

Taurine determination by capillary electrophoresis with laser-induced fluorescence detection: from clinical field to quality food applications

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Abstract In this work we describe a new method for taurine quantification in plasma by capillary electrophoresis laser-induced fluorescence detection. Taurine is derivatized with fluorescein isothiocyanate at 100°C in 20 min. These conditions allow to reduce the pre-analytical times and to derivatize quantitatively the taurine contained in the reaction mixture, contrary to the room temperature derivatization commonly adopted. FITC-taurine adduct is analyzed in an uncoated fused-silica capillary, 75 µm ID and 40 cm effective length using a 20 mmol/L tribasic sodium phosphate buffer pH 11.8, at 22 kV. To avoid the typical problems due to instability of FITC-adduct, we use the homocysteic acid as internal standard. The loss of FITC-taurine signal during the sequence analysis is compensated by the same loss of FITC-internal standard adduct, thus giving a noteworthy improvement in the assay precision. The method shows a good reproducibility of the

migration times (coefficient of variation, CV%, 1.93) and the peak areas (CV%, 3.65). Intra- and interassay CV were 4.63 and 6.44%, respectively, and analytical recovery was between 98.1 and 102.3%. Assay application was tested measuring taurine plasma levels in 50 healthy volunteers in which a mean value of 60.2 ± 17.9 µmol/L was found. Moreover, the applicability of the method was also checked on energy drinks and milk.

Keywords Taurine · FITC · Capillary Electrophoresis

Abbreviations

CE Capillary electrophoresis
LIF Laser-induced fluorescence
FITC Fluorescein isothiocyanate

Introduction

Taurine (2-amino ethanesulfonic acid) is a sulfur-containing amino acid largely distributed in mammal tissues, where it is present in millimolar concentrations, while in body fluids it is at micromolar levels (Waterfield 1994). It has been demonstrated that taurine not only has physiological functions, acting as a neurotransmitter, membrane stabilizer, antioxidant, modulator of intracellular calcium levels and osmolyte, but also possesses pharmacological properties, being able to protect the liver and benefit the gallbladder, lowers blood pressure and increases anti-arrhythmia (Tachiki et al. 1977; Perry et al. 1975; Pasantes-Morales and Cruz 1984; Milei et al. 1992; Franconi et al. 1982; Militante and Lombardini 2000; Huxtable 1992; Franconi et al. 2004). Recently, it has also been shown that taurine is an agonist of glycinergic

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neurons (Kilb et al. 2002). Taurine levels depend on age (Hardy and Norwood 1998) and on animal species; for example, human and non-human primate species and cats have a very limited ability to synthesize taurine, so they are prone to develop taurine deficiency being largely dependent on an exogenous source (Tachiki et al. 1977). Changes of taurine levels in physiological fluids and tissues also have a close relationship with many illnesses such as Alzheimer's disease, cardiovascular diseases, cancer, epilepsy, retinal degeneration, growth retardation and diabetes mellitus (Tachiki et al. 1977; Perry et al. 1975; Mou et al. 2002; Bhatnagar et al. 1990; Tenaglia and Cody 1988; Jeevanandam et al. 1990; Gray et al. 1994; Stocchi et al. 1989). Recently, it has been reported that taurine supplementation in diabetic rats prevents mortality rate without influencing the body weight (Kishida et al. 2003), whereas clinical data suggest that taurine administration could be useful in the treatment of type 1 DM (Franconi et al. 2004). At present, taurine is added to cow's milk and to soy protein-based milk used for the preparation of infant formula (Aerts and Van Assche 2002) and to parenteral nutrition solutions for premature babies (Huxtable 1992). For these reasons, literature calls for new, rapid and sensitive methods for taurine detection in human plasma and other biological fluids. Currently, available analytical methods for the determination of taurine alone or with other amino acids are based on HPLC. Detection was performed principally after *o*-phthaldialdehyde (OPA) derivatization, but other derivatizing reagents are used as well as 2,4-dinitrofluorobenzene, fluorescamine and dimethylaminonaphthalene-5-sulfonyl chloride (Mou et al. 2002). Indeed, capillary electrophoresis methods have been also described using OPA as derivatizing reagent or other reagents such as dansyl chloride, naphthalene dicarboxaldehyde (NDA) or fluorescamine (Sturman 1993; Albin et al. 1991; Amiss et al. 1990; Ueda et al. 1992; Weber et al. 1994a, 1994b). However, since both the HPLC method and capillary electrophoresis method require long derivatization times and complex instrumentation, produce excessive side products and have poor sensitivity, the application of these methods on a large number of samples is prevented. The aims of this paper is the development of an easy and sensitive taurine assay for the application on a large number of samples required for clinical studies. In doing that we used FITC as fluorophore that was largely used in the past for amino acid quantification by capillary electrophoresis (Ueda et al. 1991; Poinot et al. 2003; Bayle et al. 2004; Lacroix et al. 2005), but resolving some common troubles such as the long derivatization times and instability of adducts that were typical of the previous assay.

Materials and methods

Chemicals

Taurine, homocysteic acid, Na_3PO_4 , Na_2HPO_4 , NaOH, trichloroacetic acid (TCA), FITC (isomer I) were obtained from Sigma (St Louis, USA). The 0.45 μm membrane filters (used to filter all buffer solution before CE analysis) were purchased from Millipore (Bedford, USA). Vivaspin 500 microconcentrators (cut-off M_r 10,000, membrane MWCO PES) were obtained from Vivascience AG (Hannover, Germany).

Preparation of samples

Blood was collected after an overnight fast by venipuncture into evacuated tubes containing EDTA and immediately centrifuged at $3,000g \times 5$ min at 4°C . A volume of 50 μL of the obtained plasma was mixed with 50 μL IS homocysteic acid (200 $\mu\text{mol/L}$) and 100 μL of TCA (10%) was then added to precipitate the proteins. After centrifugation at $3,000g$ for 5 min, 10 μL of clear supernatant was mixed with 90 μL of 100 mmol/L Na_2HPO_4 of pH 9.5 and 11 μL of 15 mmol/L FITC. After 20 min incubation time at 100°C , the samples were diluted 100-fold and injected in CE.

Capillary electrophoresis

Analysis of taurine was performed by a CE system (P/ACE 5510) equipped with a laser-induced fluorescence (LIF) detector (Beckman, Palo Alto, CA, USA). The system was fitted with a 30 kV power supply with a current limit of 250 μA . Analysis was performed in an uncoated fused-silica capillary, 75 μm I.D. and 47 cm length (40 cm to the detection window), injecting 18 nL of sample (3 s \times 3.5 KPa). Separation was carried out in a 20 mmol/L tribasic sodium phosphate buffer, pH 11.8, 23°C at normal polarity 22 kV (190 μA). After each run, the capillary was rinsed for 1 min with 0.5 mmol/L NaOH and equilibrated with run buffer for 1 min.

Results and discussion

Electrophoretic conditions optimization

In the last few years, we set up several CE-LIF methods for evaluation of thiols in different biological samples by using iodoacetamidofluoresceine (IAF) as an effective fluorophore (Poinot et al. 2006; Zinellu et al. 2003, 2004, 2005a,

2005b, 2005c; Carru et al. 2004. By using our previous CE expertise, we succeeded in detecting FITC-aurine adduct in the basic conditions already employed for IAF-thiols adducts by using sodium phosphate tribasic as run buffer. To optimize the electrophoretic separation of taurine, pilot experiments were carried out by using a capillary with an effective length of 40 cm (total length 47 cm). Phosphate run buffer at different concentrations (from 5 to 50 mmol/L) and different pH values (from 10 to 12 pH units) was employed. Moreover, we tested the effect of cartridge temperatures for increments of 3°C from 20 to 50°C. As reported in Fig. 1, the optimal conditions for taurine detection, together with homocysteic acid selected as internal standard, were found by using a 20 mmol/L tri-basic sodium phosphate pH 11.8, 23°C, 22 kV at normal polarity.

Derivatization conditions optimization

Based on the literature data, FITC derivatization needs long incubation times from 14 to 17 h (Zinellu et al. 2006; Lau et al. 1998; Paez et al. 2000; Rada et al. 1999; Zhang et al. 2001; Li et al. 2003), but determination of amino acids after a 2 h derivatization procedure has been also reported (Pobozy et al. 2006; Nouadje et al. 1995). The only work describing taurine quantification by using FITC as a derivative reagent proposes a 6 h derivatization at 40°C (Arlt et al. 2001). In order to reduce the pre-analytical times, we evaluated the possibility of accelerating the reaction by increasing the incubation temperature. As reported in Fig. 2, the efficiency of derivatization strongly depends on temperature and time. Only at 80 and 100°C temperatures, reaction plateau was reached, while at room temperature and at 40°C the reaction was largely incomplete. At 100°C the reaction should be considered quantitative in about 20 min. Beyond 1 h incubation at this

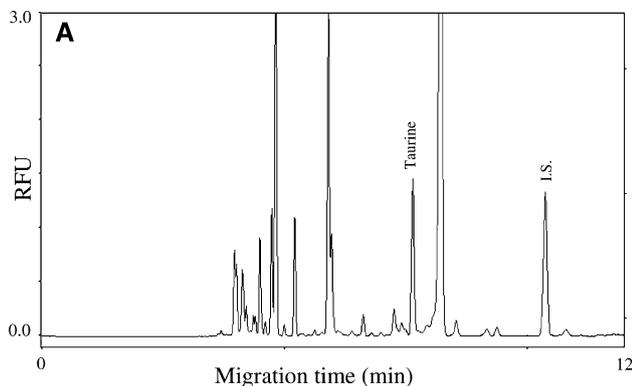


Fig. 1 Typical electropherogram of standard taurine (100 µmol/L) and homocysteic acid (100 µmol/L)

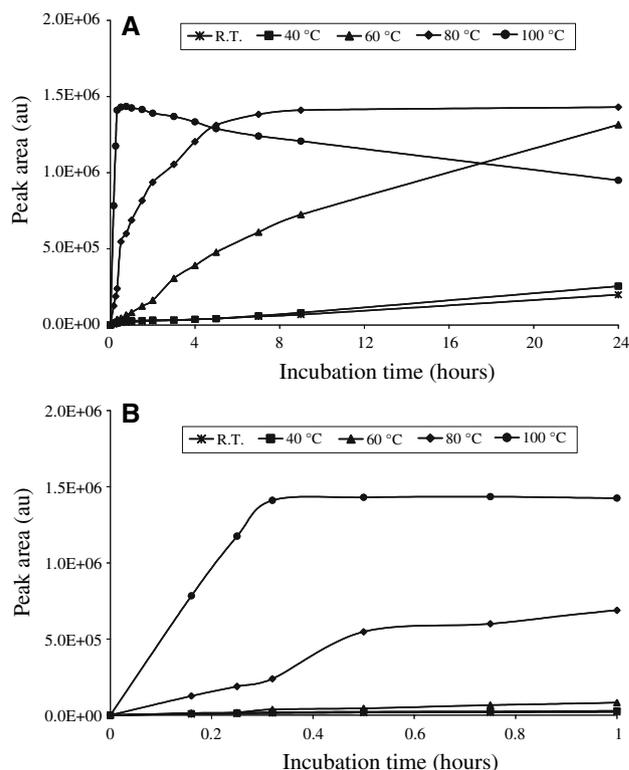


Fig. 2 Reaction trend between FITC and taurine contained in a typical plasma sample performed at different incubation temperatures in a 100 mmol/L sodium phosphate buffer pH 9.5, over 24 h (a). An expanded plot of derivatization curves during the first hour is reported in (b). Results are means of three replicates (CV% < 7%). RT room temperature

temperature, the adduct was unstable and fluorescence signal decreased. Therefore, the possibility of stabilizing the adduct through variation of pH and of phosphate buffer concentration used for sample derivatization was examined. A 100 mmol/L of phosphate buffer at pH 9.5 was the optimal condition because many additional FITC hydrolysis products appeared at higher concentrations of phosphates and higher pH values, making the identification of analyte signals more difficult (data not shown).

The quantity of FITC needed for an optimal derivatization was also investigated and Fig. 3 showed that, for a typical plasma sample, the derivatization mixture must have a final FITC concentration of 1.5 mmol/L to carry out the complete analyte derivatization.

Sample stability

We checked the stability by studying: (1) the heat stability of taurine during the incubation at 100°C; (2) the stability of FITC-aurine adduct during the sequence of analysis. First we pre-incubated the sample at 100°C in the derivatization mixture without FITC for 0, 20, 40 and 60 min.

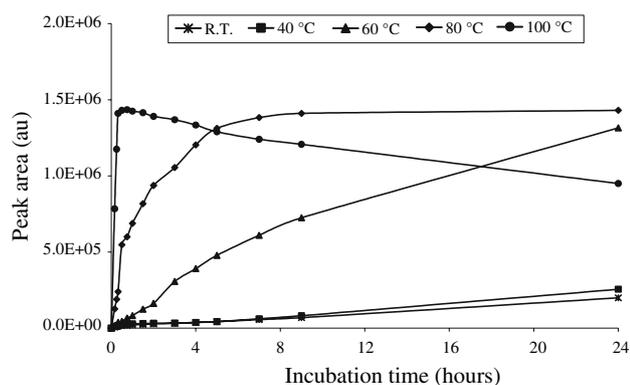


Fig. 3 Effect of FITC concentration on taurine derivatization in a typical plasma sample. Reaction was performed for 20 min at 100°C in a 100 mmol/L sodium phosphate buffer of pH 9.5. Results are means of three replicates (CV% < 7.3 %)

Successively, the sample was mixed with FITC and incubated for 20 min at 100°C and analyzed in CE. No differences were found during the first 40 min of pre-incubation, while a slight decrease (about 3%) was found after 60 min of pre-incubation. These data are consistent with previous observations in which longer incubation times at 100°C or higher temperatures were needed to detect a significant loss of taurine in the sample (Zhang et al. 1998). Since it has been largely described in literature that FITC was an unstable chromophore, we decided to use an internal standard to improve the precision of the assay. After derivatization, the samples were stored in the autosampler at room temperature. Routinely, we measured a series of 40 samples, requiring 10 h total analysis time. Although peak areas of FITC-*taurine* adduct decreased by approximately 10% over this period, this loss of response was fully compensated by the internal standard. Consequently, no significant differences between the concentrations of analytes in the quality control sample, analyzed at the beginning and at the end of each series of samples, were observed. In addition, when an autosampler with a thermostat was employed, the stability of the samples could be further improved.

Calibration, precision, accuracy and limit of quantification

Calibration curves obtained as ratio of peak areas of taurine to that of internal standard versus concentration were linear in the concentrations range tested between 10 and 200 $\mu\text{mol/L}$ (calibration curve $Y = 0.015X + 0.191$, $R^2 = 0.9999$). Injection reproducibility was calculated by injecting ten times consecutively the same standard solution. Within-run precision (intra-assay) of the method was evaluated by injecting the same biological sample 10 times

consecutively, while between-run (inter-assay) precision was determined by injecting the same biological sample on 10 consecutive days. Precision tests indicate a good repeatability of our method both for migration times (CV < 1.93%) and areas (CV < 3.65%). Moreover, a good reproducibility of intra-assay and inter-assay tests was obtained (CV < 4.63% and CV < 6.44% respectively). For the assessment of the analytical recovery, the serum pool was spiked with taurine standard solutions at three different concentrations, and the mean of recovery, evaluated by five different experiments, was between 98.1 and 102.3%. The limit of quantification (LOQ) evaluated as signal-to-noise ratio of 10 and calculated by 18-nL injection of sample, was about 1 $\mu\text{mol/L}$.

Method application

Suitability of the method was tested by measuring taurine levels in 50 healthy volunteers (18 females, 32 males; mean age 51 ± 16 years). Figure 4 a and b shows the assay ability to well discriminate among samples with different taurine levels (82 and 23 $\mu\text{mol/L}$, respectively). Mean values for all subject examined were 60.2 ± 17.9 $\mu\text{mol/L}$ and were similar to those already reported in literature (Saidi and Warthesen 1990; Mitani et al. 2006;

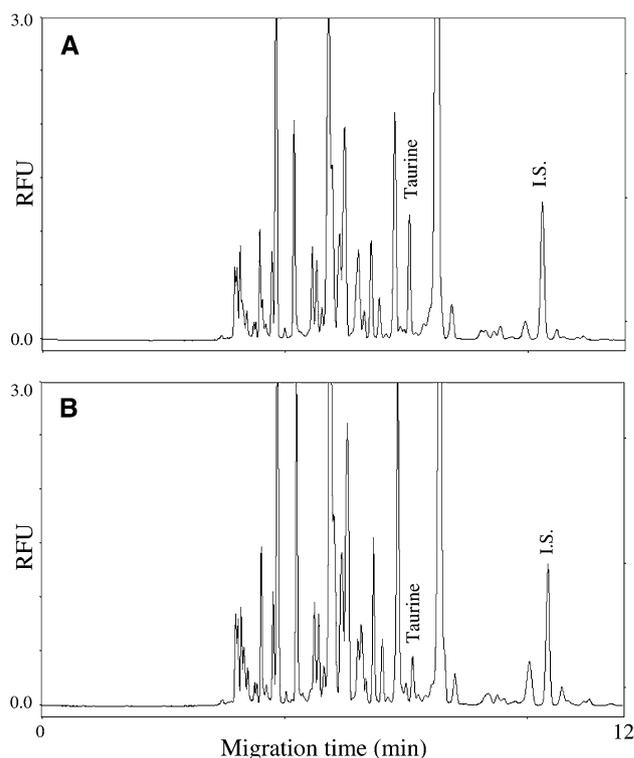


Fig. 4 Representative electropherograms of plasma samples containing elevated (a: 82 $\mu\text{mol/L}$) and low (b: 23 $\mu\text{mol/L}$) levels of taurine

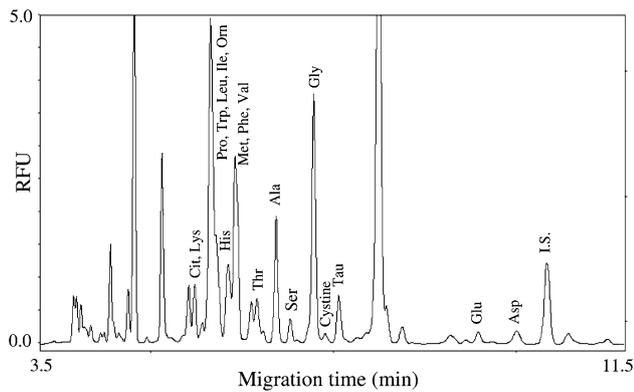


Fig. 5 Electropherogram of a plasma sample showing peak identification of some analytes following amino acid standard co-injection

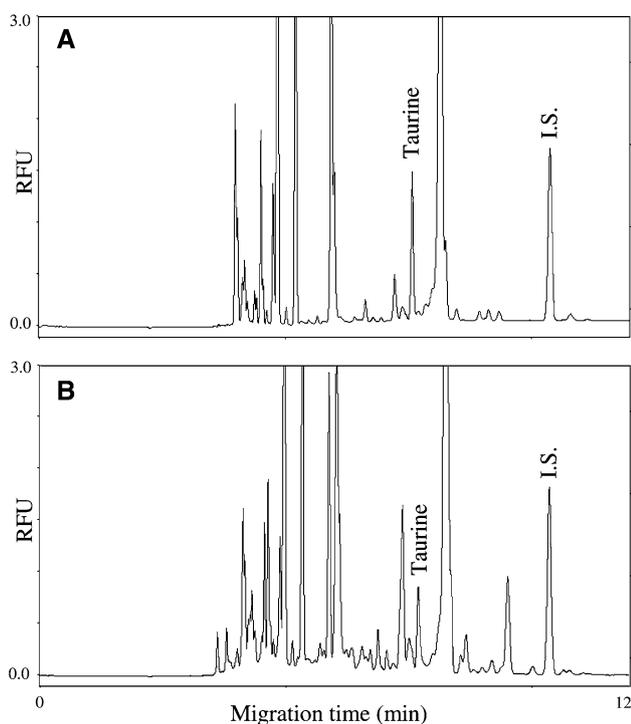


Fig. 6 Electropherogram of taurine contained in energy drink (Red Bull) (a), and Sardinian cow milk (b). The energy drink was diluted 500-fold before analysis

Ward et al. 1999; Obeid et al., 2004; Suliman et al., 2002; vom Dahl et al. 2000). No difference has been found between males ($61.7 \pm 18.1 \mu\text{mol/L}$) and females ($57.6 \pm 17.7 \mu\text{mol/L}$). Moreover, our method allows detecting simultaneously other amino acids like glutamic acid, aspartic acid, glycine, alanine and serine, as described in Fig. 5. The separation is based on the difference between the ionization of taurine and that of other amino acids, due to the fact that taurine is a sulfonic rather than a carboxylic amino acid. Increasing the migration times, by rising the run buffer concentration, allows to resolve, at least in part, also some of the other overlapped analytes.

Since the use of taurine in infant formula, nutritional supplements and energy promoting drinks has been recently more and more encouraged for its beneficial effects, a reliable method to analyze taurine in foods for quality control purpose is also required. For these reasons, we also evaluated the applicability of our assay in other samples like energy drink (Red Bull) and cow's milk in which we proved its suitability (Fig. 6). Taurine concentration in the energy drink (Fig. 6a) measured by our assay (0.43%) is near to the declared values in the drink label of 0.40%.

Concluding remarks

The CZE-LIF method here described is suitable for the analysis of taurine in human plasma and in other samples types, such as foods and drinks. Our method is characterized by high sensitivity and precision. Due to the high sensitivity guaranteed from LIF detection, 20 μL sample volume are sufficient for the analysis of plasma samples, even if we preferred to standardize the assay starting from 50 μL sample to improve the precision of the method. The pre-analytical study of the reaction between taurine and FITC allowed us to drastically reduce the derivatization times. The use of elevated temperatures permits to bring down the time of the reaction at 20 min versus the 6–14 h usually described for the reaction between FITC and amino acids at room temperature. We also described how at low temperatures the reaction is not quantitative and that this may lead to a loss of sensitivity or faulty results. The use of internal standard, which gives the possibility of compensating the typical loss of FITC signal during a sample sequence, allows increasing the method reproducibility and precision. Since taurine analysis is becoming one of the fundamental measurements of biological sciences, with applications in every aspect of biological research, clinical medicine, biotechnology (agriculture, medicine) and food technology, methods able to detect taurine in various samples are required. Thus, we proved the applicability of the assay both in plasma samples and in other kinds of sample, such as energy drink and milk.

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