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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF NEUROTRANSMITTER AMINO ACIDS IN BRAIN

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SUMMARY

Rapid and selective determination of neurotransmitter amino acids in rat and human brain is accomplished by pre-column derivatization with *o*-phthaldehyde–3-mercaptopropionic acid, reversed-phase high-performance liquid chromatographic separation, and fluorimetric detection. Aspartate, taurine, glutamate, and glycine are determined in less than 12 min with intra- and inter-assay precisions of 2.1–9.5% and 4.2–9.2%, respectively. γ-Aminobutyric acid is determined in less than 8 min with intra- and inter-assay precisions of 1.6 and 11.7%, respectively. Both assays utilize internal standards and require a minimum of sample preparation

INTRODUCTION

A variety of chromatographic methods have been developed to determine amino acids in physiological samples. One fruitful approach has been to combine reversed-phase high-performance liquid chromatography (HPLC) with pre-column *o*-phthaldehyde (OPA) derivatization. This method generally offers high sensitivities and is capable of detecting a number of primary amino acids in less than 1 h [1–8]. Interest in the neurotransmitter amino acids in brain tissue has prompted the development of several specific determinations. Gradient HPLC separation of OPA derivatives followed by electrochemical detection has permit-

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ted the analysis of brain glutamine (Gln), taurine (Tau), glutamate (Glu), aspartate (Asp), glycine (Gly), and γ-aminobutyric acid (GABA) in 7 min [9]. More recently, five of the neurotransmitters were determined electrochemically after OPA derivatization and a 30-min isocratic HPLC separation [10]. The first of these methods [9] requires a complex gradient, and the OPA–*tert.*-butyl thiol derivatives formed, although while relatively stable, are not fluorescent. In the latter method [10], the unstable OPA–β-mercaptoethanol derivatives are determined. Neither method makes use of an internal standard. We have developed rapid isocratic determinations of the stable OPA–3-mercaptopropionic acid (OPA–3MPA) derivatives of the neurotransmitters in brain. The analyses incorporate internal standards, require minimal sample preparation, and can be performed rapidly.

EXPERIMENTAL

Reagents

Amino acids, OPA, and 3MPA were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade and purchased from local suppliers. Distilled and deionized water was used throughout. Brain tissue was obtained from adult male Sprague–Dawley rats and from adult neurosurgical patients. Tissue was immediately frozen, stored at −70°C, and not thawed until homogenized.

All amino acid solutions were made up to 1 μg/μl and diluted to working solutions of 1 ng/μl with distilled water. Stock solutions were prepared each month. The OPA reaction mixture was prepared weekly by dissolving 50 mg OPA in 4.5 ml methanol and 0.5 ml borate buffer, then 50 μl of 3MPA were added and the mixture stored refrigerated in the dark. The borate buffer was made weekly by adjusting 0.5 *M* boric acid to pH 9.5 with sodium hydroxide.

High-performance liquid chromatography

The HPLC system consisted of an Altex 110A pump, a Rheodyne 7125 injector, an Altex Ultrasphere (250 mm×4.6 mm I.D.) 5-μm ODS column (all Rainin Instruments, Woburn, MA, U.S.A.), and a Shimadzu RF350 fluorometer (Shimadzu Instruments, Columbia, MD, U.S.A.). Excitation and emission wavelengths were 330 and 440 nm, respectively, with bandpasses of 20 nm. Output was recorded on a Kipp & Zonen BD-41 strip-chart recorder. For some experiments, detection was accomplished with an Aminco fluoromonitor fitted with a G.E. F4T4/BL ultraviolet lamp, Wratten No. 8 emission filter and Corning 7-51 excitation filter. For electrochemical comparison studies, a glassy carbon electrode was maintained at +0.60 V vs. Ag/AgCl using an LC-2A potentiostat (Bio-Analytical Systems, West Lafayette, IN, U.S.A.).

The mobile phase used for the determination of Asp, Glu, homoserine (Hse), Tau, and Gly consisted of 0.57 *M* acetate buffer (pH 3.75), 100 mg/l Na_2EDTA–acetonitrile (75:25, v/v). For GABA analysis, the mobile phase was 0.2 *M* acetate buffer (pH 3.8), 100 mg/l Na_2EDTA–acetonitrile (50:50, v/v).

Acetate buffers were prepared by adding 32.8 ml (0.57 *M*) or 11.5 ml (0.2 *M*) of glacial acetic acid to 925 ml of water, adjusting the pH with 3 *M* sodium hydroxide, and diluting to 1000 ml. Flow-rates were 1.0 ml/min.

Sample preparation

Brain homogenate for analysis was obtained by sonication of whole rat brain, previously frozen and stored at −80°C, with 10 volumes (v/w) of cold 1% perchloric acid. Samples were sonicated on ice for approximately 1 min. Hse and δ-aminovaleric acid (DAVA) were added as internal standards before sonication (200 μg/g added using 1 μg/μl internal standard solution). Samples from brain areas were prepared similarly, however, fixed amounts of Hse and DAVA which were only approximately 200 ng/mg were added per tube. Samples were centrifuged at 13 000 *g* for 2 min, and the supernatant was derivatized as described below.

Derivatization

Derivatization of the amino acids was modified from the technique of Godel and co-workers [5,6].

Samples and standards were derivatized by reacting 50 μl of supernatant with 200 μl methanol, 200 μl borate buffer, and 50 μl OPA–3MPA solution. Samples were vortex-mixed and allowed to stand at approximately 22°C for 2–5 min. A 20-μl aliquot then was mixed with 200 μl of 0.1 *M* phosphoric acid and 50 μl were injected onto the appropriate system.

In later analyses it was determined that the second step, dilution with phosphoric acid, was not necessary. The second step was eliminated, and 10 μl of the reaction mixture were injected directly onto the column.

Calculations

Concentrations of amino acids were determined by ratioing peak heights of analyte and internal standard in sample, multiplying by the ratio of the standard peak heights and by the concentration of the internal standard in sample: e.g., (sample Asp/Hse) × (standard Hse/Asp) × (μg Hse added/wet weight of sample in tube).

RESULTS AND DISCUSSION

The OPA–3MPA derivatives of Asp, Tau, Glu, Gly, and the internal standard Hse were separated in less than 12 min. Chromatograms of standards and rat whole brain homogenate are shown in Fig. 1. The four neurotransmitter amino acids are resolved from other peaks, and no problems were encountered with late eluting peaks. Similar chromatograms were obtained with human cortex and hippocampus. Compounds tested, and found not to interfere, included glutamine, methionine, aspargine, α-alanine, β-alanine, arginine, serine, threonine, histidine, tyrosine, tryptophan, ornithine, lysine, leucine, isoleucine, valine, phenylalanine, ethanolamine, phosphoethanolamine, and glutathione.

Complete (85–100%) and linear recoveries were obtained for Asp, Tau, Hse,

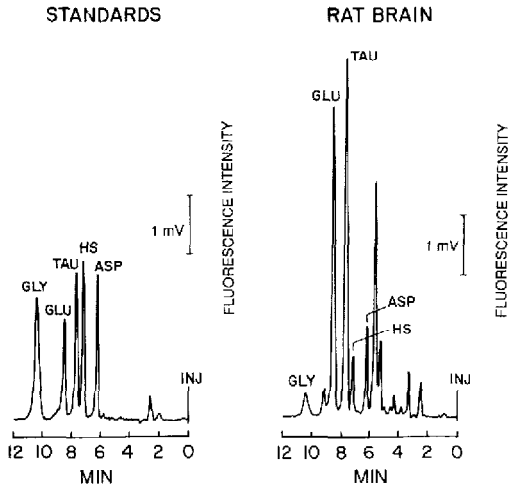

Fig. 1. Separation of Asp, Hse, Tau, Glu, and Gly in standards and rat brain homogenate. The standard mixture contained Asp, Hse (internal standard), Glu, Gly (5 ng/μl, 5 ng on-column) and Tau (2 ng/μl, 2 ng on-column); 10 μl of the reaction mixture were injected. Homogenate of whole rat brain contained the following concentrations: Asp, 352 ng/mg; Hse, 200 ng/mg; Tau, 567 ng/mg; Glu, 1835 ng/mg; Gly, 108 ng/mg; 10 μl were injected Chromatographic conditions: see Experimental

Glu, and Gly in the range 0–1500, 0–2500, 0–1500, 0–4000, and 0–500 ng/ml of tissue added, respectively. Absolute detection limits (amount giving signal twice the peak-to-peak noise level) ranged from 5 to 30 pg corresponding to concentration detection limits of less than 1 ng/mg of brain. Within-day ($n=5$) and day-to-day ($n=7$) coefficients of variation for the neurotransmitter amino acids were: Asp, 4.6 and 9.2%; Tau, 2.1 and 4.2%; Glu, 2.6 and 9.2%; and Gly, 9.5 and 5.9%.

Peak identifications were verified using electrochemical detection in series. Values obtained for brain samples analyzed with the two different detection methods were in good agreement (differences 5.0–14.9%). Substantially fewer extraneous peaks were observed using fluorimetric detection compared to electrochemical detection. Levels (ng/mg wet weight) observed in whole rat brain (Tau, 526 ± 22; Glu, 1700 ± 156; Gly, 72.4 ± 4.2; Asp, 345 ± 32; $n=7$) and in human entorhinal cortex (Tau, 85.3 ± 26.6; Glu, 1120 ± 140; Gly, 118 ± 31; Asp, 162 ± 56; $n=3$) were in approximate agreement with previous reports [10–14].

In varying the mobile phase parameters to achieve optimal separation of Asp, Tau, Hse, Glu, and Gly, some interesting differences became apparent. As could be expected, retention times decreased with increasing organic solvent concentration (Fig. 2) and increasing pH (Fig. 3) in a relatively similar pattern for most of the amino acids. However, an anomalous pH effect was observed with Tau as its retention time at first increased when pH was increased (Fig. 3). When ionic strength of the acetate buffer was increased (Fig. 4), the retention times of most of the amino acids decreased slightly; the decrease seen for arginine (Arg) was somewhat more marked. However, the retention time of Tau increased with increasing ionic strength. This fact was utilized, after selection of optimum pH and organic concentration, to shift Tau after Hse. Another benefit of the higher acetate concentration is the improved chromatography of Gly. At lower ionic strength

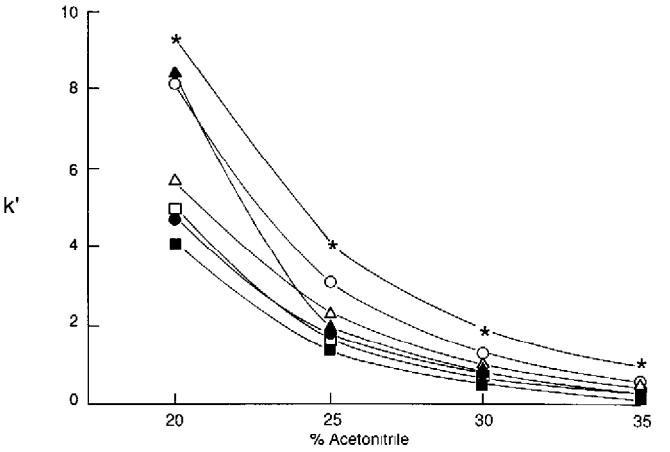

Fig. 2. k' Values of Asp, Hse, Tau, Glu, and Gly versus acetonitrile concentration of mobile phase; 0.24 M acetate buffer, pH 3.50 used in mobile phase. ■, Tau; ●, Asp; △, Hse (internal standard); ○, Glu; ★, Gly; □, Gln; ▲, Arg.

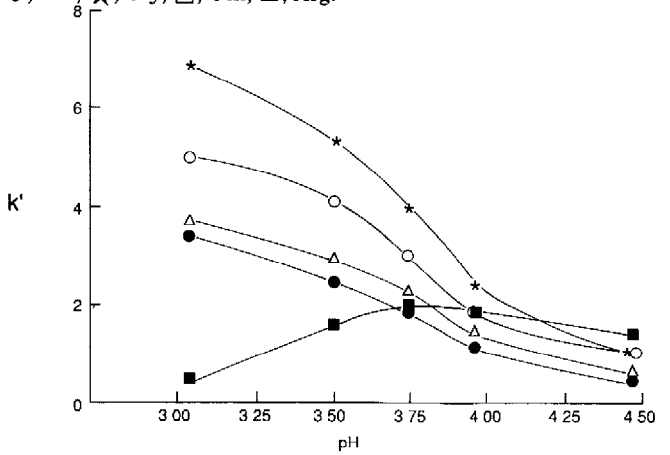

Fig. 3. k' Values of Asp, Hse, Tau, Glu, and Gly versus pH of 0.24 M acetate buffer; 25% acetonitrile used in mobile phase. ■, Tau; ●, Asp; △, Hse (internal standard); ○, Glu; ★, Gly.

the Gly peak is broad with respect to the other amino acids (chromatogram not shown). This effect is minimized at higher ionic strength.

Separation of GABA and DAVA standards and whole brain homogenate is shown in Fig. 5. The GABA and DAVA peaks are resolved from other peaks, none of the compounds tested (listed above) were found to interfere.

Complete (84–99%) and linear recoveries were obtained for GABA and DAVA upon addition of standards in the range 0–1000 ng/mg of tissue. Similar chromatograms were obtained with human cortex and hippocampus. Limits of detection for GABA and DAVA were 72 and 85 pg, respectively, resulting in a concentration detection limit for GABA of approximately 1 ng/mg of brain. Identification of the GABA and DAVA peaks were confirmed using electrochemical detection. Values were not significantly different from those obtained by fluorimetric detection. Within-day ($n=5$) and day-to-day ($n=7$) variabilities for GABA were 1.6 and 11.7%, respectively.

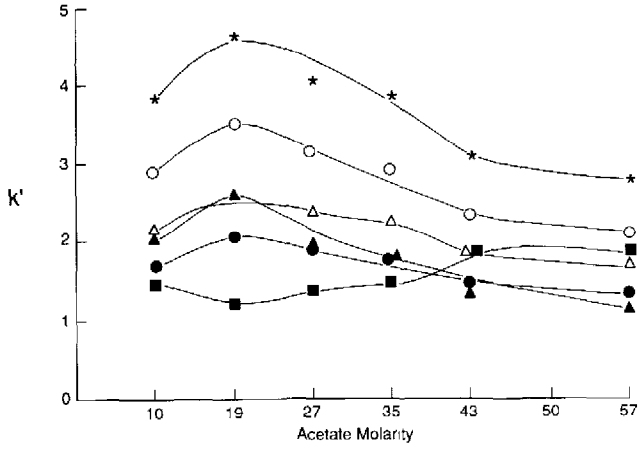

Fig. 4. k' Values for Asp, Hse, Tau, Glu, Gly, and Arg versus acetate molarity of mobile phase. Acetic acid brought to pH 3.75 with sodium hydroxide, 25% acetonitrile used in mobile phase. ■, Tau; ●, Asp; △, Hse (internal standard); ○, Glu; ★, Gly; ▲, Arg.

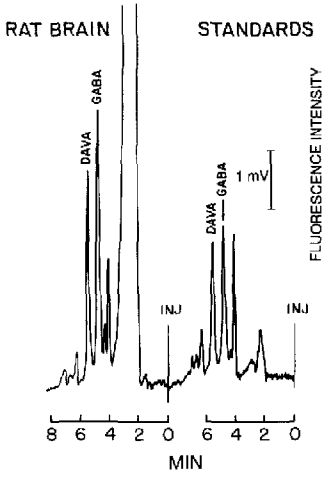

Fig. 5 Separation of GABA and DAVA, internal standard. Standards (each 10 ng) and whole rat brain homogenate (10 μl injected). Concentration of GABA and DAVA in the brain sample, 209 and 200 ng/mg, respectively. For chromatographic conditions, see Experimental.

The stabilities of the isoindoles formed by the reaction of OPA–3MPA with primary amino acids were examined in the borate–methanol reaction mixture and after neutralization with 0.1 *M* phosphoric acid. The derivatives exhibited enhanced stability in the borate–methanol reaction mixture. Several examples of the half-lives of the OPA–3MPA derivatives in 0.1 *M* phosphoric acid after an initial 2.5-min reaction period were: Asp, 1.5 min; Glu, 4.5 min; and Tau, 39.5 min. The derivatives in the borate–methanol reaction mixture had markedly longer half-lives. Fluorescence of the derivatives at 1 and 3 h post-reaction relative to the initial fluorescence were: Asp, 1.06, 1.05; Glu, 1.09, 1.03; Hse, 1.00, 0.95; Tau, 0.94, 0.82; Gly, 0.87, 0.69; GABA, 0.81, 0.73; and DAVA, 0.80, 0.71. Stability of Asp, Glu, Hse, Tau, and Gly after 1 h was excellent, and after 3 h Tau

had decreased by 18% and Gly by 31%. The ratios of analyte/internal standard are used in practice and after 1 h these varied from 87% (Gly) to 109% (Glu) of the initial ratios.

The derivatization procedure was adapted from the method of Godel and co-workers [5,6], who used a two-step procedure. They noted, however, that the dilution with 0.1 *M* phosphoric acid was not necessary when using ultra-pure reagents. In our investigation the OPA–3MPA derivatives were found to be more stable in the borate–methanol mixture than in the acid. Also, by eliminating the second step, a ten-fold dilution is avoided, resulting in lower practical detection limits of the method.

The derivatives formed with 3MPA have been found to be good compromise between stability and fluorescent intensity [3,15]. In addition to the increased stability of the derivatives, the compounds chosen as internal standards, Hse and DAVA, displayed similar decay curves to the neurotransmitters This eliminates the necessity for rigorous control of derivatization and injection, which requires automated equipment or strict operator control.

The two analyses described are able to quantitate the neurotransmitter amino acids in brain tissue with a minimum of preparation. Sources of variability are held to a minimum due to the use of isocratic HPLC systems, internal standards, and the improved stability of the OPA derivatives.

NOTE ADDED IN PROOF

See Suñol et al., Anal. Chem., 60 (1988) 649, for recent HPLC method for brain GABA.

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