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Note

γ-Aminobutyric acid, glutamate, glycine and taurine analysis using reversed-phase high-performance liquid chromatography and ultraviolet detection of dansyl chloride derivatives

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Numerous assays exist for the measurement of γ-aminobutyric acid (GABA) concentrations in brain and other tissues [1–5]. However, these methods are often technically difficult and time-consuming. Moreover, most of these procedures do not permit the simultaneous quantitation of glutamate, the precursor of GABA. Since GABA is the major inhibitory neurotransmitter in brain and glutamate is the principal excitatory amino acid in the nervous system, it is important to have a rapid and sensitive method for the determination of both compounds in discrete brain regions.

The present method permits the relatively rapid and sensitive determination of GABA and glutamate in small amounts of brain tissue. In addition, this assay permits the quantitation of the putative amino acid neurotransmitter glycine and taurine in the same tissue samples that are used for the GABA and glutamate determinations. In the present method, these compounds are extracted with an ethanol–acetic acid solution, derivatized with dansyl chloride, separated using an isocratic automated reversed-phase high-performance liquid chromatographic (HPLC) system, and quantitated following UV detection. This type of procedure had been used previously by Zecca et al. [5] for the measurement of brain GABA concentrations and their method was used as a starting point for the assay in this report.

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EXPERIMENTAL

Tissue preparation

Brains used for the GABA, glutamate, glycine, and taurine determinations were obtained from male Sprague–Dawley rats (Hilltop Labs., Scottsdale, PA, U.S.A.) that were killed by either decapitation or microwave irradiation focused on their heads for 4 s using a modified Litton® microwave oven (General Medical Engineering, Peabody, MA, U.S.A.). Then, the brains were rapidly dissected on ice-cooled glass plates. Tissues were stored at −80 °C until analyzed. Large tissue samples (> 10 mg; e.g., striatum) were homogenized using a Polytron® (Brinkman, Westbury, NY, U.S.A.) and small tissue samples (< 10 mg; e.g., substantia nigra) were sonicated using a micro-ultrasonic cell disrupter (Kontes, Vineland, NJ, U.S.A.) in 40 volumes (tissue wet mass/volume) of ethanol–water–glacial acetic acid (75:20:5, v/v). This solution contained 25 μg/ml β-aminoisobutyric acid (β-AIBA), as an internal standard. Homogenates were centrifuged at 25 000 *g* for 20 min at 4 °C in polypropylene tubes. Next, 50 μl of the resulting supernatant were transferred to 12×75 mm glass tubes and evaporated to dryness in a Savant Speed-Vac concentrator (Savant Instruments, Hicksville, NY, U.S.A.). At this point the dried extracts may be stored overnight at −20 °C.

Dansylation reaction

A 100-μl volume of 0.1 *M* sodium bicarbonate was added to each dried sample and the samples were vigorously resuspended using a vortex mixer or placed in a sonicator bath to dissolve any residue. A 200-μl volume of dansyl chloride (1.25 mg/ml in actone) was added to each sample. The tubes were vortex-mixed and then incubated at 90 °C in a benchtop oven. The tubes were not capped during the incubations, and much of the sample did evaporate during incubation. However, this did not appear to adversely affect the progress of the dansylation reaction and also served to concentrate the samples. After 30 min, the tubes were removed from the oven and centrifuged at 5000 *g* for 20 min to remove the particulate matter. The dansyl derivatives in the resulting supernatants were stable for several days at −20 °C.

Chromatography

The supernatants from the dansylation reaction were transferred to HPLC microsample vials. The HPLC system used to resolve and quantify the samples consisted of: a WISP 710B autosampler (Waters Assoc., Milford, MA, U.S.A.); a Model 112 solvent delivery module (Beckman Instruments, Berkeley, CA, U.S.A.); a 3-μm, 7.5×4.6 mm Ultrasphere® ODS reversed-phase column (Beckman); a Model 153 Analytical UV detector (254 nm) (Beckman); and a C-R3A Chromatopac integrator (Shimadzu, Silver Spring, MD, U.S.A.). The HPLC mobile phase consisted of a water–acetonitrile mixture (87:13, v/v) containing 0.15% (v/v) phosphoric acid. The flow-rate was 1.0 ml/min. The system was calibrated by dansylating a solution containing 10 μg taurine, 5 μg glycine, 5 μg glutamate, 5 μg GABA, and 2.5 μg β-AIBA, as described above, and injecting 4-μl aliquots of this standard solution of dansyl derivatives into the HPLC system. Aliquots

(4 μl) of derivatized sample, containing the internal standard β-AIBA, were also injected and quantified relative to this calibration standard.

Reagents

The assay standards were purchased from Sigma (St. Louis, MO, U.S.A.) and were stored in stock solutions of 0.1 *M* hydrochloric acid at a concentration of 1–2 mg/ml. These stock solutions were stable for at least one month when stored at 4°C. Dansyl chloride (Sigma) was prepared as a stock solution in anhydrous acetone (100 mg/ml) and stored at 4°C for up to one month. All other reagents were the highest commercial quality available. Mobile phase solutions were filtered and degassed by filtration through a 0.2-μm Nylon filter under vacuum prior to use. The mobile phase was maintained in a degassed state by placing reagent bottles in a water bath which was maintained at 35°C.

RESULTS AND DISCUSSION

Extraction and dansylation

The extraction of GABA, glutamate, glycine, and taurine was accomplished by deproteinization of tissue samples with an ethanol–water–acetic acid solution. Under the conditions used over 95% of authentic standards added to tissue homogenates were recovered. Once the extracts were dried down in a vacuum centrifuge, they were derivatized with dansyl chloride. The dansylation reaction was complete by the end of reaction incubation (30 min at 90°C). In fact, the reaction was complete before the end of the incubation (unpublished observations), but the incubation time was set at 30 min to permit the removal of the volatile acetone in the samples, thereby concentrating the samples prior to the HPLC injections. Small variations in either sample recoveries, incubation conditions, or sample evaporation did not appear to affect the final results, probably because of the use of an internal standard which was present in each sample throughout the assay.

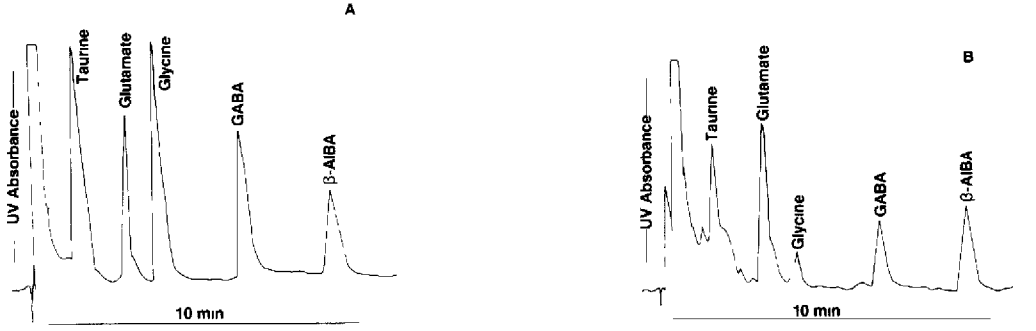

Fig. 1. Chromatograms of a sample of (A) assay standards containing 2 μg taurine and 1 μg each of glutamate, glycine, GABA, and the internal standard β-AIBA and (B) a striatal tissue extract prepared as described in the text. Full scale peak height is equal to 0.04 absorbance units.

TABLE I

CAPACITY FACTORS (k') OF VARIOUS AMINO ACIDS AND OTHER COMPOUNDS IN THE GABA HPLC ASSAY

Compounds which are measured in the assay and the internal standard, β-aminoisobutyric acid, are designated by an asterisk. k' equals the HPLC column retention time of a particular compound (t_R) minus the retention time of a non-retained compound (t_0) divided by t_0; t_0 was measured by injecting a non-polar solvent such as hexane.

Compound	k'
Cysteic acid	0.61
Glutathione	0.93
p-Ethanolamine	0.98
Asparagine	1.24
Taurine*	1.26
Methionine	1.30
Ammonia	1.71
Glutamine	1.58
Cystathionine	1.94
Leucine	1.95
Lysine	1.95
Isoleucine	1.96
Cysteine	2.01
Proline	2.14
Homocarnosine	2.43
Urea	2.45
Arginine	2.54
Hydroxyproline	2.54
Glutamic acid*	2.70
Aspartic acid	2.86
Serine	3.65
Threonine	3.65
Glycine*	4.01
Norvaline	4.55
Alanine	5.91
Valine	5.93
GABA*	6.48
ϵ-Amino-*n*-caproic acid	8.58
β-Aminoisobutyric acid*	9.25
α-Aminobutyric acid	11.42
Tryptophan	16.23
Tyrosine	22.13

Chromatography

The efficient separation of the dansyl derivatives of GABA, glutamate, glycine, taurine, and the internal standard β-AIBA is illustrated in chromatograms of assay standards (Fig. 1A) and a rat striatal tissue extract (Fig. 1B). No other compounds were present in sufficient amounts in tissue extracts to cause any interference with the quantification of the compounds that are measured by this method (Fig. 1B). Furthermore, the specificity of this assay was also examined by dansylating a number of compounds which might be present in tissue samples

and then measuring their capacity factors (k'). None of these compounds significantly overlapped with any of the assay standards (Table I). Likewise, catecholamines and serotonin, when added to extracts in concentrations in excess of those normally present in tissues, were not detected. Moreover, when the HPLC mobile phase composition was varied, no interfering peaks were detected in tissue samples (unpublished observations). In this regard, increasing the concentration of acetonitrile in the mobile phase reduces the retention time of all compounds and permits the determination of GABA concentrations with a total HPLC run time of approximately 5 min. However, the resolution of other compounds is impaired.

Assay sensitivity and reliability

The sensitivity of this method is more than sufficient to permit the quantitation of each of the compounds assayed in microgram quantities of tissue. Typically, only 4 μl of dansylated tissue extract were injected onto the HPLC column, but up to 40 μl can be injected, permitting the measurement of each of the compounds that are assayed in a few micrograms of tissue. Assay sensitivity can also be further increased by the use of microbore HPLC columns (unpublished observations). The response versus concentration curve was linear from 10 pmol to at least 100 nmol per sample for each compound assayed.

The coefficient of variation for each of the compounds measured typically was low ($<5\%$) when the same tissue sample was injected mutiple times. The high reliability of these procedures may be due to the relative simplicity of this assay and the use of the internal standard β-AIBA which reduces the effects of variations in dansylation conditions and HPLC injection volumes. In addition, the high stability of the dansyl derivatives contributes to the reliability and convenience of this assay, relative to procedures which use less stable derivatizing reagents, such as *o*-phthalaldehyde [6,7].

Tissue concentrations

The usefulness of this assay is illustrated by the data presented in Table II. Only 1.25-mg tissue samples were derivatized to obtain these data and substantially smaller tissue samples could be used. The concentrations of GABA, glutamate, glycine, and taurine in the striatum and substantia nigra of rats that were killed by either microwave irradiation (to rapidly inactivate tissue enzymes) or decapitation are shown. In addition, the effects of aminooxyacetic acid (AOAA), an inhibitor of GABA catabolism, on GABA accumulation in these two brain regions are shown. Both decapitation and AOAA produced large elevations in tissue GABA concentrations relative to microwave-irradiated control values (Table II). Substantial regional differences in the tissue concentrations of taurine, glycine, and glutamate are also evident (Table II). These data are in good agreement with those obtained using other analytical procedures [5,8–11]. Furthermore, data such as those presented in Table II can be readily obtained in a single assay with a relatively small commitment of laboratory personnel time. Thus, this method serves a need for the rapid and sensitive analysis of GABA, glutamate, glycine, and taurine in small (<1 mg) brain tissue samples.

TABLE II

EFFECTS OF DECAPITATION AND AOAA, AN INHIBITOR OF GABA CATABOLISM, ON THE CONCENTRATIONS OF TAURINE, GLUTAMATE, GLYCINE, AND GABA IN RAT STRIATUM AND SUBSTANTIA NIGRA

Groups of eight rats were injected with either saline or AOAA and killed 35 min later by either decapitation or microwave irradiation (see Experimental for details).

Sample	Concentration (mean ± S.D.) (μg/g)			
	Taurine	Glutamate	Glycine	GABA
Striatum				
Saline, i.p. (decapitated)	1028 ± 28	1443 ± 9	44 ± 2	292 ± 27
Saline, i.p. (microwaved)	1034 ± 161	1297 ± 38	46 ± 6	203 ± 32
25 mg/kg AOAA, i.p. (microwaved)	1169 ± 179	1321 ± 40	48 ± 5	302 ± 17
Substantia nigra				
Saline, i.p. (decapitated)	339 ± 16	2050 ± 38	92 ± 2	435 ± 13
Saline, i.p. (microwaved)	343 ± 10	1822 ± 192	91 ± 2	279 ± 16
25 mg/kg AOAA, i.p (microwaved)	335 ± 10	1887 ± 108	93 ± 3	489 ± 28

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