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Note

Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts

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Amino acids can be analyzed by high-performance liquid chromatography (HPLC) using a variety of systems and derivatization methods[1–10].

The purpose of the present investigation was to develop a simple and rapid assay for determination of amino acids that are substrates and products of enzymes engaged in glutamate and γ-aminobutyric acid (GABA) metabolism, such as aspartate amino transferase (E.C. 2.6.1.1), alanine amino transferase (E.C. 2.6.1.2), glutaminase (E.C. 3.5.1.2), glutamate dehydrogenase (E.C. 1.4.1.2), glutamate decarboxylase (E.C. 4.1.1.15) and GABA transferase (E.C. 2.6.1.19).

Earlier studies have focused on samples containing all amino acids and the analysis was completed approximately within 1-h intervals. Here we report a more rapid determination of glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), alanine (Ala) and GABA in the presence of the amino acids usually found in synaptosomes[11]. The time of determination was lowered to 10 min and, thus, four analyses can be performed per hour.

Separation and detection of the amino acid *o*-phthalaldehyde (OPA) derivatives can be achieved with sufficient resolution and sensitivity to follow uptake and formation of Glu, Gln, Asp, Ala and GABA by isolated synaptosomes. The separation was carried out on a reversed-phase (C_{18}) column using a gradient-elution (methanol–potassium acetate buffer) procedure.

EXPERIMENTAL

HPLC was carried out on a Spectra-Physics Model 8000 liquid chromatograph with a gradient accessory and helium degassing system. An autoinjector (CV-6-UHPa-N60); Valco Instrument, Houston, TX, U.S.A.) with 10-μl loop was used. The column effluent was monitored by an Aminco Fluoro-Colorimeter (American Instrument, Silver Spring, MD, U.S.A.) equipped with a 9-μl flow-through cell operated at an excitation wavelength of 340 nm (7-60 Corning filter) and an emission wavelength of 455 nm (2A Wratten cut-off filter). The detector was fitted with a Supergrator 2 computing integrator (Columbia Scientific Industries, Austin, TX, U.S.A.) and a Varian G-4000 one-pen chart recorder. A Normaton Spherosil XOA 600, C_{18} column (10 cm $\times$ 4 mm I.D.), particle size 5 μm (Prolabo, Paris, France), was used.

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Reagents and chemicals

The mobile phase was composed of 0.1 *M* potassium acetate, pH 5.50, and methanol (HPLC grade; Rathburn, Walkerburn, Great Britain). Individual amino acid standards were obtained through Sigma (St. Louis, MO, U.S.A.). A stock solution of each amino acid was prepared by dissolving it in 10 m*M* HCl to provide a concentration of 100 μmol/ml. Standard solutions containing 200 nmol/ml of each amino acid were prepared from the stock solutions by dilution with water. Gln stock solution was freshly prepared every day. The solutions were stored at 4°C. All other reagents were of analytical reagent grade and used without further purification.

The derivatization reagent was prepared by dissolving 10 mg *o*-phthalaldehyde (Fluoropa; Durrum, Palo Alto, CA, U.S.A.) in 500 μl absolute ethanol. To this solution 500 μl of 2-mercaptoethanol were added and then diluted up to 10 ml with 0.4 *M* boric acid (adjusted to pH 10.4). To maintain the reagent strength, 50 μl 2-mercaptoethanol were added every 2–3 days[10], and the solution was kept under nitrogen.

An ethanol extract of rat brain synaptosomes[12], which was incubated for 2 min at 37°C with 2 m*M* Gln[13], was used for the determination of amino acids.

Derivatization

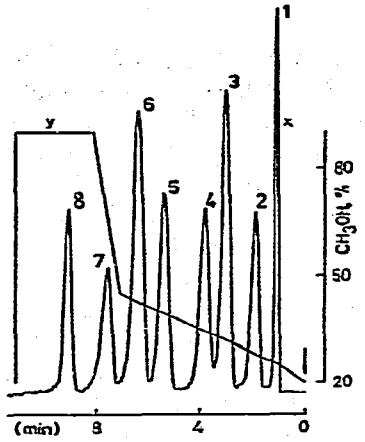
One volume (5 μl) of amino acid standard solution or synaptosomal extract was mixed with two volumes (10 μl) of the derivatization reagent solution in 100-μl Reacti-vials (Pierce, Rockford, IL, U.S.A.). The contents were mixed and injected after 90 sec at room temperature.

Chromatography

The mobile phase gradient was run from 20% to 45% methanol in two linear steps (Fig. 1) at a flow-rate of 2 ml/min. The gradient elution program was followed by a 3-min washing step (90% methanol) and, finally, the column was equilibrated with 20% methanol. The column temperature was maintained at 40°C. The relative retention times were measured from retention times which had been corrected for the void volume of the column.

RESULTS AND DISCUSSION

A typical separation of OPA derivatives of a standard solution of selected amino acids is shown in Fig. 1. In Fig. 2 is seen a chromatogram of an ethanolic extract from incubated synaptosomes[13]. The day-to-day reproducibility of the relative retention times (Table I) expressed as the coefficient of variation (C.V.) was less than 2%. The method can be used for identification of other amino acids than those listed above. The amino acids asparagine, serine, histidine, threonine, methionine sulphone, glycine, arginine and tyrosine are present in very low concentrations in the synaptosomes[11] and are completely separated from the amino acids listed in Table I (data not shown). β-Ala however overlaps to some extent with Ala. This presents no problem due to the very low concentration of β-Ala in synaptosomal extracts[11]. The OPA derivatives of tryptophan, methionine, valine, isoleucine, leucine, lysine and taurine required more than 10 min for elution and were removed from the column during the washing step.



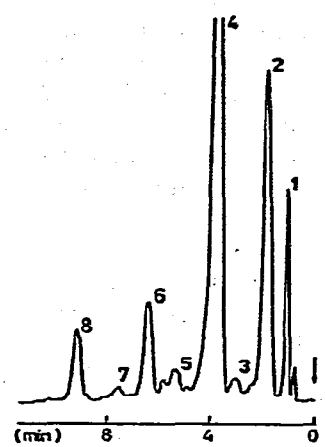


Fig. 1. Chromatogram (×) of OPA-derivatized amino acids (*ca.* 300 pmol of each). Peaks: 1 = aspartic acid; 2 = glutamic acid; 3 = serine; 4 = glutamine; 5 = glycine; 6 = alanine; 7 = histidine; 8 = GABA. Mobile phase: methanol (y)–potassium acetate, pH = 5.50. Flow-rate, 2.0 ml/min. Column temperature, 40°C.

Fig. 2. Chromatogram of an ethanolic extract (30 mg brain tissue per ml) of synaptosomes which were incubated for 2 min at 37°C with 2 m*M* glutamine and the proteins precipitated with ethanol (70% final concentration). For peaks and chromatographic conditions, see Fig. 1.

TABLE I

RELATIVE RETENTION TIMES, PEAK HEIGHTS AND COEFFICIENTS OF VARIATION (C.V.)

Concentration of each compound, 140 pmol; number of determinations, 5.

Compound	*Relative retention time**	*C.V. (%)*	*Peak height ratio***	*C.V. (%)*
Asp	0.12	1.0	1.68	3.0
Glu	0.39	0.7	0.89	2.7
Gln	1.00	0.5	1.00	0.9
Ala	1.80	1.4	0.79	3.6
Gaba	2.66	1.2	1.22	1.4

* Glutamine = 1.00.

** Defined as the ratio between peak height and amount of the compound in question, relative to that of glutamine.

The peak heights in the chromatogram were directly proportional to the amount of the OPA derivatives of the Glu, Gln, Asp, Ala and GABA in the range 20 pmol to 5000 nmol. Estimations of amino acids in synaptosomal extracts were made by the external standard method. Individual run-to-run peak-height variation, due to different degrees of derivatization, can be corrected using an internal standard (*e.g.*, asparagine) that is not present in synaptosomal extracts. Under these chromatographic conditions the coefficient of variation of the heights estimated with samples containing 140 pmol and 100 nmol of each amino acid (Glu, Gln, Asp, Ala and

GABA) was less than 5% (five determinations at each concentration). The sensitivity of the method was better than 10 pmol for all the amino acids investigated.

CONCLUSION

The HPLC method described is well suited for the determination of the amino acids present in synaptosomal extracts and is thus an excellent tool for general studies of the glutamine, glutamate and GABA metabolism. The OPA fluorimetric derivatization provides high sensitivity and the described method gives reproducible results for minimal sample preparation.

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