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Short Communication

γ-Aminobutyric acid, catecholamine and indoleamine determinations from the same brain region by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A new procedure for the measurement of γ-aminobutyric acid, norepinephrine, dopamine, serotonin and 5-hydroxyindoleacetic acid from the same brain region was developed. In general, two separate high-performance liquid chromatographic runs were performed, one for the γ-aminobutyric acid determination and one for the determination of the monoamines. The electrochemical detection of γ-aminobutyric acid was determined by a new procedure that utilized a small aliquot of the brain sample prepared for monoamine measurement. This assay was linear and parallel between 6 and 200 ng per 20-μl injection with 5-aminovaleric acid utilized as an internal standard. Inter-assay variability averaged 5% throughout the assay with γ-aminobutyric acid values in the gerbil hypothalamus of 344 μg/g. The catecholamine assay has been characterized previously and utilizes 3,4-dihydroxybenzylamine as an internal standard with less than 5% variability. Norepinephrine, dopamine, serotonin and 5-hydroxyindoleacetic acid levels in the gerbil hypothalamus averaged 2922, 729, 797 and 272 ng/g, respectively. This new protocol allows a wide range of neurochemicals to be determined and evaluated from the same brain region.

INTRODUCTION

The ability to measure catecholamines and indoleamines by high-performance liquid chromatography (HPLC) with electrochemical detection (ED) is a well known technique that is relatively simple to perform and yields excellent sensitivity [1–7]. This has allowed the determination of amine levels throughout the nervous system providing researchers with a vast amount of data on aminergic neurotransmission [2]. The monoamines that are routinely measured include norepinephrine (NE), dopamine (DA), serotonin (5HT) and the 5HT metabolite 5-hydroxyindoleacetic acid (5HIAA). Other metabolites and catabolites of these amines can also be assayed with minor adjustments to the HPLC protocols.

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Another compound within the nervous system that is found in greater quantities than the monoamines is γ-aminobutyric acid (GABA) [8,9]. This compound is believed to be the major inhibitory neurotransmitter of the central nervous system [8,9]. It has been measured by a number of methods with a recent innovation involving HPLC with ED [10–12]. The present study describes a new method for isocratic determination of GABA by HPLC–ED with the separate determination of the monoamines from the same sample of brain tissue.

EXPERIMENTAL

Equipment

A Bioanalytical Systems isocratic high-performance liquid chromatograph (LC-4B) was utilized with the mobile phase circulated by a Beckman Altex 110A pump at a flow-rate of 1.0 ml/min (GABA) or 1.5 ml/min (amines). The LC-4B electrochemical detector was set at +0.67 V at the working electrode for both assays. Two reversed-phase C_{18} columns were used, one for each determination. Both columns were maintained at a constant 40°C with a Bioanalytical Systems LC-22A column heater. The GABA assay utilized a Supelcosil (15 cm × 2.1 mm I.D.) RP LC-18 column (Cat. No. 5-7934, Supelco, Bellefonte, PA, USA) with a phosphate-buffered mobile phase containing 30% methanol and no sodium octyl sulfate. The catecholamine assay utilized a Supelcosil (25 cm × 2.1 mm I.D.) RP LC-18 column (Cat. No. 5-8298, Supelco) and a phosphate-buffered mobile phase with 3% methanol and 20 mg sodium octyl sulfate per liter.

Mobile phase

The same mobile phase was utilized for both assays with the only differences occurring in the amount of methanol, sodium octyl sulfate and the pH of the buffers. Centrally available reversed osmosis water was distilled and deionized. The water was added to 0.946 g of Na_2HPO_4, 2.80 g of citric acid and 18.6 mg of EDTA. The buffer for the catecholamine assay had 20 mg of sodium octyl sulfate added to the above reagents in 970 ml of water. This buffer has been found within a pH range of 2.6–2.8 and no further pH adjustment was necessary. The buffer for the GABA assay had no sodium octyl sulfate added; the remaining reagents were added to 700 ml of water and the resulting buffer was brought to a pH of 5.0. Both buffers were filtered through 0.45-μm filters and degassed for at least 5 h under vacuum and 40°C. The catecholamine buffer was added carefully to 30 ml of methanol and mixed by gently inversion to prevent addition of excess gas. Likewise, the GABA buffer was added carefully to 300 ml of methanol and mixed by gently inversion. Both buffers were allowed to sit overnight to allow equilibrium of the exothermic reaction.

Chemicals

All of the standards and dry reagents were obtained from Sigma (St. Louis, MO, USA). The liquid reagents were obtained from Fisher (Houston, TX, USA). The standards for the catecholamine assay were diluted to the proper concentration in 0.16 *M* perchloric acid (PCA) with 400 μl of 1 *M* $NaHSO_3$ per liter. The standards for the GABA assay were diluted to the proper concentration in 70% ethanol. The derivatizing solution for the GABA assay contained 250 mg of *o*-phthaldialdehyde in 2 ml of ethanol mixed with 46 ml of 0.4 *M* borate buffer, pH 10.4, and 2 ml of mercaptoethanol. This stock solution was diluted 1:10 with borate buffer for brain GABA determination or 1:100 for dialysate determination.

Animals

Samples of brain regions were collected from male gerbils (*Meriones unguiculatus*) prior to or after receiving, for ten weeks, daily saline or melatonin injections (25 μg per day) at 1630 h. The gerbils were lightly anesthetized with metofane and injected with 3-mercaptopropionic acid (100 mg/kg, intraperitoneally) 3 min before decapitation to prevent GABA catabolism after death. Brain regions of interest were rapidly removed, weighed to the nearest milligram and frozen rapidly in microcentrifuge tubes. The samples were stored at −70°C for up to three months.

Assay procedure

The brain regions were removed from the freezer, placed in an ice bath and received an appropriate amount of 0.16 *M* PCA containing dihydroxybenzylamine (DHBA, amine internal standard, 200 ng/ml). For hypothalamus, 100 μl of PCA containing DHBA was appropriate for samples weighing between 10 and 20 mg. Other brain regions required approximately 100 μl of PCA with DHBA per 10 mg wet weight. The tissue samples were disrupted by sonication and centrifuged at 13 000 *g* for 2 min. A 20-μl volume of the supernatant was transferred to another microcentrifuge tube that contained 60 μl of 70% ethanol with 5-aminovaleric acid (AVA, GABA internal standard, 2.5 μg/ml). The remainder of the brain supernatant was kept refrigerated in the dark until assayed for catecholamine and indoleamine content (see below).

The 80-μl mixture formed above was vortex-mixed well and a 20-μl aliquot was added to a 20-μl portion of the derivatizing solution. After 1 min, 20 μl were injected onto the HPLC column for GABA determination. It is important to mix the 80-μl solution well prior to aliquoting the 20-μl sample, since the acid and acohol components tend to separate. GABA and AVA were quantified by electrochemistry using a glassy carbon electrode at +0.67 V. Sample peak heights were compared to standard peak heights using a Hewlett-Packard integrator (3390A) (Fig. 1). The standards were processed in the same manner as the samples. The internal standard (AVA) was utilized to accommodate for changes in derivatization efficiency and detector sensitivity. Sample values were expressed as μg/g of tissue wet weight.

A 20-μl aliquot of the remainder of the brain sample supernatant was injected directly onto the HPLC column for standard amine determination [13–17] (Fig. 2). If the solvent front obscured the NE peak of the samples, then an aliquot of the supernatant was extracted onto alumina, washed and the resulting bound catecholamines resuspended in PCA [15]. This extraction procedure removed the indoleamines, so an unextracted sample was also injected onto the HPLC column

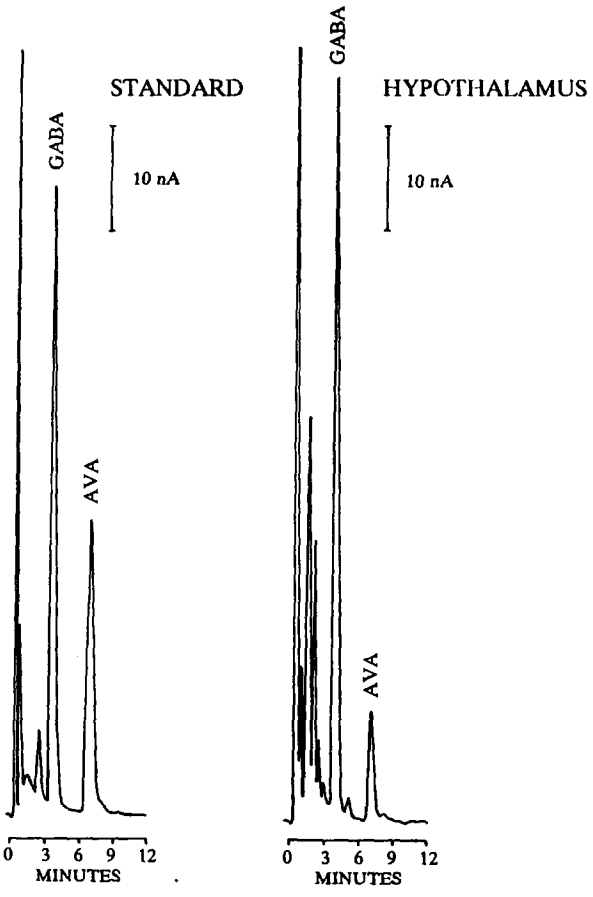

Fig. 1. Chromatograms of GABA and the internal standard, AVA, from standard and hypothalamic preparations. Retention time for GABA is 3.85 min and for AVA is 7.15 min. The standard peak heights represent 50 ng of GABA and AVA. Mobile phase characteristics and assay procedures are described in the text.

to measure these compounds. As above, the sample peak heights were compared to standard peak heights and the internal standard (DHBA) was utilized for changes in extraction efficiency and detector sensitivity. Sample values were expressed as ng/g of tissue weight.

RESULTS AND DISCUSSION

Characterization of the monoamine HPLC methodology has been performed repeatedly in the past by this laboratory and other laboratories [1-7, 13–17] (Fig. 2). The methodology for the

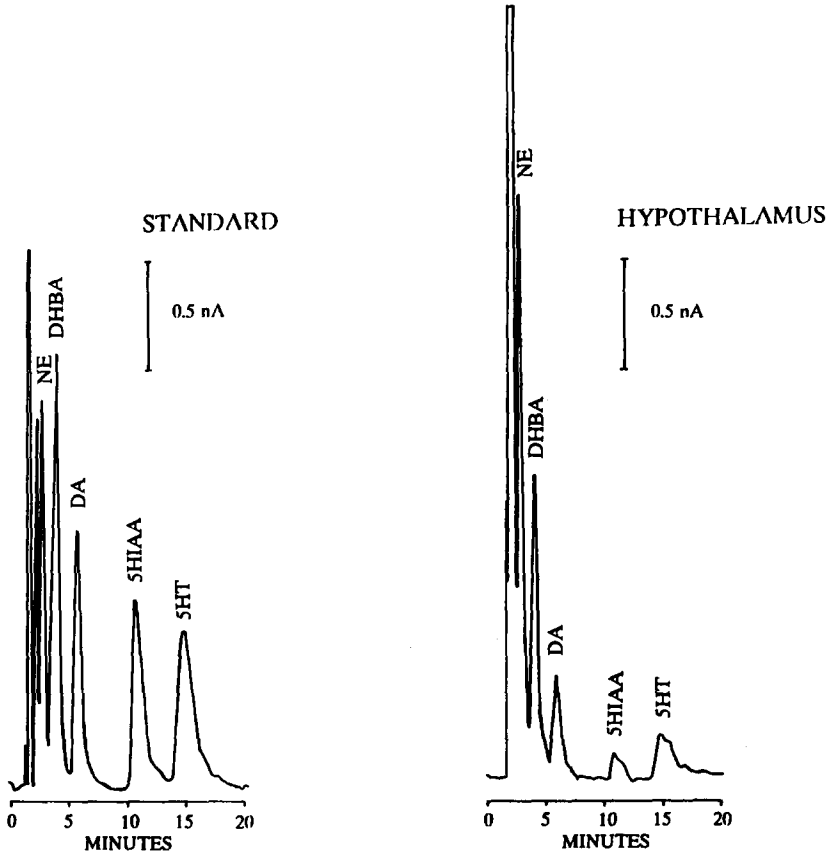

Fig. 2. Chromatograms of norepinephrine (NE), dopamine (DA), 5-hydroxyindoleacetic acid (5HIAA), serotonin (5HT) and the internal standard, dihydroxybenzylamine (DHBA), from standard and hypothalamic preparations. Retention times for the compounds are 2.97 min (NE), 4.19 min (DHBA), 6.00 min (DA), 11.27 min (5HIAA) and 15.59 min (5HT). The standard peak heights represent 2 ng of each of the amines. Mobile phase characteristics and assay procedures are described in the text.

GABA assay has been recently developed and is based on a fluorescence method [18] and other ED methodologies [10–12]. The base mobile phase for the catecholamine assay was modified for the GABA assay for ease of buffer transitions. The use of 30% methanol and a pH of 5.0 provided good peak separation and retention times (Fig. 1). A voltammogram for both GABA and AVA was constructed with an optimal voltage of +0.670 V determined for both compounds. Column temperature was adjusted and evaluated, with 40°C determined to be the most effective. GABA peak heights were unaffected by the column temperature, but AVA peak heights increased with increased column temperature. Both compounds had reduced retention times with increased column temperature. Derivatization time was also adjusted and it was determined that 1–2 min produced the best derivatization. As mentioned earlier, diluting the stock derivatizing solution 1:10 with borate buffer provided the same level of derivatizing ability with a decrease in noise and extraneous peaks. Standard curves of GABA and AVA were assayed and found to be linear and parallel between 6 and 200 ng per injection. Likewise, the same standard curves were run with the addition of 20 μl of supernatant from homogenized gerbil brain and the results were parallel to a standard curve run without addition of supernatant. Multiple runs of the

TABLE I

GABA CONTENT FROM GERBIL BRAIN REGIONS AND FROM DIALYSATES OF RAT SUPRACHIASMATIC NUCLEUS

Values are presented as means ± standard errors with at least six values in each group.

Sample	GABA content (μg/g)
Cortex	132 ± 11
Hippocampus	138 ± 9
Striatum	198 ± 27
Dialysate	200 ± 22[a]

[a] Values are in pg per 20 μl of dialysate.

standard concentrations (n = 17) were utilized to determine the percentage variability of the assay which averaged 4.99% for GABA (range = 0.11–15.68%) and 5.54% for AVA (range = 0.12–17.2%). The assay sensitivity was determined using standards and regional brain dialysates from rat suprachiasmatic nucleus. The range of values from dialysates was 143–754 pg per 20 μl of dialysate. Three times the variability of the noise produced a sensitivity of 53 pg for GABA.

The GABA assay was utilized to measure GABA in striatum, hippocampus, cortex, and brain dialysates (Table I). Pairing the GABA assay with the catecholamine assay was utilized for hypothalamic and cortical tissues. No differences in GABA or monoamine levels were observed between the treatment groups from gerbil hypothalamic tissues (Table II). The values of GABA measured in the present study (Tables I and II) were approximately ten-fold higher than previously observed values form gerbil hippocampus and striatum [19,20]. The increased GABA levels in the present study could be due to the use of 3-mercaptopropionic acid to prevent endogenous GABA degradation [18]. This compound was not used in the previous studies and, therefore, lower levels of GABA could have been determined. The catecholamine and indoleamine values observed in the present study (Table II) were similar to values observed in this and other laboratories in the past [16,17,20–22].

In conclusion, an assay methodology for the measurement of neural GABA levels has been developed and paired with an existing amine assay, so that GABA and the monoamine neurotransmitters can be determined from the same tissues. This allows for direct comparisons to be made between GABA and the monoamines while reducing the intra-animal variability obtained when these compounds are measured in multiple brain portions. The techniques described are easy to perform and can provide additional data on the neurochemistry of brain regions without the use of exhaustive experimental procedures.

TABLE II

GABA, NE, DA, 5HT AND 5HIAA CONTENT FROM GERBILS PRIOR TO AND AFTER RECEIVING DAILY INJECTIONS OF SALINE OR MELATONIN (25 μg, sc) AT 1630 h FOR TEN WEEKS

Tissues were collected and assayed for the above neurochemicals with all of the compounds determined in the same portion of hypothalamus. Values are presented as means ± standard errors.

	n	Hypothalamic content (ng/g)				
		GABA[a]	NE	DA	5HT	5HIAA
Pretreatment	5	344 ±28	2922 ±270	729 ±88	797 ±90	272 ±29
Saline	7	371 ±34	3026 ±340	1034 ±150	901 ±131	369 ±44
Melatonin	4	292 ±40	3361 ±497	1054 ±134	1013 ±179	406 ±60

[a] Values are in μg/g.

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REFERENCES

1 D. S. Chapin, R. J. Lookingland and R. E. Moore, *Curr. Sep.*, 7 (1986) 68.
2 M. F. Ganhao, J. Hattingh, M. L. Hurwitz and N. I. Pitts, *J. Chromatogr.*, 564 (1991) 55.
3 V. M. Gregory, B. Larsen and B. Benson, *J. Chromatogr.*, 345 (1985) 140.
4 I. N. Mefford, *J. Neurosci. Methods*, 3 (1981) 207.
5 L. J. Felice, J. D. Felice and P. T. Kissinger, *J. Neurochem.*, 31 (1978) 1461.
6 R. Keller, A. Oke, I. Mefford and R. N. Adams, *Life Sci.*, 19 (1976) 995.
7 C. Kotake, T. Heffner, G. Vosmer and L. Seiden, *Pharm. Biochem. Behav.*, 22 (1985) 85.
8 B. V. Manyan, *Clin. Neuropharm.*, 10 (1987) 38.
9 G. Tunnicliff and T. T. Ngo, *Neurochem. Int.*, 8 (1986) 287.
10 V. R. Roettger and M. D. Goldfinger, *Soc. Neurosci. Abstr.*, 13 (1987) 1565.
11 P. A. Shea and W. A. Jacobs, *Curr. Sep.*, 9 (1989) 57.
12 X. Xu, A. L'Helgoulac'h, E. Morier-Teisser and R. Rips, *J. Liq. Chromatogr.*, 9 (1986) 2253.
13 T. H. Champney, *J. Pineal Res.*, 9 (1990) 75.
14 T. H. Champney and R. T. Matthews, *J. Pineal Res.*, 11 (1991) 163.
15 T. H. Champney, R. W. Steger, D. S. Christie and R. J. Reiter, *Brain Res.*, 338 (1985) 25.
16 T. H. Champney, *Epilepsy Res.*, 4 (1989) 14.
17 T. H. Champney, *Neurosci. Lett.*, 120 (1990) 245.
18 G. D. Frye and G. R. Breese, *J. Pharmacol. Exp. Ther.*, 223 (1982) 750.
19 G. Delbarre, B. Delbarre, F. Calinon and A. Ferger, *J. Ocul. Pharmacol.*, 7 (1991) 147.
20 B. B. Mrsulja, B. M. Djuricic, Y. Ueki, W. D. Lust and M. Spatz, *Neurochem. Res.*, 14 (1989) 1.
21 R. Philo, R. J. Reiter and J. R. McGill, *J. Neural Trans.*, 46 (1979) 239.
22 R. Philo and R. J. Reiter, *Behav. Brain Res.*, 3 (1981) 71.