Journal of Chromatography, 227 (1982) 331—339
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1077

DETERMINATION OF γ-AMINOBUTYRIC ACID BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(Received June 15th, 1981)

SUMMARY

γ-Aminobutyric acid (GABA) has been determined in rat brain by derivatization with 2,4,6-trinitrobenzenesulfonic acid. The derivative and an internal standard, 2,4,6-trinitrophenyl-δ-aminovaleric acid, are extracted into toluene and separated by reversed-phase chromatography. Electrochemical reduction of these derivatives permits picomole measurements of GABA in microgram amounts of brain tissue.

INTRODUCTION

Electrochemical detectors for compounds separated by liquid chromatography have been shown to be extremely useful for selective determination of trace components in complex samples. This has been especially true for compounds of neurochemical interest such as catecholamines, indoleamines, and their metabolites [1]. In the determination of these classes of compounds, liquid chromatography with electrochemical detection (LC—ECD) is the method of choice because this technique is very sensitive, selective, and relatively inexpensive. The selectivity of this method arises because only compounds which can be oxidized or reduced at the potential applied to the working electrode give an electrochemical signal.

As we demonstrate in this paper, the classes of compounds which can be detected by the LC—ECD method can be extended by use of precolumn derivatization methods. Several derivatizing agents of potential utility for LC—ECD have been reviewed [2], and we have previously shown that dinitrobenzenesulfonyl chloride derivatives of amines can be detected at the picomole level [3]. These derivatization methods have been little used in LC—ECD since most of the derivatizing reagents are reduced at relatively negative potentials. Under these conditions, atmospheric oxygen in the mobile phase or sample can be a significant interference in the LC—ECD method. Recent reports describing im-

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provements in electrode materials and solution handling techniques have demonstrated that reductive LC—ECD is viable for trace analysis [3—7].

In this report, we describe a method for the determination of picomole amounts of γ-aminobutyric acid (GABA) in brain tissue using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as a derivatizing agent. Determination of GABA is important since it has been recognized as a neurotransmitter in mammalian brain. Measurements of concentrations of endogenous GABA in many types of physiological samples have been reported [8]. These include the determination of concentrations of GABA in particular brain regions [9,10], in physiological fluids [11—14] and in subcellular fractions of rat brain [15]. Discrepancies in measured GABA concentrations exist in the literature partly because free GABA levels increase during storage of samples [16,17] and, in brain tissue, because of active synthesis post mortem [9]. Discrepancy in reported GABA levels also arises since sensitive analytical methods are needed in certain applications such as the determination of free GABA in human cerebrospinal fluid or in very small brain regions. The LC—ECD derivatization method described here provides a simple method for the measurement of GABA in very small amounts of brain tissue.

The derivatizing agent that we have employed, TNBS, was introduced as a reagent for amino acid determinations several years ago [18,19]. The kinetics and mechanism of the substitution reaction [20,21] and the optimum conditions for the derivatization reactions on a macroscale have also been reported [22]. However, since detection of TNBS derivatives has been with the relatively insensitive technique of absorption spectrophotometry, this reagent has not been employed for trace analysis. As will be shown, derivatization with this reagent is quantitative even at low concentrations under proper solution conditions. TNBS can be electrochemically reduced by a $12e^-$ process at an easily accessible potential in aqueous solution. All of these features make TNBS an ideal reagent for picomole determination of GABA.

EXPERIMENTAL

Apparatus

The liquid chromatography system consisted of a buffer reservoir with a gas dispersion tube (medium frit) for nitrogen introduction to deoxygenate the mobile phase, a constant flow reciprocating pump (Model 396 Instrument Mini Pump, Milton Roy, Riviera Beach, FL, U.S.A.), and a 50-μl loop injector (Model 7010 Rheodyne, Berkeley, CA, U.S.A.). All connecting tubing was stainless steel. Pulse dampening was accomplished with a 1 m × 5.0 mm I.D. tubing between the pump and loop injector. The columns were either 25 cm × 4.6 mm I.D. Biophase (5 μm particle size) reversed-phase columns (Bioanalytical Systems, West Lafayette, IN, U.S.A.) or 50 cm × 2 mm I.D. Zipax (30 μm particle size) strong anion-exchange columns (SAX, DuPont, Wilmington, DE, U.S.A.). The mobile phase for the reversed-phase column contained 0.1 *M* sodium acetate, 0.02 *M* citric acid (pH 4.0) and 40% methanol (HPLC Grade, Fisher, Cincinnati, OH, U.S.A.). The SAX mobile phase consisted of 0.037 *M* potassium biphthalate, 0.039 *M* sodium acetate, and 0.001 *M* ethylene dinitrilotetraacetic acid disodium salt (EDTA). The flow-rate for the reversed-phase

analysis was 1.2 ml/min and for the SAX analysis was 0.8 ml/min. The separated compounds were detected amperometrically at the oxidized basal plane of pressure annealed pyrolytic graphite (PAPG) at −0.8 V vs. SCE. The detector design and characteristics have been described previously [3]. The working electrode area was 0.33 cm^2 and a 51-μm spacer was employed. Although all results in this paper are with the PAPG electrode, we have also successfully employed glassy carbon as a detector material.

Reagents

All aqueous solutions were prepared in doubly distilled water with reagent grade chemicals: (1) 0.2 *M* sodium hydroxide—0.2 *M* potassium tetraborate; (2) 2.0 *M* TNBS (Sigma St. Louis, MO, U.S.A.); (3) 0.2 *M* and 2.0 *M* perchloric acid; (4) buffer, pH 9.0 (Fisher).

Crystalline forms of 2,4,6-trinitrophenyl-γ-aminobutyric acid (TNP-GABA) and 2,4,6-trinitrophenyl-γ-aminovaleric acid (TNP-DAVA) for use as standards were prepared by a procedure similar to that of Okuyama and Satake [19]. TNBS (0.005 mol) and amino acid (0.005 mol) were added to an aqueous sodium bicarbonate solution (0.68 *M*, 35 ml). After 1 h of constant stirring and of pH maintenance at 8.5 with added sodium hydroxide (1.0 *M*), the red solution was acidified to pH 1.0 with concentrated hydrochloric acid. The bright yellow precipitate was filtered and recrystallized from a 50% ethanol—water solution. Ring substitution by the amine was verified by NMR and IR spectroscopy. The melting point for TNP-GABA was 149—151°C and for TNP-DAVA was 114—115°C, indicative of pure derivatives.

Tissue preparation

Adult male Sprague-Dawley rats were decapitated and the brain rapidly removed and placed in a dry ice—methanol solution to prevent metabolic changes of GABA post mortem [23]. The brains were then weighed, homogenized in 20 ml of perchloric acid (0.2 *M*) and subjected to sonication (Kontes Micro-Ultrasonic Cell Disrupter, Vineland, NJ, U.S.A.) for 30 sec. Subsequently, the protein was precipitated by centrifugation (Microfuge B, Beckman Instruments, Palo Alto, CA, U.S.A.) at 10,000 *g* for 4 min. The supernatant was then collected and diluted to the desired amount with perchloric acid (0.2 *M*).

Microderivatization

The pH of a sample (100 μl) in perchloric acid (0.2 *M*) is adjusted to 9.0 with the sodium hydroxide—potassium tetraborate solution (100 μl) in an Eppendorf polypropylene micro test tube (1.5 ml, Brinkman Instruments, Westbury, NY, U.S.A.). The derivatizing agent, TNBS (2.0 *M*, 20 μl), is added and the reaction is allowed to progress for 30 min. The reaction is quenched by the addition of perchloric acid (2.0 *M*, 180 μl) containing the internal standard, TNP-DAVA ($2.2 \cdot 10^{-6}$ *M*). The final pH is 0.2.

Toluene (400 μl) is added to the test tube and the mixture is vortexed for 2 min. An aliquot of the toluene layer (250 μl) is transferred to a separate tube. Fresh toluene (400 μl) is added to the reaction mixture and equilibrated as before. A second aliquot of toluene (250 μl) is removed and combined with the first toluene aliquot. The combined toluene fraction (500 μl) is reextracted

with 250 μl of pH 9.0 buffer. The aqueous solution containing the derivatized amine and internal standard is then introduced onto the reversed-phase column for separation and quantitation. Since GABA levels in brain homogenates in perchloric acid are not stable, samples are derivatized and the TNP derivatives are stored in acidic solution in the dark at 5°C.

RESULTS

Both the reversed-phase column and the SAX column with their respective mobile phases described in the Experimental section provide a suitable separation. The elution order on the reversed-phase column is TNBS, TNP-GABA, and TNP-DAVA, suggesting that the separation at pH 4.0 is primarily based on relative lipophilicity. With the SAX columns, the elution order is TNP-GABA, TNP-DAVA, and TNBS, indicative of an ionic separation mechanism. The retention times of these compounds on the SAX column are drastically reduced by the phthalate relative to an acetate buffer of identical pH, suggesting that sites for adsorption of aromatic compounds on the SAX resin are blocked by the phthalate. As will be shown, the reversed-phase column is preferable for routine analysis because of its improved resolution and faster throughput rate. However, direct injection of the reaction mixture before extraction rapidly degrades the separation efficiency of the reversed-phase column because of the massive amounts of derivatizing agent and reaction by-products present in the sample. In this work, the SAX column was useful for the development of the optimum reaction conditions because decreased resolution following direct injection is not observed.

For the reaction conditions given for the microderivatization, solutions containing known amounts of GABA are derivatized to 100% from 10^{-7} to $4 \cdot 10^{-5}$ *M* as determined by comparison of the chromatographic peak heights on the SAX column to authentic standards of known concentration (note that the concentration of the TNP-GABA which is formed in this reaction is four times less than that of the GABA because of dilutions). The yield of the derivatization reaction is extremely sensitive to pH — in more basic solutions, the derivatized GABA decomposes while at lower pH values the reaction is prohibitively slow [21]. High yields in derivatization reactions are obviously desirable to maintain accuracy and precision in any precolumn derivatization scheme.

The microextraction procedure for TNP-GABA and TNP-DAVA approaches 100% yield. Since there is some variability in this step caused by solvent evaporation, dispensing errors, etc., the addition of the internal standard is absolutely essential [24]. TNP-DAVA is an ideal compound for this role because of its structural similarity to TNP-GABA and because δ-aminovaleric acid is not present in mammalian brain. Calibration curves were generated with GABA solutions that were derivatized, extracted and chromatographed, and the peak height ratios for TNP-GABA to TNP-DAVA vs. initial GABA concentration were linear from 4—2000 pmol.

A typical reversed-phase chromatogram of a microderivatized GABA solution is shown in Fig. 1A. Peaks 7 and 9 correspond to TNP-GABA and TNP-DAVA, respectively. Derivatization of blank solutions reveals no peaks coeluting with TNP-GABA or TNP-DAVA. Peak 1 is the solvent front and

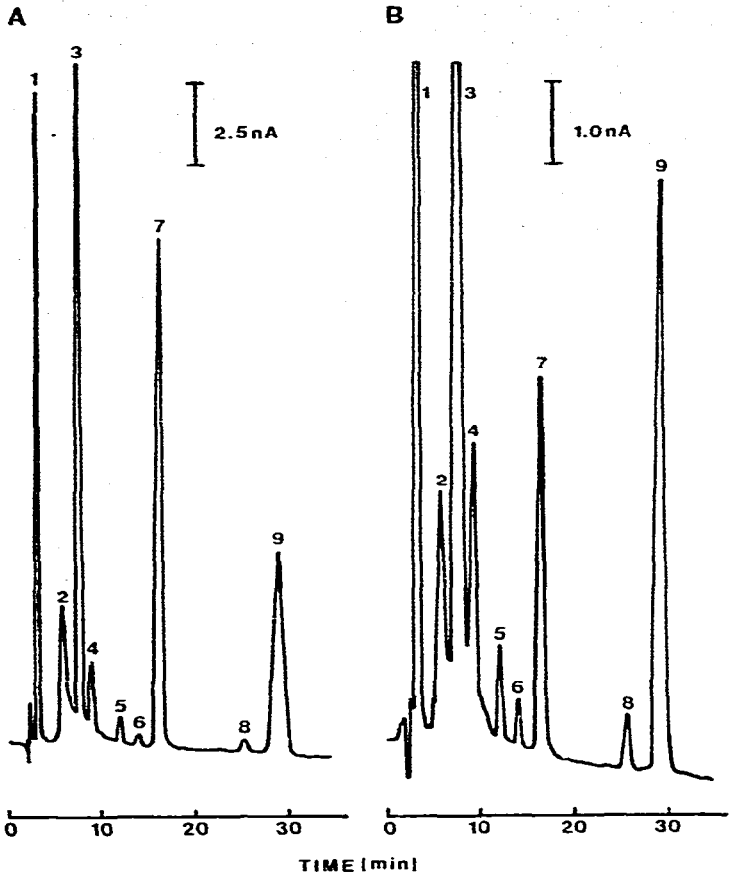

Fig. 1. Reversed-phase chromatograms. (A) Microderivatized $8 \cdot 10^{-6}$ *M* GABA solution; (B) brain homogenate that was diluted 100-fold and then microderivatized. Peaks: 1, solvent front; 2, oxygen in the sample; 3, picric acid; 4, 5, 6 and 8, reaction by-products 7 (in A), TNP-GABA (100 pmol); 7 (in B), TNP-GABA (25.8 pmol); 9, TNP-DAVA (50 pmol).

peaks 4, 5, 6, and 8 are due to reaction by-products. Peak 3 is due to picric acid that is generated in the derivatization. Peak 2 is attributed to oxygen in the sample. This peak is not present with UV detection, decreases upon deoxygenation of the sample with nitrogen and increases with cold samples were oxygen solubility is greater. Fig. 1B shows a typical chromatogram of a brain homogenate that was diluted 100-fold (corresponding to 0.084 mg wet tissue) and then microderivatized. Peak 7 corresponds to 25.8 pmol of TNP-GABA and peak 9 corresponds to 50 pmol of TNP-DAVA. Fig. 2 shows a typical chromatogram for a brain homogenate that was diluted 1000-fold and then microderivatized. Peak 7 corresponds to 2.3 pmol of TNP-GABA and peak 9 corresponds to 50 pmol of TNP-DAVA. GABA levels were determined in five whole rat brain homogenates. The mean and standard error of the mean are reported in Table I for dilutions of 10, 100, and 1000. The GABA levels are comparable to whole rat brain levels as determined by other methods [17,25].

One possible problem in this separation scheme is that some unknown com-

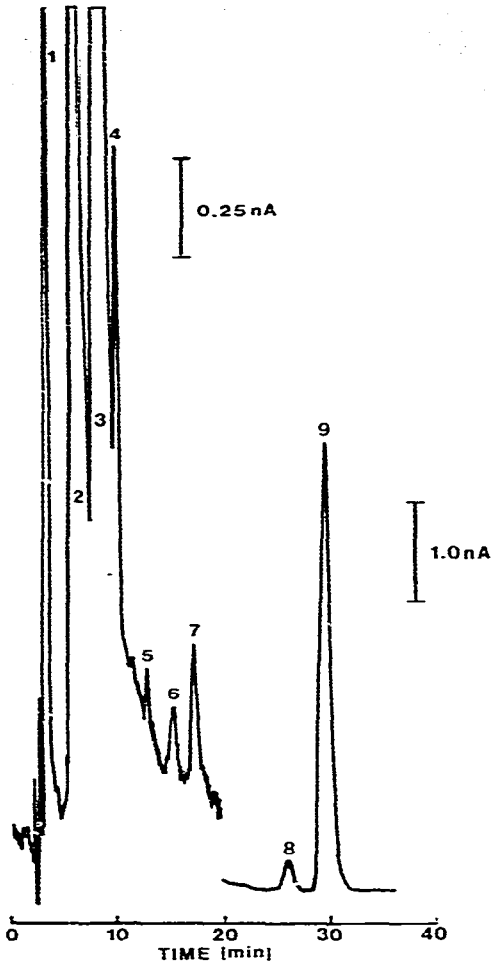

Fig. 2. Reversed-phase chromatogram of a brain homogenate that was diluted 1000-fold and then microderivatized. Peaks: 1, solvent front; 2, oxygen in the sample; 3, picric acid; 4, 5, 6 and 8, reaction by-products; 7, TNP-GABA (2.3 pmol); 9, TNP-DAVA (50 pmol).

TABLE I

WHOLE RAT BRAIN GABA LEVELS

Homogenate dilution	10	100	1000
Mean (μmol/g)	2.06	2.09	1.88
S.E.M. (n=5)	0.085	0.085	0.12

pound may coelute with TNP-GABA or TNP-DAVA in the derivatized brain homogenates. Removal of TNP-DAVA reveals no peak at the retention time corresponding to the retention time of TNP-DAVA. Our confirmation that no other electroactive compounds coelute with TNP-GABA relies on the difference in separation mechanism of the strong anion-exchange column and the reversed-phase column. Derivatized, extracted rat brain homogenates (diluted 10-fold) of three rats were collected at the time corresponding to that for TNP-GABA. The collected samples were then directly injected onto the strong anion-exchange column and the reversed-phase column and the concentrations compared (Table II). The slight difference in the concentrations of the TNP-GABA in the collected fractions is attributed to measurement error since these concentrations are near the detection limit on the SAX column. These results give strong assurance that peak 7 in the deirvatized rat brain homogenate is TNP-GABA.

TABLE II

REINJECTION DATA

Brain	Concentration ($\times 10^{-7}$ *M*)		Difference (%)
	SAX column	Reversed-phase column	
1	2.72	2.77	1.8
2	2.29	2.42	5.4
3	2.22	2.34	5.1

As shown in Fig. 2, electroreduction of TNP-GABA following reversed-phase separation is a very sensitive method for the determination of GABA in brain samples. This sensitivity is achieved in part because the oxygen level in the mobile phase is maintained at a very constant, low level with constant nitrogen bubbling in the buffer reservoir. The chief interference is oxygen in the sample which is retained on the reversed-phase column. (Oxygen in the sample is not a problem with the SAX column because it is unretained.) This can be removed by deoxygenating the sample with nitrogen, however, care must be exercised in the duration of this procedure to ensure sample reproducibility. The approach we have used is to find the potential where the ratio of the oxygen signal to that of the TNP derivatives is minimized. This ratio varies with electrode material and mobile phase composition and must be optimized for each detector. The limiting factors in the precision of this method arise from variability in reagent dispensing, extraction, and extent of reaction yield. Of course, these problems are common to all precolumn derivatization methods and the high efficiency of the reaction and the extraction for this scheme minimizes the errors.

The range of concentration of GABA that can be determined via this method is directly competitive with other methods given in the literature. Derivatization followed by gas chromatographic separation has been employed as have gas chromatography—mass spectrometry (GC-MS) techniques [12,26—31]. A radioreceptor assay has been widely used and compared with a GC—MS method

[13,32]. Both of these methods have a detection limit of 10 pmol. An improved radioreceptor assay has been developed emphasizing ^{3}[H]muscimol [10]. Liquid chromatographic separation of amino acids followed by post-column derivatization with *o*-phthalaldehyde has been widely used and is reported to give limits of detection in the picomole range [9, 11, 33—35]. Pre-column derivatization with *o*-phthalaldehyde followed by reversed-phase high-performance liquid chromatography separation has been reported with low picomole sensitivity [36].

The LC—ECD method described here for the measurement of GABA is simple and reliable. The high yield derivatization of GABA followed by extraction and separation by reversed-phase LC—ECD permits trace quantitation of this compound. This method for GABA determination in brain homogenates should be of great utility because of the widespread use of LC—ECD methods in neurochemical laboratories. The reaction and extraction conditions have been optimized for GABA; other primary amines can also be derivatized by TNBS and we are currently exploring the utility of this general scheme for these compounds.

ACKNOWLEDGEMENTS

Support of this research was provided by the National Science Foundation (BNS-81-00044). R. M. W. is the recipient of a Research Career Development Award from the National Institutes of Health. The technical aid of C.R. Troyer is greatly appreciated.

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