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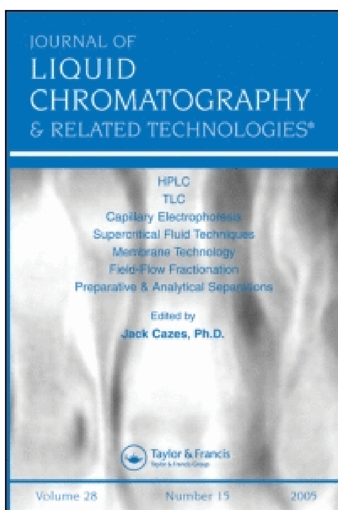

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Separation and Quantitation of O-Phthalaldehyde Derivatives of Taurine and Related Compounds in a High Performance Liquid Chromatography (HPLC) System

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SEPARATION AND QUANTITATION OF O-PHTHALALDEHYDE DERIVATIVES OF TAURINE AND RELATED COMPOUNDS IN A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) SYSTEM

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ABSTRACT

A sensitive specific assay for taurine using high performance liquid chromatograpy and fluorescence measurement is described. The method employs precolumn derivatization with o-phthalaldehyde in the presence of ethanethiol. Taurine is clearly separated from other amino acids including its precursors hypotaurine and cysteine sulfinic acid. The fluorescence peak height is linear between 1 and 100 picomoles of taurine. There is clear separation of taurine from a contaminant of other taurine assays, α-glycerophosphoryl ethanolamine.

INTRODUCTION

Taurine (2-aminoethanesulfonic acid) occurs in large amounts in the central nervous system (1,2), as well as in other tissues such as the heart (1,3). There is increasing evidence that taurine plays an important role in the body. A dietary deficiency of taurine has been shown to cause blindness and retinal degeneration in cats (4,5), indicating it is an essential amino acid in this species. Taurine levels in affected tissues have been reported to be altered in several different pathological conditions. These include epilepsy (6,7) cardiac failure (3) and muscular dystrophy (8). The role of taurine, apart from bile salt metabolism, remains unknown. It has been suggested that taurine may have a role as a neurotransmitter (9,10) or as a membrane stabilizer (11,12).

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A specific quantitative taurine assay is of importance in understanding the physiological role of taurine in the body. It is thus of concern that variable tissue levels of taurine have been reported in the same species when different taurine assays are used (1,13). Moreover acid hydrolysis of the same extracts resulted in decreased levels of taurine measured (13,14). Since authentic taurine is stable after hydrolysis (15), it is likely that there is a contaminant that may have led to overestimatiion of some of the taurine levels reported. A recent paper by Tachiki and Baxter (16) has examined this problem. They reported that in commonly used taurine assays, using amino acid analysis with a single column, the taurine fraction is contaminated by α-glycerophosphoryl ethanolamine. This was a major contaminant in amphibian brain, but also of probable significance in mammalian brain (14,16). Hence the taurine distribution in different tissues has to be reassessed.

Thus there is a need for a specific assay for taurine that separates it from α-glycerophosphoryl ethanolamine, as well as from structurally related compounds. The combination of HPLC and fluorescence detection is a powerful new tool for the assay of amino acids. We report a new specific HPLC fluorescence assay for taurine in the picomolar range.

The method described here utilizes the reaction of amino acids with a mixture of o-phthalaldehyde (OPT) and ethanethiol (ETSH) to form a highly fluorescent isoindole (Figure 1).

Ethanethiol was used rather than 2-mercaptoethanol, as it has been reported to be more stable in the above reaction than the latter (17). This method involves precolumn derivatization of the amino acids, followed by HPLC separation on a reversed phase column which eliminated interference by the compounds listed above.

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REACTION OF OPTA WITH ETSH AND AMINO ACID

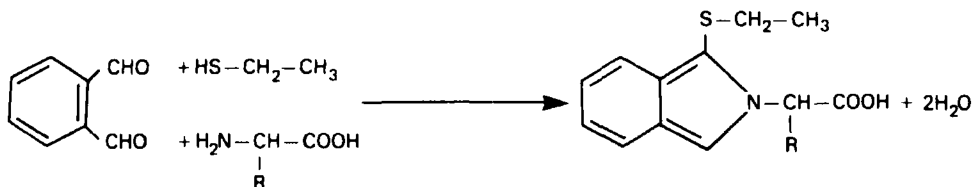

Figure 1. The reaction of o-phthalaldehyde (OPT) with ethanethiol (ETSH) and amino acid to yield a fluorescent isoindole.

MATERIALS

Apparatus

The high performance liquid chromatographic system (Waters Associates, Milford, MA) consisted of two pumps, (model 6000 A), a solvent programmer (model 660), and an injector (model UGK). A fluorometric detector (Schoefell Westwood, NJ. model FS-970) with a 5 μl flow cell was employed. The samples were excited at 229nm and a cut off filter (470nm, Schott Optical Glass Co., Duryea, Pennsylvania) was used on the emission side. It was equipped with a μ Bondapak C_{18} colum (Waters Associates).

Chemicals

The following solvents were used, Acetonitrile HPLC grade (Fisher Scientific Co., Fair Lawn, NJ.) and Methyl alcohol HPLC grade (Waters Associates, Milford, MA). Water was purified using a Milli-Q Water Purification System (Millipore Corp. Bedford, MA). The following chemicals were purchased: o-phthalaldehyde (Pierce Chemical Co. Rockford, IL): ethanethiol (Aldrich Chemical Co. Milwaukee, WI); Na_2HPO_4 ACS grade (Fisher Scientific Co.); $NaHPO_4$ $7H_2O$ ACS grade (Baker Chemical Co. Phillipsburg, NJ); taurine, hypotaurine, L-cysteinesulfinic acid, L-cysteic acid, L-cysteine, cysteamine, β-alanine, L-arginine, L-tyrosine, o-phosphoryl ethanolamine, and L-α-glycerophosphoryl ethanolamine (Sigma Chemical Co., St. Louis, MO). O-phthalaldehyde was made up in absolute ethanol, 100mg/10 ml, and stored at -20°C.

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Methods

The method used is modification of the method reported by Stuart, and co-workers (18).

Preparation of Phosphate Buffer

Stock buffer solution was made by weighing out 5.796g of NaH_2 PO_4 and 28.95 gm of $Na_2HPO_47H_2O$. They were dissolved in distilled deionized water from a Milli-Q purification system (Millipore Corporation Bedford MA) and made up to 1 litre in a volumetric flask. Buffer for use was mixed freshly by diluting it 4 fold in water, it was filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA), and then degassed before use on the column. The buffer pH was 7.12, and 0.150 M phosphate, 0.258 M sodium.

Preparation of Borate Buffer

Boric acid was added to 1 litre of hot water to form a saturated solution. The solution was allowed to cool, filtered and then pH adjusted to between 9.2 and 9.5 with NaOH.

Preparation of O-phthalaldehyde/Ethanethiol Derivativing Mixture and Amino Acid Derivative

Daily, ethanethiol was first diluted 10 fold in methyl alcohol. 25 μl (0.25 mg) of ophthalaldehyde was added to 4.5 ml of methyl alcohol, to which 0.5 ml of borate buffer was added. 5 μl of diluted ethanethiol was then added to the mixture, which was mixed well and kept wrapped in aluminium foil till it was used. 200 μl of this derivatizing mixture was then added to a solution made up of 0.4 ml of amino acid solution in methyl alcohol, and 0.4 ml of borate buffer. The mixture was vortexed and after a 10 minute period a 10 μl aliquot was added to 390 μl of sodium phosphate buffer for application to the HPLC column. It should be noted that all operations employing ethanethiol additions were made in a well ventilated hood to avoid the extremely pungent smell of this compound.

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The C_{18} reverse phase column was equilibrated with 9% acetonitrile in phosphate buffer (pH 7.12). 200 μl of the amino acid derivative in phosphate buffer was injected on the C_{18} reverse phase column using a Waters low volume flow loop. The sample was eluted with a linear gradient 9-22% acetonitrile over a 15 minute period, started at the time of injection (see Figure 3), followed by an isocratic period of 15 minutes of 22% acetonitrile. This was followed by a column wash with 50% acetonitrile. At the end of each day the column was washed first with 10% methyl alcohol in water to remove all traces of phosphate from the column, then with 75% methyl alcohol (in H_2O).

Results and Discussion

The o-phthalaldehyde/ethanethiol (OPT/ETSH) derivatization was shown to be a slow reaction reaching equilibruim after 10 minutes (Figure 2). The reaction was slightly slower when the amount of amino acid was increased 10 fold. Hence it is important to wait at least 10 minutes before diluting the sample for chromatography.

The chromatographic system used clearly separates OPT-taurine (Retention time, R_t, 24.5 min) from other related compounds and from OPT-α-glycerophosphoryl ethanolamine (R_t 18.5 min) (Figure 3). The method was highly reproducible, and the OPT-taurine peak

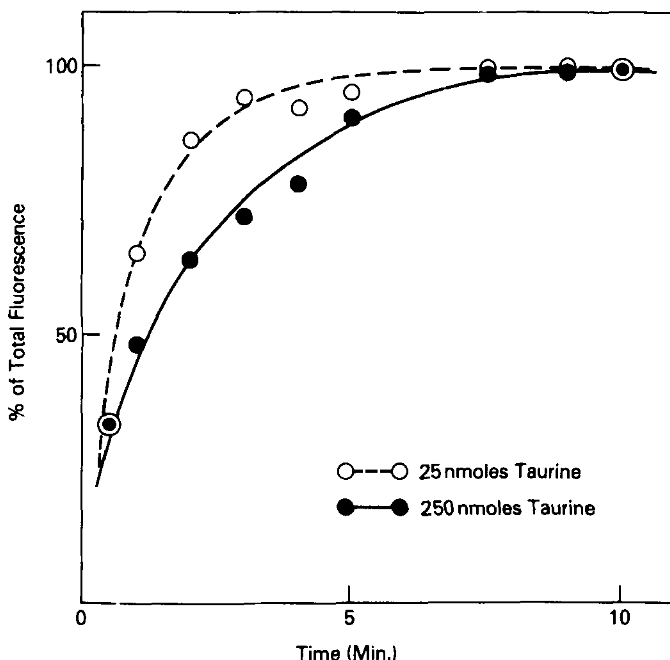

Figure 2. Time course of o-phthalaldehyde-ethanethiol derivatization of taurine at two different amounts of taurine. Excitation wavelength 340 nm, emission 470 nm.

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was found to have little variation in its retention time (Figure 4). The method also provides a quantitative assay for taurine. The assay was found to measure as little as 1 picomole of taurine applied to the column, and was linear between 1-100 picomoles (Figure 5). OPT-hypotaurine was also found to give a linear fluorescence response for increasing concentrations of the amino acid from 1-100 picomoles. However the fluorescence response of OPT-β-alanine was linear between 1 and 10 picomoles. The figure also shows that the fluorescence quantum yield of the derivatives is different with different amino acids. Such a difference for fluorescence yield has been described (20) and is probably due to the configuration of the individual fluorescent isoindole formed.

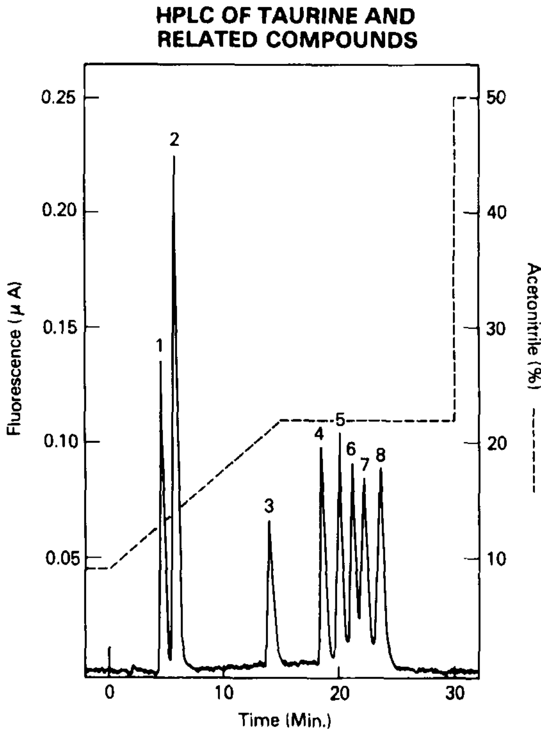

Figure 3. A gradient elution profile of the o-phthalaldehyde ethanethiol derivatized taurine, other free amino acids, and α-glycerophosphoryl ethanolamine. Conditions: column Solvent A. pH 7.12, sodium phosphate buffer, solvent B - CH_3CN with a linear program from 9-22% CH_3CN, followed by a 15 minute isocratic hold, before 50% CH_3CN. Flow rate 2.0 ml/min. For derivatization conditions see text. Amino acids are: 1. cysteic acid 2. cysteinesulfinic acid 3. o-phosphoryl ethanolamine 4. αglycerophosphoryl ethanolamine 5. arginine 6. tyrosine, β-alanine 7. hypotaurine 8. taurine

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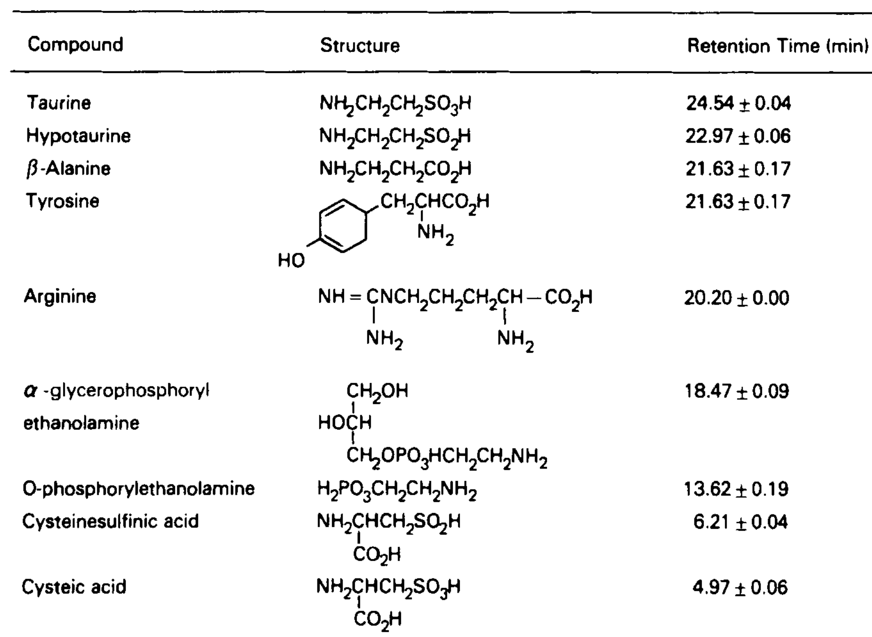
Two of the compounds tested, cysteine and cysteamine yielded no fluorescent derivative with o-phthalaldehyde and ethanethiol. This has been reported elsewhere (19). These compounds can be treated to give flurorescent derivatives, for example after oxidation of the sulphydryl group (19, 20).

The linearity of the fluorenscence response of OPT-taurine was maintained even in the presence of other amino acids. Hence the method should be useful with biological samples which would contain

THE EFFECT OF CHEMICAL STRUCTURE ON RETENTION TIME



Compound	Structure	Retention Time (min)
Taurine	$NH_2CH_2CH_2SO_3H$	24.54 ± 0.04
Hypotaurine	$NH_2CH_2CH_2SO_2H$	22.97 ± 0.06
β-Alanine	$NH_2CH_2CH_2CO_2H$	21.63 ± 0.17
Tyrosine	$HO-C_6H_4-CH_2CH(NH_2)CO_2H$	21.63 ± 0.17
Arginine	$NH=C(NH_2)NCH_2CH_2CH_2CH(NH_2)-CO_2H$	20.20 ± 0.00
α-glycerophosphoryl ethanolamine	$CH_2OH-HOCH-CH_2OPO_3HCH_2CH_2NH_2$	18.47 ± 0.09
O-phosphorylethanolamine	$H_2PO_3CH_2CH_2NH_2$	13.62 ± 0.19
Cysteinesulfinic acid	$NH_2CH(CO_2H)CH_2SO_2H$	6.21 ± 0.04
Cysteic acid	$NH_2CH(CO_2H)CH_2SO_3H$	4.97 ± 0.06

Figure 4. Shows the retention time (R_t) for taurine and other primary amines. Rt is time in minutes after injection for the fluorescent peak. Results are means for 6 samples $\pm$ SEM.

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a number of different amino acids. However it is important to maintain an excess of reagent, ie of OPT-ETSH. At least a 2 fold molar escess of derivatizing reagent to the total amount of free amino groups was necessary to obtain a linear response. In order to minimize day to day variations in response it was also necessary to standardize the devivatization procedure as to time and temperature. Intra assay variations were minimized by using the same stock solution of OPT, which was stored at -20°C, protected from light. The slope of the fluorescence response was almost identical between assays. However at least 2 known concentrations of taurine were tested in each assay. The slope of the fluorescence response does change when a different stock solution of OPT is used, which makes it important to construct a complete standard curve with each new stock solution.

The method described here provides several advantages compared with other methods for quantitation of taurine. 1. The use of both fluorescence and HPLC combine to give a method that is both

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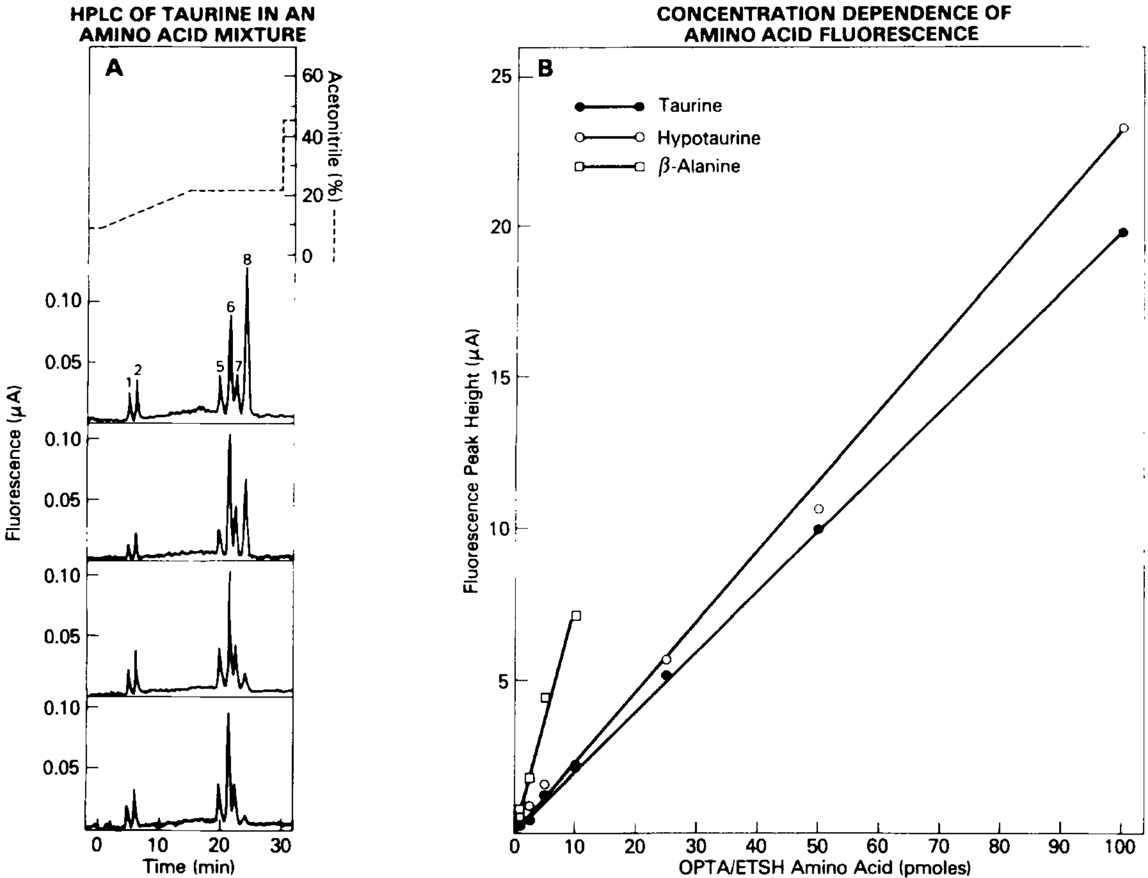

Figure 5. A. Shows taurine peak height with increasing taurine concentration from 1-25 picomoles in the presence of other amino acids. Peaks are numbered as in Figure 3.
B. The graph shows fluorescence yield versus taurine concentration from 1-100 picomoles and for hypotaurine, and β-alanine.

highly sensitive and specific because it gives a clear separation of taurine from related compounds. 2. The sensitivity of measurement (as little as 1 picomole of taurine) is about 500 fold greater than for commonly used ion exchange assays (16). 3. The method has the advantage of separating taurine from its precursors hypotaurine and cysteine sulfinic acid, compared with other reported methods where this is not known (18,21). Recently Baskin et al. Using a fatty acid analysis column and post-column derivatization with o-phthalaldehyde (Baskin et al., (22)) found that taurine and its precursors were separated. However in that method taurine was not very well retained by the column and this could provide problems with biological samples, since the elution volume of taurine is close to the void volume. In the case of the present method, the use of pre-column derivatization obviates the need for the use of additional apparatus necessary for mixing the reagent and also reduces background fluorescence. The linearity observed with other amino acids hypotaurine and β-alanine suggests that this method can also have wide use in the quantitative analysis of other primary amines.

In conclusion the method described here should prove valuable as a sensitive specific assay for taurine, especially with small tissue samples and biological fluids. Furthermore, this method does discriminate between the common interfering substances encountered in these samples.

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