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Note

Determination of γ-aminobutyric acid in brain areas by high-performance liquid chromatography of dansyl derivatives with ultraviolet detection

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Many different methods for the estimation of γ-aminobutyric acid (GABA) in biological samples have been published [1—7]. Unfortunately, even though some are sensitive, most of those are expensive and time-consuming. Gas chromatography coupled with mass spectrometry [4] involves expensive instrumentation and derivatization. For gas chromatography with an electron-capture detector [2] a prior ion-exchange separation is needed. Furthermore, the experimental conditions employed for the preparation of N-trifluoroacetyl-O-hexafluoro-isopropyl derivatives must be carefully controlled since these derivatives are rapidly decomposed by traces of water in the ethyl acetate used to dissolve the dried residues.

In the present work, GABA was estimated in brain tissue by a simple and rapid dansylation reaction followed by high-performance liquid chromatography (HPLC) with UV detection. This procedure was applied to the determination of GABA levels under physiological conditions and after injection of aminooxyacetic acid (AOAA).

MATERIALS AND METHODS

Reagents and solutions

Valine, GABA and acetonitrile for liquid chromatography were obtained from Merck (Darmstadt, G.F.R.). All other chemicals and reagents were of analytical grade (Carlo Erba, Milan, Italy). Stock solutions of GABA and valine were prepared at a concentration of 1 mg/ml in 0.1 *M* hydrochloric acid and stored at 5°C.

A sodium bicarbonate solution was obtained by dissolving 0.84 g of sodium bicarbonate in 100 ml of water. Dansyl chloride stock solution was prepared

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by dissolving 100 mg in 1 ml of anhydrous acetone. The working solution was 50 μl of stock solution diluted in 4 ml of acetone.

Liquid chromatography

A Series 2/2 Perkin-Elmer liquid chromatograph with a variable-wavelength UV detector LC-75 (Perkin-Elmer, Norwalk, CT, U.S.A.) and Autocontrol system was employed. The injection valve was a Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). The system was connected with a Hitachi-Perkin-Elmer Model 56 recorder. An RP-8 column (10 μm particle size; 25 cm × 4.6 mm I.D.) from Perkin-Elmer was operating at room temperature. The mobile phase was an acetonitrile–water (35:65) mixture containing 0.15% by volume H_3PO_4; the flow-rate was 1.5 ml/min. The column effluent was monitored at 254 nm.

Animals

Male Sprague-Dawley Charles River rats (150—170 g) were used. Rats were killed by microwave radiation or decapitation 2.5 min after injection of 3-mercaptopropionic acid (MPA; 100 mg/kg, intraperitoneally) dissolved in 0.154 *M* sodium hydroxide. This treatment prevents the post-mortem increase in GABA [8].

After decapitation of MPA-treated rats, the brains were quickly removed and placed on an ice-cold Petri dish; the hypothalamus, the brain stem and the striatum were dissected out, frozen on dry ice and stored at —20°C until assayed.

AOAA was injected intraperitoneally at a dose of 20 mg/kg. Injections were given in a volume of 5 ml/kg. The animals were killed by exposing their heads for 3—4 sec to high-energy microwave radiations (oven: 2.0 kW, 2.45 GHz, 75 W/cm^2; Medical Engineering Consultants, U.S.A.) [9] 2 h after injection of AOAA, and the striata were dissected out.

Analysis

Tissues were placed in 10-ml plastic tubes containing appropriate volumes of valine solution (1000 μg/g of sample) and 15 volumes of 75° ethanol. The mixture was then homogenized by a Politron and centrifuged at 1500 *g* for 10 min at 4°C. Volumes ranging from 50 to 200 μl of supernatant, depending on the expected tissue GABA concentration, were introduced into 10-ml screw-capped tubes and dried under a flow of nitrogen at 50°C.

Internal standards (IS) were prepared by adding known amounts of authentic GABA (250—1000 ng) to aliquots (50—200 μl) of supernatant pool containing appropriate volumes of valine solution and assayed in parallel with the tissue samples. The pool aliquots (50—200 μl) without added GABA served as a tissue blank (TB) or for the standards.

For derivatization 50 μl of bicarbonate solution and 100 μl of working dansyl chloride solution were added to the dried residues. The tubes were then placed on a dry block and heated for 15 min at 90°C; then 3—5 μl of this solution were injected into the chromatograph.

Calibration curve

One standard curve was constructed with increasing amounts of pure GABA

(250, 500, 1000, 1500 ng) each plus 1000 ng of valine. A second standard curve was obtained by adding the same quantities of GABA and valine to tissue extracts prepared as described before. The relationship between the peak height ratios and the amount of GABA was found to be linear over the range shown (250—1500 ng).

Recovery

Overall recovery was gauged by comparison with data obtained by HPLC analysis (assuming 100% recovery of derivatives) of the residue obtained when standard solutions of authentic compounds equal in amount to those added to striatal homogenate aliquots were taken to dryness directly. Each sample was run in duplicate.

RESULTS

Recovery after addition of GABA (200 μg/g) to homogenates was found to be 96 ± 5%. The sensitivity of the method was 1—2 ng of injected dansyl-GABA, with a signal-to-noise ratio of 2.

Amino acids that are present in the central nervous system in amounts comparable with those of GABA were tested (glutamic acid, taurine and glycine), but they did not give any peaks interfering with GABA determination. GABA concentrations were determined by the peak height ratio using valine as a marker. The amounts (μg/g) of GABA in the brain areas were calculated as follows

$$\text{GABA } (\mu g/g) = \frac{R_S \times V_H \times A_G}{(R_{IS} - R_{TB}) \times V_S \times W_T}$$

where R_S = peak height ratio of sample, R_{IS} = peak height ratio of internal standard, R_{TB} = peak height ratio of tissue blank, W_T = tissue weight (g), V_H = volume of ethanol added for homogenization, A_G = μg of GABA added to the internal standard, V_S = volume of supernatant used for derivatization.

Fig. 1 shows typical chromatograms for a reagent blank and for the tissue extract samples. In the sample chromatogram the peaks corresponding to taurine, glycine and glutamic acid overlapped the solvent front.

Table I shows the distribution of GABA concentration (μg/g) in different brain regions.

In Table II are the physiological levels of striatal GABA and the levels after intraperitoneal injection of AOAA (20 mg/kg). Fig. 2 shows the calibration curve obtained with pure standard and the curve obtained when known amounts of GABA were added to rat striatal extract.

The same tissue homogenate tested at three different times gave a coefficient of variation of 5.4%.

DISCUSSION

A dansylation method for GABA estimation in biological samples was developed by Neuhoff and Weise [1] based on the formation of unlabelled or

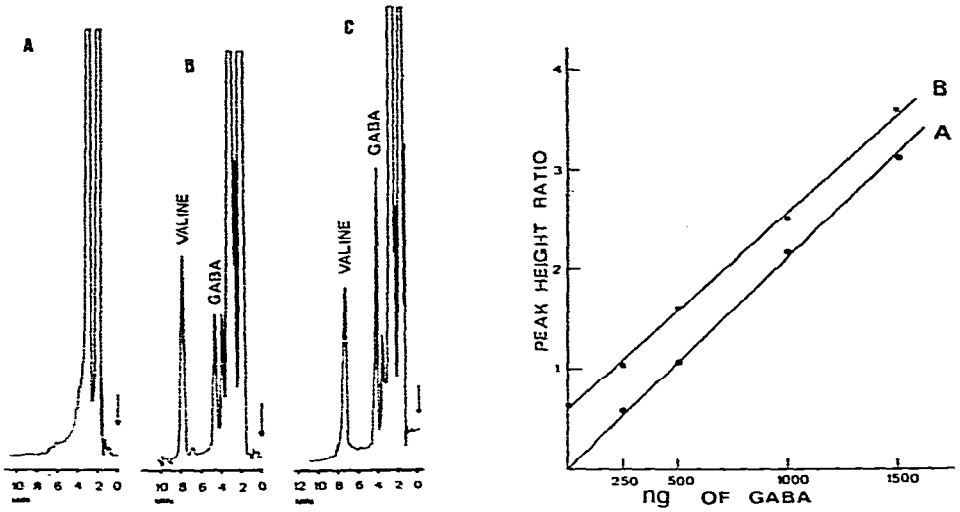

Fig. 1. (A) Chromatograms of blank reagent, (B) striatum extract with 500 μg/g valine added, and (C) the same extract after addition of 0.5 μg of GABA.

Fig. 2. Standard curves for pure GABA + valine (A) and with a striatum extract (B).

TABLE I

GABA CONCENTRATIONS IN RAT BRAIN AREAS

Animals were sacrificed by decapitation 2.5 min after injection of MPA (100 mg/kg, intraperitoneally). Values are mean ± S.E.M. of at least six determinations.

Sample	GABA (μg/g)
Brain stem	177.5 ± 9.5
Hypothalamus	513.2 ± 29.0
Striatum	238.5 ± 13.9

TABLE II

EFFECT OF INTRAPERITONEAL ADMINISTRATION OF AOAA ON STRIATUM GABA CONCENTRATIONS

Each value represents the mean ± S.E.M. of 5—7 determinations. Animals were sacrificed with high-energy microwave radiation. Striatum GABA concentrations were measured 2 h after AOAA or saline administration.

Treatment	GABA (μg/g)
Saline	181.3 ± 8.2
AOAA	502.5 ± 23.5*

*Difference from saline-treated rats $p < 0.001$, Student's t-test.

labelled dansyl derivatives, which are then separated by thin-layer chromatography. Other groups [10–12] have determined amino acids except GABA by utilizing pre-column dansyl derivatization followed by liquid chromatographic separation and detection by fluorescence. The precision, accuracy and rapidity of HPLC methodology provides an efficient means for the measurement of GABA in tissue samples. We have examined the possibility of using the pre-column dansylation technique and HPLC with UV detection.

In the UV spectrum of dansyl-GABA, recorded by means of the Autocontrol system, there are two absorbance maxima, at 220 and 287 nm. We chose to operate at 254 nm, this being the wavelength accessible also with fixed-wavelength detectors.

The precision of the method can be seen from the coefficient of variation — 5.4%. The sensitivity is satisfactory and the specificity greater than that of any other method except gas–liquid chromatography–mass spectrometry (GLC–MS).

The method is simple, reasonably rapid and has good recovery and reproducibility and apparently a high specificity. The analyses of rat brain areas gave values that are in agreement with determinations carried out by GLC–MS [4].

Treatment of rats with AOAA has been reported to elevate GABA levels markedly [13]. We also found this in the present study, in which AOAA (20 mg/kg, intraperitoneally) elevated the GABA concentration in the rat striatum about three-fold.

GABA is assumed to play an important role in the regulation of different functions, including feeding [14], heart rate [15], respiration [16], pituitary hormone release [17] and analgesia [18]. In addition, the involvement of GABA in pathological changes associated with Huntington's chorea, Parkinsonism and epilepsy adds clinical significance to the investigation of the role of GABA in the brain [19]. Thus, the availability of a new, sensitive, specific, rapid and inexpensive method for the analysis of GABA in brain areas will aid further research into the role of GABA in brain functions.

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