Journal of Chromatography, 337 (1985) 391—396
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2390

Note

Determination of neurotransmitter amino acids by high-performance liquid chromatography with fluorescence detection

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(First received May 24th, 1984; revised manuscript received September 26th, 1984)

The analysis of amino acids in brain tissue has been performed by several chromatographic methods. Some amino acids are contained in the central nervous system at very low concentrations (< 0.1 μmol/g); therefore pre- and postcolumn derivatization procedures have been developed. Ion-exchange liquid chromatography has been applied for this purpose by using postcolumn derivatization and detection as fluorescent compounds. With this method all amino acids could be separated by using an anion-exchange column for acid and neutral amino acids and a cation-exchange column for basic amino acids [1]. The determination of some amino acids has been described using separation on a cation-exchange resin and postcolumn fluorescence detection of the *o*-phthaldialdehyde (OPT) derivatives [2].

Thin-layer electrophoretic separation has been shown to be useful for the quantitation of some amino acids in brain tissue [3]. Other authors [4] determined amino acids in several brain areas by converting them to their dinitrophenyl (DNP) derivatives, which were then separated by two-dimensional thin-layer chromatography. These procedures are time-consuming [1, 2, 4], suitable only for a few amino acids [2, 3], require complex sample preparation [4], or employ expensive equipment [1].

Korf and Venema [5] applied the high-performance liquid chromatographic (HPLC) method of Jones et al. [6] for the quantitation of twelve amino acids by OPT precolumn derivatization and fluorescence detection, but in their paper several column, instrumental and derivatization conditions are not given.

The need for a simple, rapid, accurate and sensitive method for the determination of amino acids in brain tissue has led us to develop and to describe here an HPLC analysis with fluorescence detection of Dns derivatives, using the

0378-4347/85/$03.30

precolumn derivatization method mentioned in our previous work [7] for the determination of γ-aminobutyric acid. This procedure has been applied to the determination of amino acid concentrations in some brain areas under physiological conditions.

MATERIALS AND METHODS

Reagents and solutions

Amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). All other solvents and chemical reagents were of liquid chromatography and analytical grade (Carlo Erba, Milan, Italy).

Stock solutions of amino acids (10 μmol/ml in 0.1 *M* hydrochloric acid) were stored at 5°C.

A 0.1 *M* sodium bicarbonate solution in water was employed. Dns chloride stock solution (100 mg/ml in anhydrous acetone) was stored at 5°C protected from light. The working solution was 100 μl of stock solution diluted in 2 ml of acetone.

Liquid chromatography

A Series 2/2 Perkin-Elmer (Norwalk, CT, U.S.A.) liquid chromatograph with a variable-wavelength spectrofluorimeter 650 (Perkin-Elmer) and a variable-wavelength ultraviolet detector LC-75 (Perkin-Elmer) were employed. The injection valve was a Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). The system was connected to a Hitachi—Perkin-Elmer Model 56 recorder. Two RP-8 columns (10 and 5 μm particle size, 25 and 12.5 cm long) (Perkin-Elmer) were operated at room temperature.

Gradient elution was performed by using mixture A (acetonitrile—isopropanol, 90:10) and mixture B (0.011 *M* sodium acetate—0.008 *M* acetic acid). The pH of mixture B was adjusted to 3.00 with phosphoric acid. The flow-rate was 0.8 ml/min. The column effluent was monitored with the fluorescence detector at 345 nm for excitation and 545 nm for emission. The bandwidths of both monochromators were selected at 10 nm. Mixtures A and B were degassed in an ultrasonic bath for 5 min. The multi-step gradient elution of the mobile phase was carried out as described in Table I. The column was initially equilibrated with the starting mobile phase for 15 min. At the end of the separation the column was washed with mixture A for 7 min.

TABLE I

MOBILE PHASE COMPOSITION DURING GRADIENT ELUTION

Time (min)	Mixture A (%)
0	10
15	25
18	34
24	34
39	100
43	100

Animals and analysis

Male Sprague—Dawley rats (Charles River, 180—200 g) were killed by exposing their heads to microwave radiation. Brains were immediately removed, then striata were taken frozen on dry ice and stored at −30° C until assayed. 5-Aminovaleric acid (5-AVA) as internal marker (600 μg/g of sample) was added to the tissues which were homogenized with 15—20 vols. of 85% methanol.

After centrifugation at 1500 *g* for 10 min a 100-μl volume of supernatant was introduced into a screw-capped tube and dried under a nitrogen flow at 50° C. Derivatization was carried out by addition of 50 μl of bicarbonate solution and 100 μl of working Dns chloride solution followed by heating for 15 min at 90° C. The resulting solution (3—5 μl) was injected into the chromatograph. Calibration curves were constructed for each amino acid by adding to tissue extracts increasing amounts of all amino acids and the internal marker. Recovery was evaluated by using striatum homogenates, which were divided into two aliquots. One aliquot was analysed directly while the other was analysed after addition of amino acids in amounts similar to the expected tissue content. The results were then compared with those obtained by analysis of pure amino acids.

RESULTS

Recovery of the amino acids tested was found to be in the range 93 ± 7%. The sensitivity of the method varied for each amino acid, depending on the structure, ranging from 0.1 pmol for γ-aminobutyric acid to 4 pmol for glutamine. Amino acid concentrations in samples were determined by using calibration curves for each amino acid. Peak height ratios using 5-AVA as a marker were plotted against the amount of amino acid added to tissue extracts and the relationship was found to be linear over the ranges tested.

When the same tissue extract was independently analysed at three different times, the mean percentage standard deviation between trials was 9%.

The chromatogram of blank reagent (Fig. 1A) does not show interfering peaks with added amino acids.

Fig. 1B shows a chromatogram for a standard pool with a concentration of 0.1 μmol/ml for each amino acid except taurine (0.4 μmol/ml) and glutamine (0.8 μmol/ml). The separation is quite satisfactory even though the peak of aspartic acid overlaps that of histidine. The latter is present at very low levels in brain tissue and therefore does not significantly affect the height of the aspartic acid peak. Lysine shows an unusually long retention time which may be the result of lysine binding to two Dns groups.

Fig. 1C shows the chromatogram of a striatum extract processed according to the method described above. It is seen that besides the reagent peaks there are unidentified peaks. These could be attributed to other amine compounds present in brain tissue, because at the pH of reaction only these may react. Table II gives the concentrations of amino acids (μmol/g wet tissue) found in striatum; these are in a good agreement with data previously published [1, 3, 5]. Although the use of a 10-μm (25 cm) column resulted in an unsatisfactory separation between aspartic acid and glutamine, the use of a 5-μm (12.5 cm)

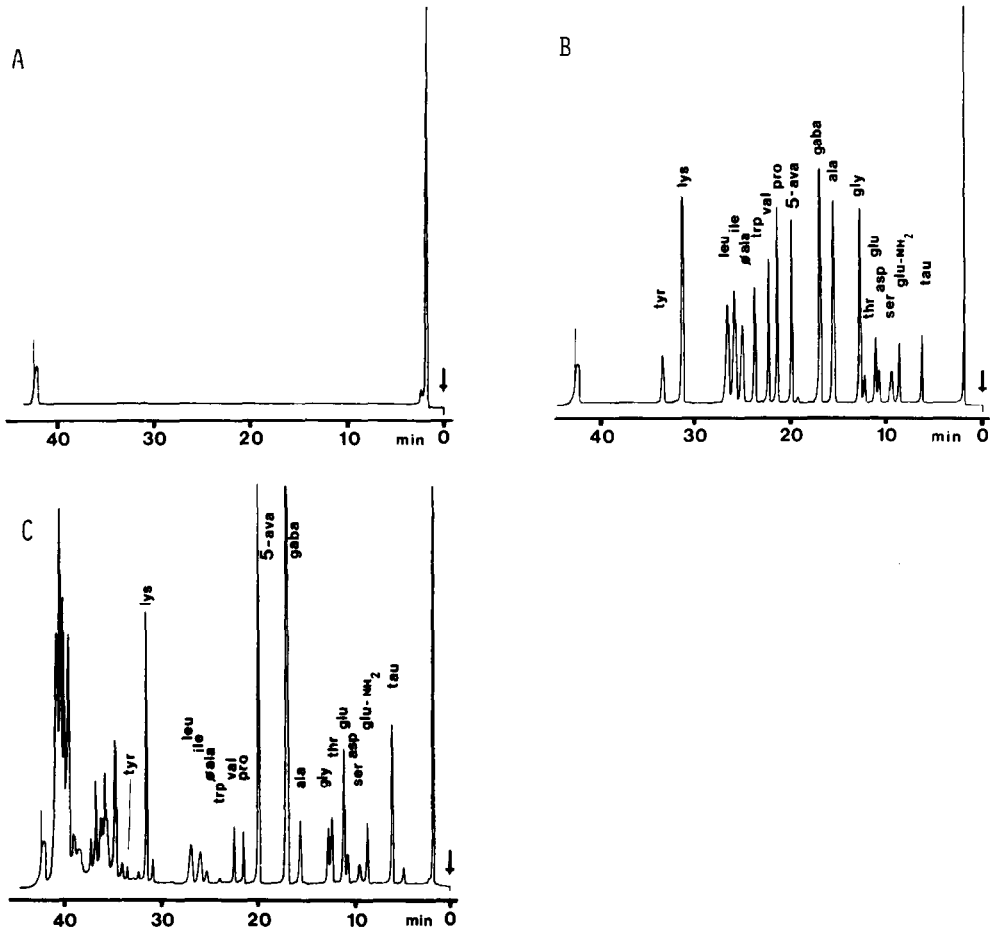

Fig. 1. Chromatograms of (A) blank reagent; (B) standard pool of seventeen derivatized amino acids; (C) derivatized striatum extract with 600 μg/g 5-AVA added. For abbreviations see Table II.

TABLE II

AMINO ACID CONCENTRATIONS IN RAT STRIATUM

Values are given as nmol amino acid per mg tissue ± S.E.M.

Amino acid	Concentration (nmol/mg)	Amino acid	Concentration (nmol/mg)
Taurine (tau)	7.81 ± 0.51	Proline (pro)	0.08 ± 0.01
Glutamine (glu-NH_2)	3.83 ± 0.47	Valine (val)	0.15 ± 0.03
Serine (ser)	1.54 ± 0.09	Tryptophan (trp)	0.09 ± 0.01
Aspartic acid (asp)	1.62 ± 0.14	Phenylalanine (ϕala)	0.03 ± 0.01
Glutamic acid (glu)	5.53 ± 0.32	Isoleucine (ile)	0.06 ± 0.01
Threonine (thr)	0.48 ± 0.04	Leucine (leu)	0.06 ± 0.01
Glycine (gly)	1.12 ± 0.07	Lysine (lys)	0.75 ± 0.06
Alanine (ala)	1.08 ± 0.06	Tyrosine (tyr)	0.15 ± 0.01
γ-Aminobutyric acid (gaba)	2.21 ± 0.36		

column gave the desired separation and had the additional advantages of lower void volume, shorter analysis time and lower solvent consumption. Some amino acids tested with this method (methionine, histidine) are present at very low levels in brain tissue and their peaks are not seen in the chromatogram of striatum (Fig. 1C). When a comparative analysis of striatum extract was made using this method and the amino acid analyser, good agreement was obtained. It is advisable to inject into the chromatograph volumes less than 10 μl, to avoid alteration in the pH of the mobile phase which results in variability of peak retention times.

This assay is highly sensitive and was applied with satisfactory results to several brain nuclei (suprachiasmatic, habenular region, dorsal and median raphe regions, periaqueductal region) with dry weights ranging from 0.5 to 2 mg. However, even smaller amounts of tissue could be analysed.

DISCUSSION

The HPLC determination of amino acids as their Dns derivatives in biological samples other than brain tissue is well known [8—13]. However, their analysis in brain tissue involves specific problems such as the presence of typical amino acids (γ-aminobutyric acid and taurine), a suitable internal standard, sensitivity, interfering compounds, etc. The use of an internal standard such as 5-AVA was found to be very important, since it is a non-endogenous amino acid and elutes well without interference in the chromatogram.

In this work the column used was selected carefully by testing different columns purchased from several manufacturers. Packing materials with nominally the same characteristics showed very different separations, by comparing plate number, peak symmetry, retention times and resolution. Such tests were not performed in other reports on the HPLC analysis of brain amino acids [5—7]. The different results observed in these tests could be explained by the lack of such packing parameters as the carbon loading, area bonded, pore diameters and other important characteristics. Unfortunately, such data are not given by the manufacturers.

The difference in response of amino acids is due to the different reactivity to Dns chloride and to the number of amino groups which undergo the reaction. The detector response was maximum for γ-aminobutyric acid, as its amino group is less subjected to inductive and steric hindrance than α-amino groups. Lysine shows a higher fluorescence response than other amino acids; this may result from two Dns-bonded groups.

The sensitivity of the method was sufficiently high to enable the analysis of the smallest brain areas; this possibility was not reported in previous studies [1—5].

In comparison to the method of Korf and Venema [5] it should be pointed out that the Dns derivatives used here are stable for many hours, whereas some OPT derivatives are extensively decomposed after 5—10 min. Moreover, the present procedure allows the analysis of seventeen amino acids, employing an appropriate internal standard.

Our method is the most rapid which allows the determination of seventeen amino acids in brain tissue. Indeed it takes only 35 min for the separation and

one sample can be injected every 55 min. The preparation of the sample is fast, simple and does not exceed 90 min. The precision of the method was also good as demonstrated by the low standard deviation.

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