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Note

Determination of amino acids in the brain by high-performance liquid chromatography with isocratic elution and electrochemical detection

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Several methods are available for the analysis of the naturally occurring amino acids by high-performance liquid chromatography (HPLC)[1–5]. The formation of the *o*-phthaldehyde (OPA)–mercaptoethanol derivatives, their separation on a reversed-phase column, and detection by fluorescence is one of the primary methods used[4,5]. The latter methodology requires a gradient maker, usually two pumps and careful attention to details of timing because of the instability of the derivatives formed[5,6]. During a study of amino acids in the brain, we sought a simpler and less expensive method which avoided the use of unstable derivatives. We devised a procedure for quantitating amino acids in the brain which only requires a single HPLC pump and a step gradient. This procedure is a modification of that devised by Allison *et al.*[6] for the electrochemical detection and quantitation of OPA–*tert.*-butyl thiol derivatives of amino acids. Separation of the amino acids derivatives is achieved by varying the organic phase or by including the cationic ion pair tetrabutylammonium phosphate (TBAP) with a step gradient for elution.

EXPERIMENTAL

Equipment and materials

The equipment consisted of a Bioanalytical Systems (BAS) Model PM30A pump, a Rheodyne 7125 injector, a BAS Model 4B electrochemical detector and a Houston Instruments Omniscribe D-5000 chart recorder. The column was stainless steel (25 cm × 4.6 mm I.D.) filled with Biophase ODS 5-μm (BAS) and protected with a Spheri-10, RP-18, 10-μm guard column (Brownlee Labs.). Similar results were obtained with a Waters μBondapak C_{18} reversed-phase column (10 cm × 4.6 mm I.D.). A low volume electrochemical cell containing a glassy-carbon electrode with a potential of 700 mV was used for derivative detection. A three-way slider valve (Rainin Instruments) was used for switching between solvents. The gradient controller (Autochrom Model III OPG/S), used in a limited number of experiments, pre-mixed the solvents before entering the dual piston pump. This arrangement avoided the necessity of a second solvent pump.

The mobile phases consisted of 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile, 0–2 m*M* TBAP and 0–10% tetrahydrofuran (THF). The

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pumping rate was 2.5 ml/min. All the organic solvents were HPLC quality (Fisher) and used without filtration. TBAP and *tert.*-butyl thiol were purchased from Aldrich. All other solutions were filtered through nylon 66 filters (Rainin Instruments). The chemicals were of reagent grade and used without further purification.

Peak identification of glutamate, glutamine and γ-aminobutyric acid (GABA) was confirmed by enzyme digestion. Glutamate was converted to GABA with glutamate decarboxylase (Sigma). To 15 μl of sample were added 60 nanomoles magnesium sulfate, 3 nanomoles EDTA, 0.1 unit of enzyme and 750 nanomoles citrate buffer, pH 5.0 in a total volume of 30 μl. The sample was incubated at 37°C for 30 min and then reacted with 90 μl of the OPA–thiol reagent. The GABase preparation (Sigma) contains glutaminase activity, therefore, the results with pure glutaminase are not presented. GABA and glutamine in the rat cerebellum were digested with 0.1 unit GABase, 600 nanomoles α-ketoglutarate and 750 nanomoles borax buffer, pH 8.5 in a total volume of 30 μl as above. Control samples lacked the enzyme or replaced the brain extract with pure amino acid standards.

Sample preparation

The amino acid standards were dissolved in water and diluted to 1 m*M* with methanol–water (1:1) containing 1 m*M* EDTA and kept at 4°C. The brain samples were sonicated in 1 *M* perchloric acid with α-aminobutyric acid (AABA) added as an internal standard. The solution was centrifuged and the supernatant was neutralized with two volumes of 0.5 *M* potassium bicarbonate. The precipitate was removed by a second centrifugation and the neutralized supernatant filtered before analysis.

The amino acids derivatives were prepared by the method of Allison *et al.*[6]. The OPA–thiol reagent was made as follows: 27 mg of OPA was dissolved in 2 ml of methanol and 20 μl of *tert.*-butyl thiol (2-methyl-2 propanethiol) was added followed by the addition of 4.5 ml of 100 m*M* sodium tetraborate decahydrate (borax), pH 9.5. The OPA–thiol reagent was prepared fresh each week and kept tightly sealed to prevent evaporation of the thiol. Heating was required to solubilize the sodium tetraborate decahydrate. Both solutions were stored at room temperature. A volume of 20 μl the amino acid solution was mixed gently in a 500-μl plastic centrifuge tube with 100 μl of the OPA–thiol reagent using a plastic pipet tip. The tube was capped until injection. Excessive shaking resulted in a diminished aspartate and glutamate peak and the appearance of an additional peak prior to the first amino acid peak. After 2 min an aliquot was injected into the high-performance liquid chromatograph. Injection of the reagent solution without amino acids resulted in a peak labeled RSH. The RSH corresponds to the free thiol. Its retention times was less affected by the concentration of organic phase or by the addition of the ion pair reagent than were amino acids derivatives.

RESULTS AND DISCUSSION

The separation of the OPA–*tert.*-butyl thiol derivatives of the most common amino acids found in the brain is shown in Fig. 1. The inclusion of the ion pair reagent, TBAP, was necessary to separate aspartate and glutamate. Separation of aspartate and glutamate in the absence of TBAP was achieved if tetrahydrofuran

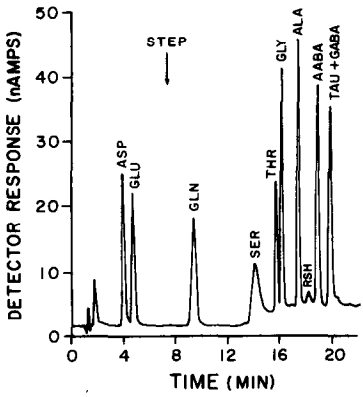


Fig. 1. Separation of OPA–*tert.*-butyl thiol derivatives of amino acids with a step gradient. The column was initially equilibrated with 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile, 2% THF and 1 m*M* TBAP. At 7 min the mobile phase was changed to include 10% THF. The quantity of each amino acid injected was 166 picomoles.

was deleted from the mobile phase and the concentration of acetonitrile was decreased to 20% or less. However, the retention time of glutamine increased to 16 min with the remaining amino acids eluting even later. Therefore, the step gradient used in Fig. 1 achieved both separation of aspartate from glutamate as well elution of the remaining derivatives in about 20 min.

The methodology is amenable to alteration of either the ion pair or the organic solvent phase. In Fig. 2 a simple gradient, in the absence of TBAP, resolved all compounds of interest except the amino acid pair threonine/glycine.

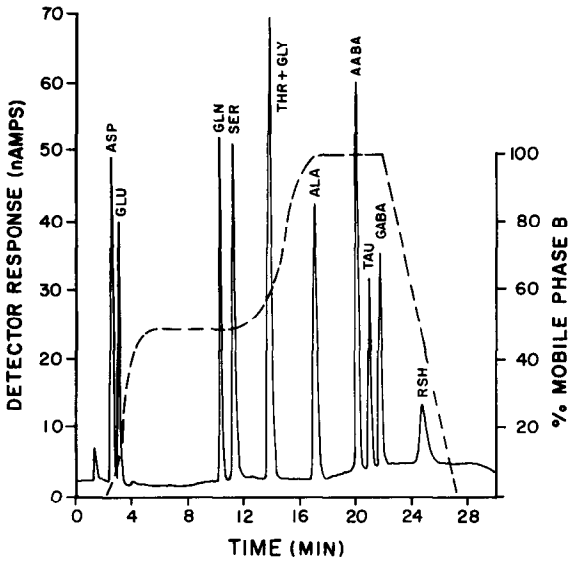
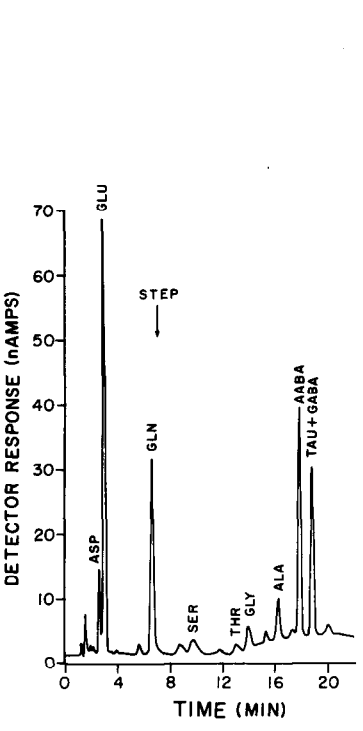


Fig. 2. Elution of OPA–*tert.*-butyl thiol amino acid derivatives with a step gradient in the absence of TBAP. The organic component of mobile phase A was 20% acetonitrile and for mobile phase B, 20% acetonitrile and 10% THF.

The application of the step gradient from Fig. 1 with an extract of rat cerebellum is shown in Fig. 3. Treatment of the rat brain extract with glutamate decarboxylase resulted in a 90–100% decrease in the glutamate peak height with concomitant increase in the taurine–GABA peak. Fig. 4A shows the cerebellum chromatogram using isocratic elution with an elevated concentration of organic solvent in the mobile phase. Under this third set of conditions taurine and GABA are resolved in less than 8 min, but two double peaks are observed: aspartate-glutamate and threonine-glycine. Treatment of the brain extract with glutaminsae plus GABAse resulted in the disappearance of the glutamine and GABA peaks and an increase in the aspartate-glutamate peak.

In an extension of the findings of Allison *et al.*[6], we tested the stability of ten OPA–*tert.*-butyl thiol derivatives for 60 min before injection into the HPLC. No decrease in peak height was observed unlike that reported for mercaptoethanol derivatives[5,6]. When eluted under the conditions used in Fig. 1, the OPA–mercaptoethanol derivatives are eluted as overlapping peaks in less than 4 min. If should be noted that Allison *et al.*[6] reported that although the electrochemical response was similar for both classes of thiol derivatives, the intensity of fluorescence of the



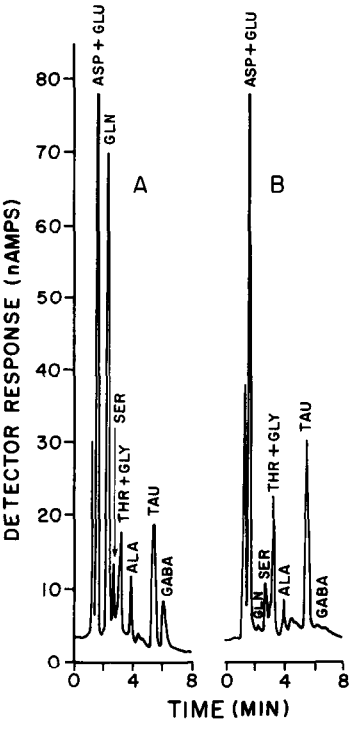


Fig. 3. Amino acid pattern in rat cerebellum. The conditions as in Fig. 1. The volume injected corresponded to 330 ng of tissue, wet weight.

Fig. 4. Rat cerebellum extract treated with GABAse and glutaminase. The composition of the mobile phase was 50 m*M* potassium phosphate, pH 7.0, 1 m*M* EDTA, 25% acetonitrile and 10% THF. (A) Untreated rat cerebellum extract. (B) Rat cerebellum extract treated with GABAse containing glutaminase activity. The volume injected corresponded to 375 ng of tissue, wet weight.

OPA–*tert.*-butyl thiol derivatives was only 10–15% as great as that of the OPA–mercaptoethanol derivatives.

This methodology is applicable for quantitating amino acids below 20 picomoles per injection. The sensitivity is limited by the shift in base line observed at 15 min (see Fig. 1) which prevented using full scale ranges below 20 nA for peaks eluting after this time. The sensitivity for the first five amino acids can be increased several fold if the subsequent amino acid peaks are allowed to go off scale or no step gradient is utilized. The regression coefficients of the lines found by plotting nanoamp response versus pmoles injected for nine test compounds was 0.991 ± 0.007 (mean ± S.D.).

In conclusion, a method for separating and quantitating amino acids by HPLC is described. It utilizes electrochemical detection of stable amino acid derivatives separated by isocratic or simple step gradient thereby eliminating the need for a gradient maker and a second pump. Asparate and glutamate are separated either by inclusion of the cationic ion pair TBAP or by reducing the proportion of the organic phase. These methods were utilized to separate amino acids in rat brain tissue. The glutamate, glutamine and GABA peaks were verified by enzymatic digestion.

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