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Note

Determination of γ-aminobutyric acid by reversed-phase high-performance liquid chromatography and pre-column labeling for fluorescence detection

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The role of γ-aminobutyric acid (GABA) in biological processes has received much attention since its discovery in the brain nearly 30 years ago [1, 2]. It plays an important role in polyamine metabolism [3—6]. In the central nervous system it acts as a major inhibitory neurotransmitter and may have a role in various neurological and mental disorders [7—9].

The quantitation of GABA has been accomplished by a variety of methods [10] which usually require chromatographic separation combined with derivatization [11—17]. The application of high-performance liquid chromatography (HPLC) for the analysis of dansylated GABA has not been demonstrated. This report describes a method of GABA analysis involving pre-column derivatization with dansyl chloride and separation by reversed-phase HPLC.

MATERIALS AND METHODS

Chromatographic equipment

A Series-3B high-performance liquid chromatograph equipped with a Rheodyne 7125 injection valve was used in combination with a Model LC-1000 fluorescence detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The analyses were performed on a 25 × 0.46 cm column packed with 5-μm C-8 reversed-phase packing, LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.). Chromatograms were recorded on a 10 in. recorder (Beckman Instruments, Fullerton, CA, U.S.A.) set at 1 mV. Sample injections were made using a Microliter S701 syringe (Hamilton, Reno, NV, U.S.A.).

Reagents and standards

GABA (Calbiochem-Behring, LaJolla, CA, U.S.A.), glutamic acid, guanidino-

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acetic acid (Sigma, St. Louis, MO, U.S.A.), γ-guanidinobutyric acid and β-hydroxy-γ-aminobutyric acid (Aldrich, Milwaukee, WI, U.S.A.) were used as supplied without further purification. Spectrophotometric grade acetone, toluene (Aldrich) and acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were used. All other chemicals were analytical reagent grade. 4-Amino-*n*-[U-^{14}C] butyric acid (226 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Assay

The dansylation procedure is similar to that described by Seiler [10]. All samples were prepared in 0.4 *M* perchloric acid, centrifuged and the supernatant saved for dansylation. Three times the sample volume of a solution of dansylchloride in acetone (20 mg/ml) was added to the sample and the solution saturated with solid Na_2CO_3. The tubes were agitated at room temperature overnight. Excess dansylchloride was removed by addition of 5 mg of proline dissolved in 20 μl water. The dansyl-amides were extracted with 6 ml toluene. The toluene and aqueous phase were separated by centrifugation. The extracted toluene phase was washed with a 6-ml solution of saturated $NaHCO_3$ to eliminate dansyl-amino acids. The toluene phase was separated and evaporated to dryness. The residue was dissolved in 1 ml acetone and 0.5 ml of 2 *M* potassium hydroxide was added. After 30 min the reaction mixture was saturated with $NaHCO_3$ and extracted twice with 6 ml toluene. The toluene phase was removed and discarded while 1 ml of acetone plus 0.1 ml acetic anhydride were added to the aqueous phase. After 90 min the samples were extracted with 6 ml toluene. The toluene was evaporated and samples redissolved in acetone.

Chromatography

All analyses were performed using a mobile phase consisting of 55% acetonitrile in water with a flow-rate of 60 ml/h. All chromatography was carried out at room temperature (ca. 23°C).

RESULTS

The adjusted retention time for GABA, β-hydroxy-γ-aminobutyric acid and γ-guanidinobutyric acid (Table I) indicated good separation of γ-amino acids with this procedure. Glutamic acid and guanidinoacetic acid were absent in the final extraction solutions since no fluorescent peaks were observed. Dansylated

TABLE I

ADJUSTED RETENTION TIMES OF γ-AMINO ACIDS AFTER REACTION WITH EXCESS DANSYL CHLORIDE

γ-Amino acid	t'_R (min)
γ-Aminobutyric acid	6.5
β-Hydroxy-γ-aminobutyric acid	3.2
Glutamic acid	—
γ-Guanidinobutyric acid	5.8
Guanidinoacetic acid	—

γ-amino acids were also assayed by thin-layer chromatography (TLC) [10]. This technique was used to confirm the results obtained with HPLC.

The extraction efficiency was determined using ^{14}C-radiolabeled GABA. Standards were prepared with a known amount of radioactive GABA and the assay carried out. The radioactivity was determined in an aliquot of the final extraction solution. Recovery of GABA was 87 ± 5%. This compares to previously reported values [10].

Fig. 1 represents chromatograms of a standard, blank and a rabbit brain tissue sample obtained with this assay procedure. GABA was well resolved from other peaks, which may be due to contaminants present in the GABA standard or derivatization reagent. Peak height plotted against concentration was linear over a range from 20 pmole to 2 nmole. The detection limit was determined by injecting a 100-pmole GABA standard. It was calculated that 20 pmole of dansylated GABA could be detected at twice the signal-to-noise ratio. Therefore, this method is sensitive enough to detect GABA present in 10—15 μg of rabbit cerebrum.

The accuracy of this method was determined by comparing the GABA concentration found in rabbit cerebrum with established techniques. The results obtained with the present method differed by + 1.0% when compared to TLC [10] and −5.5% when compared to that of an amino acid analyzer [11]. The precision was calculated to be ± 4.8% after analyzing eight separate samples of rabbit cerebrum.

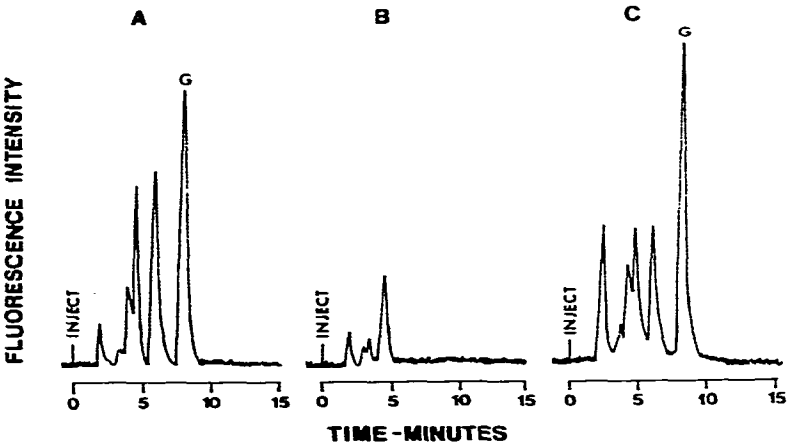

Fig. 1. Chromatograms obtained from (A) a 333 pmole dansylated GABA standard, (B) reagent blank and (C) homogenized rabbit cerebrum (ca. 200 μg). Peak G corresponds to the dansylated GABA product.

DISCUSSION

GABA is frequently assayed by using ion-exchange HPLC coupled with post-column derivatization and fluorescence detection [11—16]. This involves minimal sample preparation with detection limits ranging from 1—50 pmole. The assay time varies from 15 min to 2 h and requires a dedicated instrument and special buffers. On the other hand, reversed-phase HPLC which is applica-

ble to a wide spectrum of methods, requires minimal effort to change assay conditions.

The separation and assay of dansylated polyamines has been reported by Seiler using reversed-phase HPLC [18]. In this report we describe a reversed-phase HPLC method which allows quantitation of the GABA derivative, dansyl-oxopyrrolidine. The procedure allows rapid separation (less than 15 min) with no column equilibration necessary since an isocratic mobile phase is employed.

HPLC is a favorable technique when compared to TLC since it combines the sensitivity of the dansylation procedure with a technique suitable for automation. Since separation and quantitation are completed in one step the total analysis time is shortened. The overall resolution is greatly improved due to the development of high efficiency columns and general instrumentation technology. Radioactive measurements can easily be made on collected fractions.

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