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Note

Determination of calcium acetylhomotaurinate by liquid chromatography with fluorimetric and electrochemical detection

C. CHABENAT, P. LADURE, D. BLANC-CONTINSOUZA, F. BOISMARE and P. BOUCLY*

Laboratoire de Pharmacochimie, U.E.R. de Médecine et Pharmacie de Rouen, Avenue de l'Université, 76800 St.-Etienne-du-Rouvray (France)

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Calcium acetylhomotaurinate (Ca AOTA, Fig. 1) is a molecule derived from 3-aminopropanesulphonic acid by esterification of the amine function. Then the product is salified by calcium. Previous authors have considered the hypothesis that this new molecule, acting by 4-aminobutyric acid (GABA)-ergic receptor stimulation, reduces the voluntary alcohol intake in animals [1]. The hypothesis has been confirmed by similar observations in humans [2]. Thus, the availability of analytical techniques for the study of the pharmacokinetics of this molecule is important. However, this molecule does not have a specific reaction site, and its high aqueous solubility makes its concentration by solvent extraction difficult. One suggested analytical method involves the hydrolysis of Ca AOTA to homotaurine, a molecule which, because of its free primary amine function, can be determined by fluorescence detection after derivatization. The sensitivity of this method is significantly higher than that of the direct UV detection of Ca AOTA [3].

Amino acids in protein hydrolysates have been analysed by high-performance liquid chromatography (HPLC) as *o*-phthalaldehyde (OPA)-thiol derivatives [4–7]. In the past few years, compounds containing primary amino groups have been determined with high sensitivity and selectivity after reaction with OPA [8,9]. After hydrolysis of Ca AOTA, the method employs precolumn derivatization with OPA, with 2-mercaptoethanol used as the reducing agent.

EXPERIMENTAL

Reagents

All reagents were of analytical grade. All aqueous solutions were prepared in doubly distilled water. Ca AOTA and homotaurine were obtained from Meram

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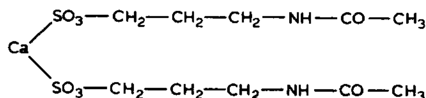

Fig. 1. Chemical structure of Ca AOTA.

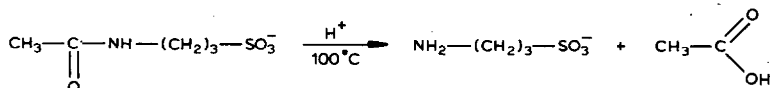

Fig. 2. Hydrolysis of Ca AOTA.

(Paris, France); OPA, methanol and 2-mercaptoethanol were purchased from E. Merck (Darmstadt, F.R.G.).

OPA reagent

A 2 g/l solution of OPA was prepared by dissolving 20 mg of OPA in 0.4 ml of ethanol. The working OPA reagent was prepared by mixing with 20 μl of 2-mercaptoethanol and 9.6 ml of 0.4 *M* borate buffer (pH 10.4). The mixture was stored in the dark at 4°C.

Reference solutions

Stock standard solutions of Ca AOTA and homotaurine were prepared by dissolving appropriate amounts in water. Working standards were freshly prepared in drug-free plasma from the stock solution to yield concentrations from 1 to 40 μg/ml.

Sample preparation

The protein precipitation and hydrolysis, transforming Ca AOTA into homotaurine, were performed before the derivatization procedure.

To 0.5 ml of each plasma sample, 0.5 ml of trichloroacetic acid (20%) was added. The mixture was vortexed and allowed to stand for 10 min. The samples were centrifuged at 1600 *g* for 15 min and the supernatant was collected. An aliquot (0.5 ml) was transferred to a separate tube, and 2 ml of 4 *M* hydrochloric acid were added. All tubes were heated for 2 h at 100°C, and the liquid phase was then dried in a rotary evaporator at 40°C.

The residue was dissolved in 100 μl of 1.22 *M* sodium hydroxide and 200 μl of 0.4 *M* borate buffer (pH 10.4); the mixture was derivatized by addition of 150 μl of OPA reagent and, after vortex-mixing for 20 s, 20 μl of mixture were injected into the HPLC system.

Chromatography

The chromatographic separation was performed isocratically at room temperature with a single-piston reciprocating pump (Model 110 A, Beckman Instruments, Berkeley, CA, U.S.A.). Injections were made with a syringe-loading injector with a 20-μl loop. The analyses were performed on a 30 cm $\times$ 3.9 mm I.D. stain-

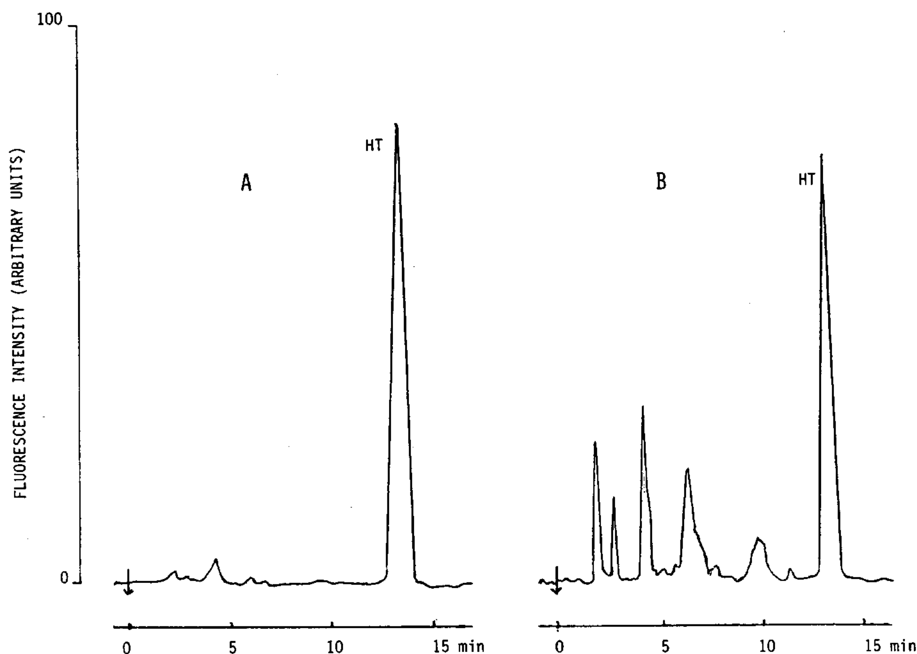

Fig. 3. Chromatograms of (A) OPA-2-mercaptoethanol homotaurine (HT) standard, 2.78 μg/ml (0.5 μA), and (B) OPA-2-mercaptoethanol Ca AOTA (8 μg/ml), hydrolysed to homotaurine (0.5 μA).

less-steel column packed with μBondapak phenyl, 10 μm (Waters Assoc., Milford, MA, U.S.A.).

A model FS 970 LC fluorometer (Kratos, Karlsruhe, F.R.G.) was used as the detector; it was operated with a deuterium lamp, excited at 330 nm with a 418-nm emission filter.

An electrochemical detector Model 641 VA (Metrohm, Switzerland) was placed just after the fluorescence detector; the potential of the working electrode was maintained at 0.70 V vs. Ag/AgCl, and the sensitivity was 50 nA full scale.

The mobile phase was a 62:38 (v/v) mixture of solvent A and solvent B. Solvent A contained 7.9 g/l sodium dihydrogen phosphate monohydrate (pH 5.7). Solvent B was obtained by dissolving 6.9 g of sodium dihydrogen monohydrate in 250 ml of water and making up to 1 l with methanol. After mixing, the mobile phase was filtered through Millipore membrane filter (0.45 μm) (Millipore, Bedford, MA, U.S.A.).

The assays were performed at room temperature and a flow-rate of 2 ml/min. The peaks were recorded on a strip chart recorder (Kipp and Zonen, Delft, The Netherlands).

RESULTS AND DISCUSSION

The hydrolysis under acidic conditions transforms Ca AOTA into homotaurine, with formation of acetic acid (Fig. 2). This deacetylation to form a free

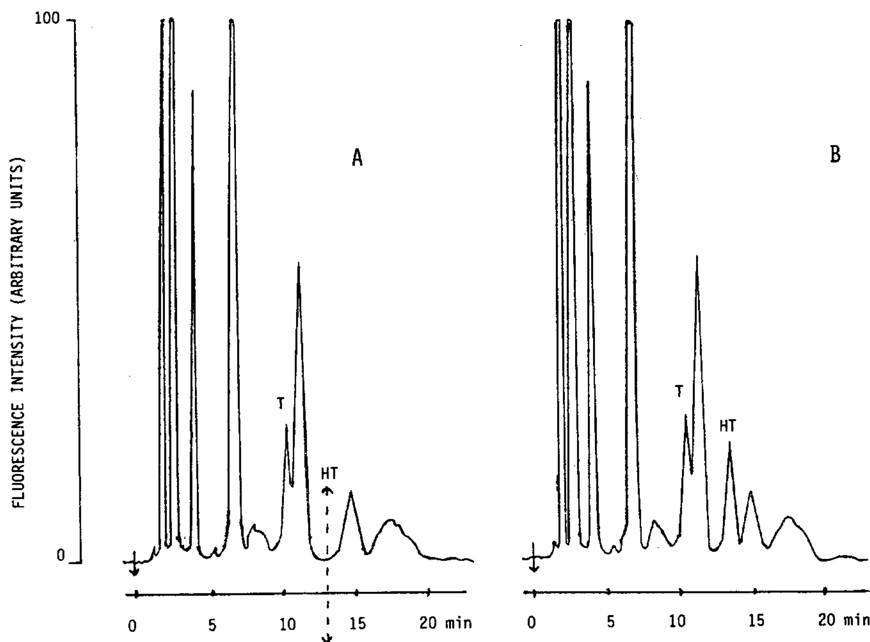

Fig. 4. Chromatograms of OPA-2-mercaptoethanol serum samples, after hydrolysis to homotaurine (1 μA), (A) without Ca AOTA, and (B) spiked with Ca AOTA (40 μg/ml).

primary amine function allowed us to use the classical derivatization reaction [8–14] for the fluorimetric detection of species with a primary amine function. Under alkaline conditions and with a reducing reagent (2-mercaptoethanol) the resulting derivative was fluorescent after UV excitation.

The addition of 2-mercaptoethanol improves the stability of the fluorescent derivatives, but it was still necessary to ensure the same reaction time for all samples in order to obtain good reproducibility.

The instability of the OPA derivatives was attributed to the presence of water in the reaction mixture. In a recent paper [15], a procedure using a non-aqueous OPA–mercaptoethanol reagent was described; the fluorescent derivatives of the drugs were stable at room temperature. However, the solubility requirement in pure methanol is not met in the case of Ca AOTA.

Recently, it was reported by Allison et al. [13] that OPA–2-mercaptoethanol derivatives of amino acids undergo anodic oxidation at moderate potentials, so that liquid chromatography–electrochemistry may be used for their determination.

The chromatograms obtained with fluorimetric and electrochemical detection of OPA–mercaptoethanol homotaurine derivative were similar. The chromatogram of the standard homotaurine solution and that of the standard Ca AOTA solution after hydrolysis and derivatization are reproduced in Fig. 3.

The efficiency of the hydrolysis reaction was determined by comparing the areas of the homotaurine working solution to that of the Ca AOTA solution. This latter solution had half the concentration of the former and the mean (±S.D.) efficiency was 93±3%.

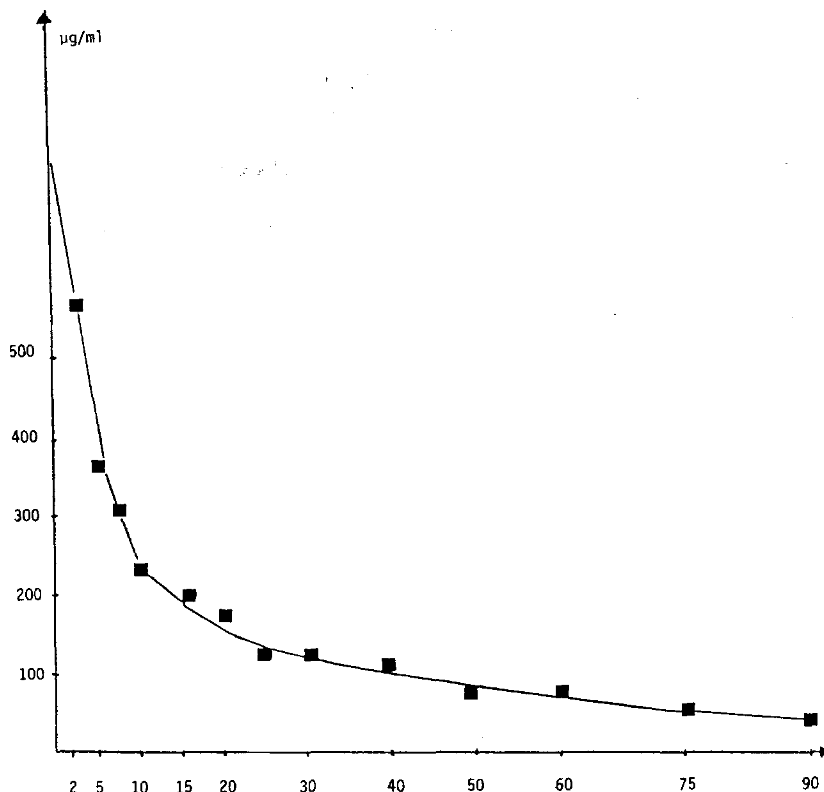

Fig. 5. Plasma concentration–time plot for Ca AOTA injected into a dog (100 mg/kg).

Frequent analyses of serum samples spiked with Ca AOTA compensated for the lack of an internal standard (Fig. 4). Each calibration enabled us to calculate the usual linear regression parameters ($y=3.512x-0.3843$ with $r=0.996$). The variation in these parameters from one series to another was consistently below 5%. The calibration curves were linear, and they passed through the origin. The assay proved to be sensitive with a detection limit of 10 ng of Ca AOTA per ml of plasma at a signal-to-noise ratio of 2. Assay precision was evaluated, and the within-day coefficient of variation was less than 5%. No degradation of Ca AOTA in plasma was observed up to four days.

As shown in the figures, homotaurine was eluted within 15 min, which means that this method is suitable for measuring a large number of samples.

This method has been used for Ca AOTA determination in the serum of dogs pretreated intravenously with this drug. Fig. 5 shows a characteristic plasma level versus time profile following intravenous administration of 100 mg of Ca AOTA per kg to a dog.

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