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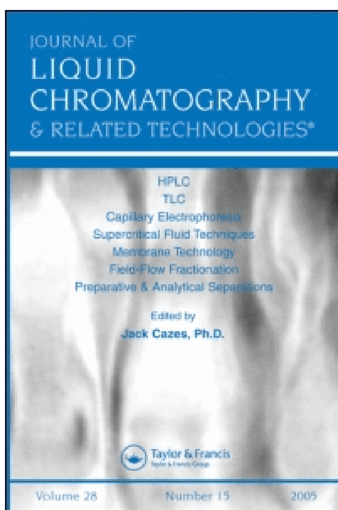

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SEPARATION OF ORTHOPHTHALALDEHYDE/ETHANETHIOL DERIVATIVES OF TAURINE AND CLOSELY ELUTING AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In studies of the reverse phase, HPLC analysis of amino acids employing precolumn derivatization with o-phthalaldehyde and ethanethiol, it was shown that α-amino-n-butyric acid, β-amino-isobutyric acid and taurine coeluted in the acetonitrile/aqueous phosphate solvent system. By using a ternary solvent system of acetonitrile/tetrahydrofuran/aqueous phosphate buffer and efficient 5- and 10-μm octadecylsilane packings, the co-elution problem has been resolved. This modified chromatographic system is now being used to quantitatively determine taurine and other closely eluting amino acids in a variety of physiological fluids in order to clarify the role of taurine in human development.

INTRODUCTION

There have been many recent reports describing the reverse phase, HPLC separation followed by fluorescent detection of derivatized amino acids and amino sulfonic acids (1-9). The method involves performing a precolumn derivatization of the amino acids (I) with o-phthalaldehyde (OPA)(II) in the presence of a thiol (III) to form highly fluorescent substituted isoindoles (IV) as

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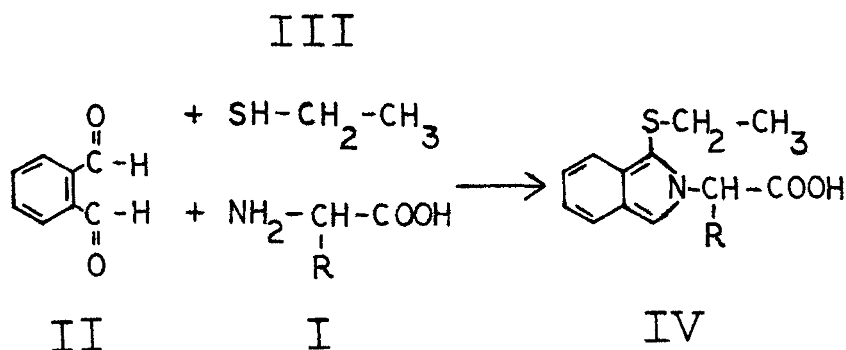

The OPA/ethanethiol (ETSH)(III) derivatization reaction, first described by Simons and Johnson (10), was used in earlier reports (1,2,4). It was found that the fluorescent products of this reaction are more stable in comparison to those formed in the OPA/2-mercaptoethanol derivatization reaction. The fluorescent isoindoles formed by the reaction of OPA/ETSH with primary amino acids are formed within seconds at room temperature and in most cases are quite stable in aqueous solution for periods of minutes to hours.

In a recent paper, Hill, Burnworth, Skea and Pfeifer (4) have presented information to characterize the optimal experimental parameters necessary for using the precolumn OPA/ETSH derivatization reaction. They reported that there is a linear increase in peak area versus amino acid concentration over a range from 5 to 500 nmol/mL for each of the twenty amino acid standards studied. Linear regression correlation coefficients of 0.999 or better were obtained. Reasonable agreements for fifteen of the amino acids

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between the values obtained by the conventional ion-exchange method, using a Beckman 119C Amino Acid Analyser and the precolumn OPA/ETSH HPLC fluorescent method was reported. Of importance to this report is the fact that Hill, et al., (4) reported that in their acetonitrile/aqueous phosphate system that α-amino-n-butyric acid (α-ABA) and β-aminoisobutyric acid (β-ABA) co-eluted with taurine.

Thalmann et al. (7), reported the use of the precolumn OPA/ETSH method to determine the levels of nineteen of the free amino acids found in the inner ear and cerebrospinal fluids of guinea pigs. They choose to omit reporting the levels of taurine because, in communication with Hill, they reported the coelution of α-ABA and β-ABA with taurine under their specific assay conditions. Turnell and Cooper (8) had reported a clinical study employing the precolumn OPA/2-mercaptoethanol derivatization reaction followed by the reverse phase HPLC separation and fluorescent detection of an estimated thirty-one amino acids in human serum and urine. Acceptable accurate and precise values of all of the common amino acids were obtained except for taurine whose value was reported to be altered by co-elution with β-aminoisobutyric acid (β-ABA). In their chromatographic system, α-ABA was found to elute several minutes after taurine from the 5-μm Ultrasphere ODS (Altex Scientific Co.) column. A mixed gradient solvent system of water/stock sodium proprionate solution/acetonitrile (72/20/8 by vol.) to water/acetonitrile/methanol/dimethylsulfoxide (42/30/25/3 by vol.) was employed. An interesting table in their

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publication detailed the stability, in terms of half-life, for the fluorescent products of amino acids/OPA/2-mercaptoethanol (8).

Wheler and Russell (9) reported the use of the precolumn OPA/ETSH HPLC-fluorescent method to separate taurine, from its precursor hypotaurine. The method was also able to separate cysteine sulfinic acid and α-glycerophosphoryl ethanolamine known to be present in mammalian brain fluids. They reported that their method provided upto a 500-fold improvement in sensitivity over the ion-exchange, post-column ninhydrin absorption method (9). However, it appears that these authors were not aware of the co-elution problem of α-ABA and β-ABA with taurine in their specific assay conditions.

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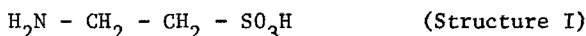
Structurally, taurine (TAU), 2-aminoethanesulfonic acid,



$$H_2N - CH_2 - CH_2 - SO_3H \qquad \text{(Structure I)}$$

is a β-amino sulfonic acid. It is readily separated and detected along with the many amino acids in physiological fluids. Taurine is unusual because it occurs in high concentrations in mammalian tissue; it is chemically and metabolically stable, yet it is not a constituent of proteins. Three books (11-13), a marked increase of references to taurine in the literature, and an upcoming review chapter on the liquid chromatographic determination of taurine (14) attest to the growing recognition of the interest in determining the biological functions of taurine.

Normal plasma concentrations of taurine in adult humans have been reported as 59 nmol/mL, with a standard deviation of ± 12 nmol/mL and a range of 41 to 78 nmol/mL (15,16). In the same

study, α-amino-n-butyric acid had a mean value of 20 nmol/mL with a standard deviation of 6 nmol/mL and a range of 9-35 nmol/mL. No values were reported for β-aminoisobutyric; its level is expected to be at least 10-fold lower than for taurine.

High levels of taurine are found in the plasma of human newborns. Within a few days, the taurine concentration in the plasma decreases about three-fold, with a concurrent increase excretion in the urine. The importance of TAU in the developmental changes of humans continues to be an active area of research making the use of high performance liquid chromatography with fluorescence detection a very attractive analytical method to use.

This report details how, by using a ternary solvent system of tetrahydrofuran/acetonitrile/aqueous phosphate system, as has been suggested earlier for the separation of threonine from glycine (4), and by the use of efficient 5-μm octadecylsilane bonded packings, resolution of α-ABA, β-ABA and taurine as their OPA/ETSH derivatives has been achieved.

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MATERIALS

A Waters Associates Liquid Chromatographic System equipped with a Model 6000A pump, and a Model U6K injector was used. The fluorescent detector was a Schoeffel/Kratos FS-970 Spectrofluoro Monitor with a standard 5-μL cell. This detector was adjusted to the following parameters: excitation wavelength - 229 nm; emission cut-off filter - 480 nm; time constant - 0.5s; a fine sensitivity setting of 5.00 on the 1.0 μA full-scale range setting.

The chromatograms were recorded using a two pen, 10-mv, electronic integrating recorder, Houston Instr. OmniScribe Model 5213-15, connected in parallel to a Hewlett-Packard Reporting Integrator, Model 3390A, on which the peak retention times, peak areas, ratio of peak area/height and relative peak areas were obtained.

Three different reverse-phase, octadecysilane columns were used and compared. A μBondapak C_{18} column (Waters Assoc. Part No. 27324, Serial No. 13909) was packed with 10 μm, irregularly shaped particles and had column dimensions of 30 cm x 3.9 mm i.d. The second column was a Partisil PXS 10 ODS-3 column, (Whatman Inc. Part No. 4228-111, Serial No. 1P3513) which was packed with 10 μm irregularly shaped silica and had column dimensions of 25 cm x 4.6 mm i.d. The third column was a Partisil PXS 5 ODS-3 column (Whatman Inc. Part No. 4238-111, Serial No. IS2004) which was packed with 5 μm irregularly shaped and also had column dimensions of 25 cm x 4.6 mm. i.d. In front of each one of the analytical columns, a guard column, hand-packed with 37-75 μm C_{18}-Corasil packing having dimensions of 5 cm x 4.6 mm i.d., was used. The temperature of the analytical column was controlled by a constant temperature, 27.5 ± .5°C, circulating water bath (Model T3, P.M. Tamson, Holland). The temperature was monitored by a Model 49TA digital thermometer from YSI Scientific. The column jacket was a precisely grooved aluminum piece made by the University of Connecticut, Chemistry Department's machine shop. The column conditioning recently described by Hill, et al., (4) was carefully followed.

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Chemicals

Individual crystalline samples of taurine (TAU), DL-*β*-aminoisobutyric (*β*-ABA), and ethanethiol (ETSH) were obtained from Aldrich Chemical Co. DL-*α*-amino-*n*-butyric (*α*-ABA) was obtained from Sigma Chemical Co. The *o*-phthalaldehyde (OPA) (Fluoropa) and solid amino acid standard H (Part No. 20088) were obtained from Pierce Chemical Co. The acetonitrile (UV grade), methanol and tetrahydrofuran (UV grade) were of HPLC quality and obtained from either Burdick and Jackson Laboratories, Inc. or the MCB Manufacturing Chemists, Inc. The water used in preparing the reverse phase chromatographic solutions was first condensed from steam, then passed thru two mixed-ion exchange resins, one organic removal cartridge followed by a 0.20 μm submicron filter (NANOPURE, 4 module with pump, Sybron/Barnstead). The resulting water would qualify as reagent grade, type I-ASTM standard water having a specific conductivity greater than 10 Mohms/cm. All solutions and samples involved in the liquid chromatographic separation were filtered through 0.22 μm Millipore filters prior to use.

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METHODS

Preparation of Solutions

Stock solutions, 1.00 x 10^{-4} M of each individual amino acid were carefully prepared in a 100-mL volumetric flask. The amino acids were dissolved and diluted with 0.1N aqueous hydrochloric acid solution. The *o*-phthalaldehyde solution was prepared by dissolving 1.00 g of OPA into 50 mL of methanol. This stock

OPA solution was protected from the light and kept refrigerated at +1°C when not in use. A dilute ethanethiol solution was prepared by adding 500 μL of ethanethiol to 25 ml of methanol, and the resulting solution was passed thru a Sep-Pak® C_{18} cartridge, Waters Assoc., in order to remove impurities. Because of the smell and the volatility of ETSH, all handling of these solutions should be done in an efficient hood. To reduce volatilization, it was found that the diluted ETSH/methanol solutions could be kept for periods of 3 to 5 days at -18°C. The derivatization of the amino acid standards was performed as follows: 20-100 nmol of the amino acid solutions in volumes of 200-1000 μL were placed in a 10-mL amber volumetric flask having a molded teflon stopper, exactly 1.00 mL of a borate buffer (4), 1.00 mL of the OPA solution and 1.00 mL of the ETSH solution were added. The resulting solution was diluted to the 10 mL mark with methanol, shaken and allowed to remain at room temperature for at least 10 min for completion of the reaction, and then analyzed by injecting 10 μL onto the HPLC system.

The tetrahydrofuran/acetonitrile/aqueous phosphate (10/10/80 by volume) solvent mixture was prepared as follows: to prevent the precipitation of the phosphate salt in tetrahydrofuran, it was found necessary to first mix the tetrahydrofuran with the acetonitrile. The stock aqueous phosphate buffer was prepared by mixing 14.10 g of $NaH_2PO_4 \cdot H_2O$ and 36.76 g of Na_2HPO_4 in reagent grade water and diluting to 1.0L. The resulting aqueous buffer solution had a pH of 7.15. Upon dilution, the solvent system consisted of

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10 percent by volume of tetrahydrofuran, 10 percent acetonitrile and 80 percent aqueous phosphate buffer. This diluted solvent system was calculated to be 31.0 mM in sodium ion and 18.1 mM in phosphate ion. Previous reports have shown that it is important to control the phosphate concentration of the solvent system in order to optimize the separation of closely eluting peaks (1,4).

RESULTS AND DISCUSSION

Figure 1 shows the isocratic, acetonitrile/aqueous phosphate buffer (20/80 by vol) elution of the OPA/ETSH derivatized products of arginine (ARG), taurine (TAU), α-amino-n-butyric acid (α-ABA) and β-aminoisobutyric acid (β-ABA). The flow rate was 1.0 mL/min and the column was a μBondapak C_{18}. It should be noted at this optimized flow rate and without the use of a gradient, β-ABA co-eluted with TAU.

Various combinations of a ternary solvent system, acetonitrile/tetrahydrofuran/aqueous phosphate buffer were used. It was found that while maintaining a final organic volume ratio of 20 percent by volume, an interesting selectivity for the separation of α-ABA, β-ABA and Taurine could be achieved. By keeping the acetonitrile volume between 10 to 13 percent and by adjusting the tetrahydrofuran volume between 10 to 7 percent, β-ABA can be made to elute after α-ABA and taurine. This selectivity is important when studying the composition of physiological fluids because the concentrations of β-ABA, are often very low (15), and hence it is important to have β-ABA well separated from other

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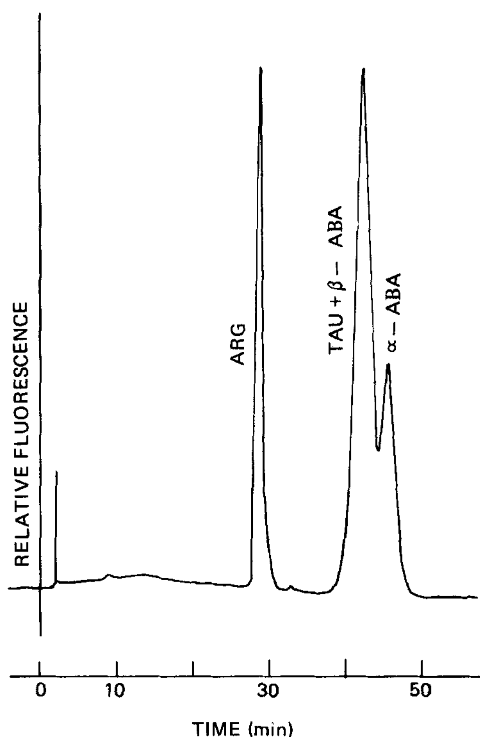

FIGURE 1. Isocratic Elution Profile of the OPA/ETSH derivatized arginine, taurine, α-amino-n-butyric and β-aminoisobutyric acids. Conditions: Column, μBondapak C_{18}, Solvent 20 percent acetonitrile/80 percent aqueous phosphate buffer, flow rate 1.0 mL/min.

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closely eluting, derivatized amino acids. In each case, the individual derivatized standards was chromatographed separately to provide peak identification.

Figure 2 shows the improved resolution that is achieved upon using the efficient, 5- or 10-μm ODS columns. The chromatographic system used to compare the three reverse phase columns was identical in all other respects, involving isocratic elution at a flow rate of 1.0 mL/min. Table 1 summarizes the capacity factors, in

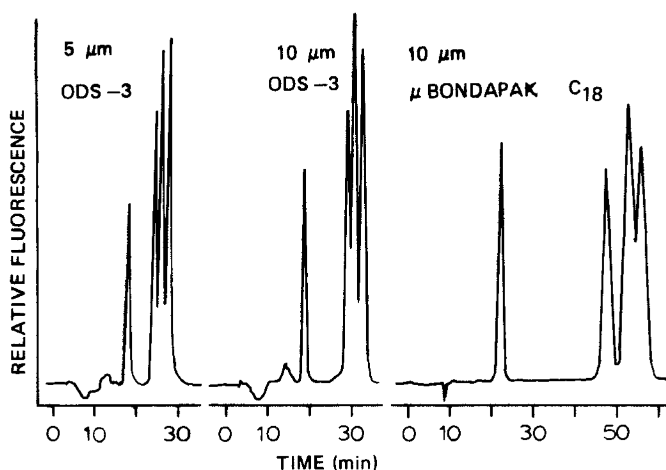

FIGURE 2. Comparison of the Elution Profiles from three different octadecylsilane, reverse phase columns, the 5- and 10-µm ODS-3 and the µBondapak C_{18}. Elution order of the OPA/ETSH derivatized compounds are in all cases arginine, α-Amino-n-butyric, taurine then β-aminoisobutyric acid. Solvent 10 percent acetonitrile/10 percent tetrahydrofuran/80 percent aqueous phosphate buffer, flow rate 1.0 mL/min for each column.

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terms of k' for the three closely eluting, OPA/ETSH derivatives of α-ABA, β-ABA and taurine. Also included in Table 1 is the capacity factor for derivatized arginine which was used as a reference point. It has been shown that the retention time of the arginine does not vary with the change in the aqueous buffer concentration because the ARG/OPA/ETSH derivative is a zwitterion at the pH of 7.15 in the aqueous phosphate buffer system (3,4).

Figure 3 depicts the isocratic elution order of a series of standard OPA/ETSH derivatized amino acids marketed by the Pierce Chemical Co. as amino acid standard H, co-injected with derivatized α-ABA, β-ABA and TAU. It can be seen that with a ternary solvent system of acetonitrile/tetrahydrofuran/aqueous phosphate

TABLE 1

Capacity Factors For Taurine and Closely Eluting Amino Acids on Different C_{18} Columns[1]

Amino Acid	5 μm ODS-3	10 μm ODS-3	10μm μBondapak
Arginine	3.9	4.5	7.0
α-amino-n-butyric acid	5.7	7.6	15.7
taurine	6.1	8.1	16.9
β-aminoisobutyric acid	6.7	8.7	17.5

1. Isochratic solvent conditions, acetonitrile/tetrahydrofuran/ aqueous phosphate buffer, pH=7.15, 31.0 mM Na and 18.1 mM PO_4, 10/10/80 by vol, flow rate 1.0 mL/min.

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buffer and with the use of the efficient 5- or 10-μm reverse phase packings, adequate resolution of α-ABA, β-ABA and TAU is possible. This modification of Hill's et al., (4) solvent system and the use of 5-μm octadecylsilane packings is presently being used to resolve α-amino-n-butyric acid, β-aminoisobutyric acid and taurine in various psysiological fluids.

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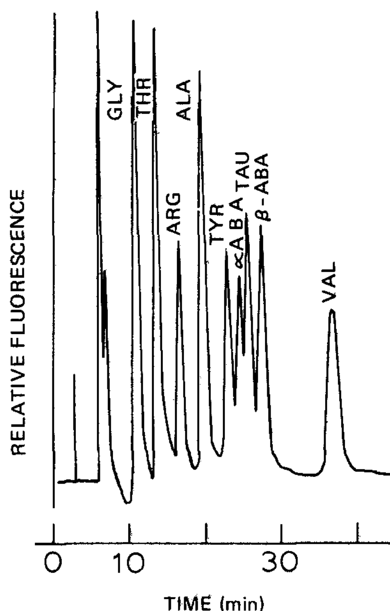

FIGURE 3. Isocratic Elution Profile of Certain of the OPA/ETSH derivatized amino acids: Column 5-um ODS-3, Solvent 10 percent acetonitrile, 10 percent tetrahydrofuran, 80 percent aqueous phosphate buffer, flow rate 1.0 mL/min.

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the nature of the ODS-3 columns. Portions of this paper were presented at the 34th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, N.J., March 1983, Paper no. 829. Financial support for purchase of the instrumentation used in this research was provided by the National Institutes of Health Research Grant No. ES-01056, the Department of the Interior, Office of Water Resources and Technology Grant No. A-089-CONN and the University of Connecticut Research Foundation Grant 5171-35-453, for which publication costs for this paper were also received.

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