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High sensitivity HPLC method for analysis of *in vivo* extracellular GABA using optimized fluorescence parameters for *o*-phthalaldehyde (OPA)/sulfite derivatives

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ABSTRACT

Reversed-phase HPLC with derivatization using *o*-phthalaldehyde (OPA) and sulfite allows electrochemical detection of γ-aminobutyric acid (GABA) in microdialysis samples. However, OPA/sulfite derivatives have been reported to produce lower fluorescent yield than OPA derivatives using organic thiols as the nucleophile. To overcome this limitation we examined excitation and emission spectra, reaction time, pH, and concentration of reagents in the derivatization solution. Optimal detection parameters were determined as λ_{ex} = 220 nm and λ_{em} = 385 nm for maximal fluorescence. The derivatization reaction occurred immediately and the product was stable up to 10 min. A pH of 10.4 for the borate buffer used in the derivatization solution was significantly better than lower pH. Increasing the amount of sulfite combined with diluting the derivatization solution in borate buffer resulted in complete separation of the GABA peak from contaminants without any loss in signal. Controlling the temperature of the detector at 15 °C significantly improved sensitivity with a detection limit of approximately 1 nM. To validate this assay, we performed microdialysis in the dorsal striatum and ventral tegmental area (VTA) of adult Long Evans rats. GABA concentrations in dialysates were determined using external standards and standard additions, in order to further confirm interfering peaks were not present in biological samples. Within the dorsal striatum (n = 4), basal GABA concentrations were 12.9 ± 2.2 and 14.5 ± 2.2 nM (external and additions, respectively). Respective basal GABA concentrations in the VTA (n = 3) were 4.6 ± 1.1 and 5.1 ± 0.6 nM. Thus, we have developed a novel, sensitive fluorescence method to determine GABA in microdialysates using HPLC of an OPA/sulfite derivative.

1. Introduction

γ-Aminobutyric acid (GABA) is the primary amino acid neurotransmitter involved in inhibitory synaptic transmission and alterations in GABAergic signaling contribute to many neurological conditions including epilepsy, schizophrenia and anxiety disorders [1]. Microdialysis is a technique commonly used in basic and clinical neuroscience to measure the concentration of GABA in the extracellular space [2,3]. Many separation approaches exist for analysis of amino acid neurotransmitters, although high performance liquid chromatography (HPLC) remains one of the most widely used in neuroscience [3]. However, GABA is neither fluorescent nor electroactive and therefore requires a derivatization procedure for detection by these methods [3,4].

One of the most well characterized derivatization reagents used for analysis of amino acid neurotransmitters is *o*-phthalaldehyde (OPA), which reacts with amines in the presence of a nucleophile to form electroactive and/or fluorescent isoindole derivatives [4–7]. The use of OPA in conjunction with thiols (e.g. 2-mercaptoethanol (MCE), 3-mercaptopropionic acid (MPA)) has been widely used for both electrochemical (ECD) or fluorescence detection (FLD) of low concentrations of GABA in microdialysis samples [8–14]. However, thiol derivatives can be unstable [6,15]. This can be particularly problematic when quantifying GABA in microdialysis samples due to highly specific chromatographic conditions required for separation [13]. OPA/N-acetyl-L-cysteine (NAC) derivatives are more stable than other thiols and this method can be used for detection of enantiomeric amino acids, however there is a pungent odor associated with using thiol derivatives

Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; CE-LIF, capillary electrophoresis with laser-induced fluorescence; FLD, fluorescence detection; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; LOD, limit of detection; MCE, 2-mercaptoethanol; MPA, 3-mercaptopropionic acid; NAC, N-acetyl-L-cysteine; OPA, *o*-phthaldialdehyde; PEEK, polyetheretherketone; VTA, ventral tegmental area; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength

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An alternative to thiols is using OPA in the presence of a sulfite group as the nucleophile to form an N-alkyl-1-isoindole sulfonate derivative that has been reported to be more stable than thiol-formed derivatives [19,20]. OPA/sulfite derivatization has been successfully used in multiple applications to measure GABA in brain microdialysates using ECD [20–23]. These ECD methods require high working potentials (0.7–0.85 V) for GABA detection and can result in more frequent maintenance of the electrochemical cell. In contrast, FLD offers advantages in ease of operation and stability over long periods of time. However, OPA/sulfite derivatives were reported to be significantly less fluorescent than derivatives using OPA/thiol combinations and not useful for high sensitivity analysis of GABA in microdialysis samples [24].

Previous studies using FLD quantified GABA in tissue or brain microdialysates using OPA/thiol derivatives reported ranges of 330–365 nm and 420–530 nm for excitation (λ_{ex}) and emission (λ_{em}) wavelengths, respectively [25–28]. Recently, excitation and emission spectra were thoroughly examined for amino acids derivatized with OPA/MPA, including GABA. These authors observed the maximal signal for the OPA/MPA derivative of GABA occurred at λ_{ex} = 229 nm and λ_{em} = 450 nm, which resulted in a sixfold greater signal compared to previous wavelengths used [29]. Therefore, we hypothesized the low fluorescence yield previously reported for OPA/sulfite derivatives may have been due to suboptimal detection parameters.

Thus, the aim of the present study was to develop a sensitive method for quantification of GABA in brain microdialysis samples using HPLC-FLD of an OPA/sulfite derivative. We determined optimal detection wavelengths, derivatization components, and reaction procedures for analysis of GABA. Additionally, we used *in vivo* microdialysis to quantify extracellular GABA in the dorsal striatum or ventral tegmental area (VTA) of Long Evans rats using external standards and standard additions to validate our method.

2. Materials and methods

2.1. Reagents

GABA, GABase from Pseudomonas fluorescens, OPA, sodium dihydrogen phosphate dihydrate, sodium sulfite and sodium tetraborate decahydrate were obtained from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). Methanol was purchased from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ, USA) and absolute ethanol from AAPER (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA). Artificial cerebral spinal fluid (ACSF) for microdialysis experiments consisted of 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.4 mM D-glucose. All solutions were made with deionized water obtained from a Milli-Q system (Millipore, Billerica, MA, USA) and filtered using 0.2 μm nylon filters (Pall Corp., Ann Arbor, MI, USA).

2.2. Instrumentation

2.2.1. Liquid chromatography

HPLC separation was achieved using a Luna C18(2) column (150 × 1.0 mm, 3 μm; Phenomenex, Torrance, CA, USA), Antec LC110 pump (Antec Leyden, Zoeterwoude, Netherlands), in-line degasser, column compartment (maintained at 40 °C) equipped with a manual injector (9725i Rheodyne, Cotati, CA, USA) and polyether-etherketone (PEEK) 20 μl injection sample loop. Mobile phase was pumped at 0.05–0.1 ml/min. Injection volume varied from 5 to 10 μl using Hamilton syringes (Models 1702 or 705, Hamilton Co., Reno, NV, USA).

2.2.2. Mobile phase

The mobile phase consisted of 0.1 M sodium dihydrogen phosphate dihydrate ($NaH_2PO4{\cdot}2H_2O$) adjusted to pH 4.5 using 1 M phosphoric acid and 10–18% (v/v) methanol added to optimize chromatography.

2.2.3. Fluorescence detection

Analyte detection was achieved using a Jasco FP-4020 fluorescence detector with a 12.7 μl analytical flow cell (Jasco Corp., Tokyo, Japan). The detector gain was set to 1000, and the response time was 3 s. We attached a borosilicate glass coverslip to the flow cell to serve as an emission filter (Fisher Scientific, Fair Lawn, NJ, USA). According to the manufacturer, the glass coverslip achieves maximal transmittance (≥90%) at 360 nm. Slit widths of 20 nm for excitation and 40 nm for emission were used.

Optimal wavelengths were determined by performing excitation and emission scans using the Jasco FP-4020. Differences in signal of the GABA derivatization product (1 μM) and the mobile phase were calculated for corresponding scans. Scans were triggered manually after the peak passed a threshold point of half the total signal of the GABA peak, and were taken at a scan speed of 200 nm/s. Scans were performed at a gain set at 100 to ensure outputs were on scale.

Chromeleon 6.8 Chromatography Data System software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition and analysis. All experiments were run at minimum in triplicate across days. Chromatographic peaks were required to have a signal to background noise ratio of at least 3:1 for analysis.

2.3. Derivatization procedure

The derivatization working solution was made by dissolving 11 mg *o*-phthaldialdehyde (OPA) in 250 μl absolute ethanol, 250 μl 1 M sodium sulfite (sulfite; Na_2SO_3) and 4.5 ml 0.1 M sodium tetraborate decahydrate (borate; $Na_2B_4O_7{\cdot}10H_2O$; adjusted to pH 10.4 with 5 M NaOH) according to the methods of Smith and Sharp [21]. During the optimization experiments, final volumes of sulfite and borate buffer used in working solution were adjusted to improve chromatography (detailed in Section 3.3). The sulfite solution was made every 2 days and the borate solution made every 7 days. Both solutions were stored in glass at room temperature. The OPA/sulfite working solution was stored in covered plastic vials at 4 °C up to 24 h. Stock GABA standards were prepared by dissolving GABA in deionized water at a concentration of 1 mM and stored at 4 °C for up to 1 month. Standard dilutions were made fresh daily in either water or borate buffer based on the experiment. Polypropylene centrifuge tubes were used for standard and sample tubes to reduce loss of GABA by adsorption [20,30].

The derivatization procedure consisted of combining 9 μl of GABA standard or sample with 2 μl borate and then adding 0.5 μl OPA working solution. Experiments for optimization used 500 nM GABA, and any exceptions to this are indicated in the results.

The additional borate added to the reaction mixture was used to obtain more reproducible separation. This reaction mixture was manually mixed with a pipette and incubated at room temperature in darkness.

2.4. In vivo microdialysis procedures

Adult, male Long Evans rats (n = 7; 290–301 g upon arrival; Charles River Laboratories, Raleigh, NC, USA) were surgically implanted with a 21 gauge guide cannula (Plastics One, Roanoke, VA, USA) above the dorsal striatum (in mm relative to bregma and skull surface: 0.0 antero-posterior, +3.7 lateral, −3.0 ventral) or ventral tegmental area (VTA; −5.8 antero-posterior, +2.1 lateral, −4.6 ventral, angled 10° toward midline) using procedures similar to our previous studies [31]. The dorsoventral coordinate represents the bottom of the guide cannula, and the probes for either brain region extend 4.0 mm below the cannula when seated into the guide. Animals were allowed at least 5 days of recovery prior to microdialysis experiments.

Microdialysis probes (length of probe active area: dorsal striatum