve was found to be linear over the investigated concentration range, with correlation coefficients >0.9995. The LoD, defined as the lowest analyte concentration that can be clearly detected, was estimated as three times the signal-to-noise ratio; the relevant value was 0.050 μM, considerably lower than the values (~0.8–40 μM) established by using chromatographic methods in which pre-column derivatization of taurine is employed [16–18]. The precision was tested by repeating the same egg sample analysis four times. The relative standard deviation within a day was 2.3%, while this value increases up to 4.6% when the same experiment was repeated on five different days. The recovery data were evaluated by spiking a representative sample of egg yolk with pure taurine at the level of 50–100% of the measured content; results ranged from 96.0 to 97.5%.

A representative chromatographic profile of a yolk sample is shown in Fig. 1 and the taurine levels found in both yolk and albumen are listed in Table 1. The taurine peak was identified by retention time and standard addition; moreover the taurine peak purity in the samples analysed was confirmed by performing a series of chromatographic runs in various experimental conditions (data not shown), namely using a different mobile phase and a second anion-exchange column, as reported in the Section 2. Whereas significantly higher level of taurine were obtained in yolk with a content of 750 ± 10 μg/100 g of freeze dried matter (FDM), a lower level was observed in albumen, where the taurine content was 98 ± 6 μg/100 g of FDM. Although the chromatogram is relatively complex, due to the high variety of free amino acids and other substances naturally present in the sample, the chromatographic conditions allow a quite good separation of taurine from endogenous compounds. We found only one reference dealing with the taurine content in eggs [19], but no evidence of taurine was reported in eggs as well as in bean, rice and other plant food; so no comparison with the present data is possible.

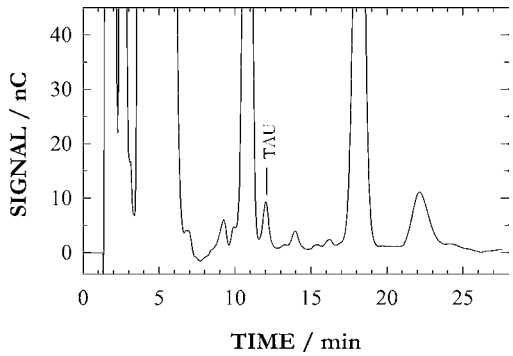

Fig. 1. Separation in HPAEC with integrated pulsed amperometric detection of a egg yolk sample. Eluent, 100 mM NaOH + 1 mM $Ba(OAc)_2$ at a flow rate of 1.0 mL/min. Column and guard column, Dionex CarboPac PA1. Column temperature, 15 °C. Volume injected, 50 μL. A six potential waveform previously described [12] was applied at a gold working electrode.

3.2. Assay of taurine in biological samples

As already mentioned, taurine is involved in a variety of physiological functions, pharmacological actions and pathological conditions [20]. Taurine is present in relatively high concentrations (i.e. up to 65 mM) in biological fluids and tissues [21]. Recent evidence suggests that the level of taurine in plasma may be a useful indicator of myocardial stroke [22]. On this basis, the developed HPAEC-IPAD method was employed to determine the taurine level in some samples of human serum, obtained from normal volunteer subjects (four men and four women). A typical chromatographic separation of taurine in a sample of human serum is shown in Fig. 2. Taking into account the complexity of the sample, a good separation of taurine along with a well-resolved peak at 12.1 min was obtained. The sample was diluted four-fold with water before injection. The free taurine level in serum samples was 98 ± 12 μmol/L, which is in good

Table 1
Occurrence of taurine (mean value ± S.D.) in real samples evaluated by HPAEC-IPAD[a]

Sample	Taurine content (n)[b]
Egg white[c]	98 ± 6 (5)
Egg yolk[c]	750 ± 10 (5)
Human serum[d]	98 ± 12 (8)
Human urine[d]	1200 ± 450 (8)
Red bull energy drink[e]	4.06 ± 0.05 (3)

[a] The chromatographic conditions are those described in Fig. 1.
[b] Number of replicates.
[c] Concentration value expressed as μg/100 g of freeze dried matter.
[d] Concentration value expressed as μmol/L.
[e] Concentration value expressed as g/L of product.

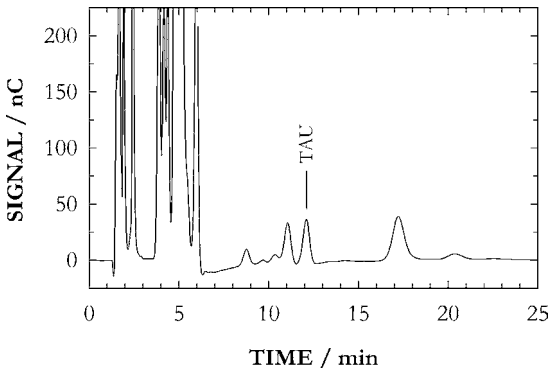

Fig. 2. HPAEC-IPAD chromatogram of a human serum sample (healthy volunteer), diluted 1:4 with water. Other experimental conditions as in Fig. 1.

agreement with that reported by Tcherkas et al. [23], namely 102 ± 17 μmol/L in healthy human subjects.

According to Jacobsen and Smith [24], when the level of taurine in the human serum exceeds 250 μmol/L, a possible metabolic dysfunction is present. Taurine is considered to be a major-product of sulphur amino acid metabolism [25], which may be excreted in the urine. As reported by Waterfield et al. [26–28] the level of urinary taurine may provide a useful, non-invasive marker of hepatotoxicity and alteration in sulphur amino acid and protein metabolism, because these conditions affect urinary and liver concentration of taurine. Urine samples from eight healthy volunteers (four males and four females) were analysed. In Fig. 3, a typical chromatogram is illustrated. Again, the peak of taurine is well resolved and the signal can be used for quantification purposes. The extraction efficiency in biological samples, both urine and serum, was evaluated from the recovery of taurine, using spiked and unspiked samples treated in the same way throughout the whole procedure. Recoveries of taurine were equal to $94 \pm 4\%$. The average level of taurine in human urine samples was in the range of 0.6–1.6 mmol/L, and no significant differences were observed between urine samples of male and female. These values are slightly higher than data reported by Waterfield [21], ranging from 0.02 to 1.0 mmol/L. As reported in our previous work [12] and

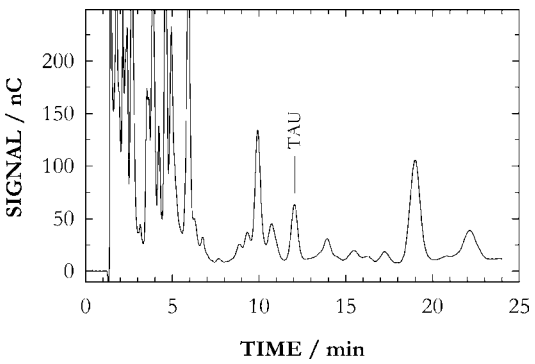

Fig. 3. Separation by HPAEC-IPAD of a 27-year-old woman urine sample (healthy volunteer), diluted 1:40 with water. Other conditions as in Fig. 1.

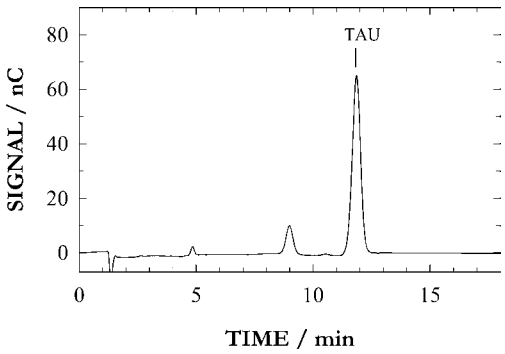

Fig. 4. HPAEC-IPAD chromatogram of a commercial pharmaceutical formulation. Other conditions as in Fig. 1.

as already described for egg samples, the identity and peak purity of taurine in urine samples were confirmed by performing a series of chromatographic runs in different elution conditions.

3.3. Determination of taurine in beverages and pharmaceutical preparations

As noted in the introduction, taurine can be used in a variety of clinical treatments. Fig. 4 shows the chromatographic separation of taurine in a pharmaceutical formulation, administered to care for ischemic pathological disorders. Upon simple dilution with water (1:10,000 final dilution) and filtration on 0.2 μm membrane filters, the content of taurine was easily established, 213 ± 10 mg ($n = 5$) per vial, which corresponds to ~106% of the labelled amount.

Fig. 5 is an additional illustration of this powerful technique. This figure shows the excellent selectivity of HPAEC-IPAD when applied to a soft drink containing some water-soluble compounds (taurine, glucuronolactone, caffeine, vitamins and simple carbohydrates). In the last ten years, there has been an increasing demand for non-alcoholic drinks, such as Red-Bull, that are marked to athletes. Such drinks contain useful substances like taurine, which favours the cardiac contractility [29,30]. Apparently, upon extreme physical exertion, the body no longer

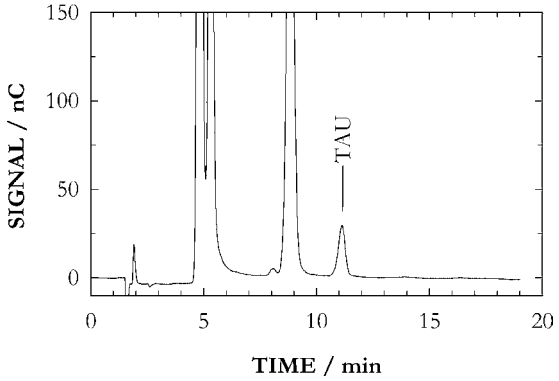

Fig. 5. HPAEC-IPAD separation of a Red Bull energy drink sample. Column temperature, 25 °C. Other experimental conditions as in Fig. 1.

produces the required amounts of taurine, and a relative deficiency occurs. Taurine acts as a transmitter, and also has detoxifying properties. It seems that the cardiac effects of taurine are probably due to its ability to protect the heart from the adverse consequences of either excessive or inadequate calcium ion levels. As can be seen in Fig. 5, the chromatographic profile is quite straightforward, with taurine well separated from the other peaks; a taurine content of 4.06 ± 0.05 g/L ($n = 3$) was established, in good agreement with the labelled value, which is 4.0 g/L. This result confirms that HPAEC-IPAD can be considered as a useful and cost effective alternative to fluorescent detection following chemical derivatization of taurine for the quality control of taurine-containing non alcoholic drinks.

4. Conclusions

The usefulness of the HPAEC-IPAD technique to determine taurine in foodstuffs, beverages and biological samples, without resorting to derivatization reactions, was demonstrated. The experimental conditions are well suited to satisfy the demand for accurate, reliable and rapid detection of taurine in complex matrices with minimal sample preparation. The primary advantages of this method are accuracy, simplicity and sensitivity, allowing the determination of taurine in different types of samples including human serum and urine.

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