Development and Validation of a Specific RP-HPLC Method for the Estimation of γ-Aminobutyric Acid in Rat Brain Tissue Samples Using Benzoyl Chloride Derivatization and PDA Detection

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Summary. A new, rapid, and specific reversed phase high-performance liquid chromatographic (RP-HPLC) method involving precolumn derivatization with benzoyl chloride was developed and validated for the estimation of γ-aminobutyric acid (GABA) in rat brain tissue preparations. The derivatization product of GABA was identified by melting point, infrared, and proton nuclear magnetic resonance (1H NMR) spectroscopy to be n-benzoyl GABA. Various parameters which influenced derivatization and elusion were optimized. The chromatographic system consisted of C-18 column with ultraviolet (UV)–photodiode array detection ranging from 210 to 400 nm. Elution with an isocratic mobile phase consisting of 0.025 M disodium hydrogen phosphate buffer–methanol (65:35, v/v; pH 6) at a flow rate of 1 mL min⁻¹ yielded sharp and specific peak of n-benzoyl GABA within 7 min. The method was validated with respect to the linearity, accuracy, precision, sensitivity, selectivity, and stability, wherein the benzoyl derivative of GABA showed stability for 2 months. The lower limit of detection was 0.5 nmol L⁻¹. This novel derivatization procedure for the estimation of GABA with benzoyl chloride was also applied for rat brain tissue preparations that gave highly specific peak and good component recovery. The results show that the method for the determination of GABA by benzoylation using RP-HPLC has good linearity, accuracy, precision, sensitivity, and specificity and is simple and economical to perform.

Key Words: GABA, benzoyl chloride, RP-HPLC, PDA, brain tissues, validation

Introduction

GABA (γ-aminobutyric acid) is the primary endogenous amino acid neurotransmitter exerting inhibitory physiological functions in the central nervous system and diverse protective effects in neurochemical abnormalities [1]. Therefore, trace level measurements of GABA in the brain are especially essential in studying the role of this neurotransmitter in neurophysiology,
behavioral effects, pathology, and disease diagnosis since its changes have
been associated with various neuronal disorders including epilepsy, anxi-
ety, sedation, schizophrenia, etc. [2]. Reversed phase high-performance liq-
uid chromatography (RP-HPLC) has proved to be a valuable tool in assessing
dysregulation of the GABAergic systems from a variety of samples gen-
erated from in vivo microdialysis or from post mortem tissues [3, 4]. Vari-
ous chromatographic methods have been established to estimate the end-
genous levels of several biological amines including GABA, wherein
most of them produced several interference peaks from other biological
amines including glutamate, taurine, glycine, aspartate, etc., and these
methods are compromised either with the increased retention time or with
the poor resolution of GABA peak specifically.

Low extracellular concentration, absence of chromophore or fluorophore,
and, moreover, inherent electrochemical inactivity have made the
detection of GABA quite challenging. In order to circumvent these limita-
tions with the estimation of GABA, a precolumn derivatization coupled
with HPLC–fluorescence/electrochemical detection has been in use for the
detection and/or quantification of this amino acid in microdialysate sam-

cles, brain tissue extracts, and other biological matrix preparations. This
precolumn derivatization was found to transform GABA into detectable
forms, and therefore, derivatizing agents like o-phthalaldehyde (OPA) with
β-mercaptoethanol or sodium sulphite have been widely used despite the
poor stability of the electroactive and/or fluorescent derivatives [4–8]. Use
of several other derivatizing agents like dansyl chloride, naphthalene-2,3-
dicarboxaldehyde, fluorescamine, phenyl isothiocyanate, 5-(4,6-dichloro-
striazin-2-ylamino) fluorescein, benzylamine with 1,2-diphenylethylened-
diamine, etc. has also been documented but those methods also carry stabil-
ity issues and longer run times [4, 9–11]. The reduced efficiency in GABA
analysis with existing gradient elution methods, enhanced run times, tede-
sious analytical procedure, and poor resolution due to interference from deri-
vatizing agents themselves and other matrix components have led to the op-
timization of the chromatographic methods to ensure the detection of
GABA with good resolution [3, 4, 7, 8, 12]. The modern methods also make
the chromatographic estimation more expensive with the use of costly deri-
vatizing agents like OPA and intricate technical requirements using gradi-
ent pumps, mass spectroscopic identification, microfluidic electrophoresis
chip, microchip electrophoresis, and monolithic column chromatography
coupled with chemiluminescence detection [4, 10, 11, 13, 14].

Notwithstanding the existence of numerous conventional estimation
methods for the crucial biological amines like GABA, there is an ever-
increasing demand to improve throughput, resolution and to obtain un-
equivocal identification of analyte in intricate biological matrix. The benzoy-
lation procedures have been widely acknowledged to derivatize amino group containing substances, which proceeds in presence of water and gives ultraviolet (UV)-active derivatives suitable for the extraction with organic solvents [15]. A review of literature has rarely shown any isocratic method of GABA in combination with benzoyl derivative and photodiode array (PDA) detection. Moreover, this economical and specific method facilitates the UV–PDA detection of stable GABA derivative in biological samples with enhanced throughput. Moreover, the simple UV detection was reported to be helpful for the detection of amino acids like GABA, and the electrochemical detection tends to lack reproducibility mainly because of hysteretic degradation of the electrode [14, 16].

The present study was aimed to develop a simple, specific, faster, and economical isocratic HPLC–PDA protocol for the detection and quantification of GABA in biological tissues using a single derivatizing agent (benzoyl chloride), in order to improve GABA derivative stability. Further, this work comprises optimization on derivatization conditions, chromatographic parameters, validation tests, and application of this improved HPLC–PDA method for preliminary GABA quantification method in discrete areas of rat brain tissue preparations.

**Experimental**

**Chemicals and Reagents**

All the solvents and reagents used in this study are of HPLC grade. GABA was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Carbon tetrachloride (CCl₄), chloroform (CHCl₃), and tris buffer were procured from Qualigens Fine Chemicals (Mumbai, India); benzoyl chloride (C₆H₅COCl) and sodium hydroxide, from S.D. Fine Chemicals (Mumbai, India); and dipotassium hydrogen phosphate, chloric (VII) acid, hydrochloric acid, methanol, acetonitrile, and HPLC grade water, from Merck (Mumbai, India). The mobile phase used in the HPLC system was vacuum-filtered daily through a 0.22-μm filter (Millipore).

**Instrumentation**

Melting point was determined on LabIndia microprocessor controlled melting point apparatus. An infrared spectrum was recorded on a Hitachi 270-50 double beam infrared (IR) spectrometer having a range of 4000–250 cm⁻¹. A UV spectrum was recorded on a Varian Cary 3E UV–visible spectrophotometer. Proton nuclear magnetic resonance (¹H NMR) spectra were re-
corded on a Bruker MSL 300 instrument at 300 MHz. Thin-layer chromatography (TLC) was run on commercially precoated plates (Merck, Kieselgel 60F254).

The chromatographic system consisted of a Waters Delta 600 equipped with a Waters 600 controller pump, a PDA detector Model Waters 2996 which was set at 210 to 400 nm, and a degasser module (all from Waters, Milford, MA). Chromatographic detection of GABA was achieved at 30 °C, using an RP-Inertsil ODS-3 C-18 column (250 × 4.6 mm I.D.; 5 μm particle size) (GL Sciences, Inc. USA). The mobile phase was an isocratic mixture of 0.025 M disodium hydrogen phosphate buffer:methanol (65:35, v/v; pH 6), the precolumn mixing being performed online by the inbuilt 600E Multi-solvent delivery unit operated by system software. The components of the mobile phase were reinstated and filtered daily. The column was equilibrated and eluted under these isocratic conditions with a flow rate of 1 mL min⁻¹ at 30 °C. Multiple wavelength detection with UV spectra was used for analyzing the samples. The quantitation of GABA was performed at 230 nm, and the peak maxima for GABA derivative occurred at the same wavelength. The chromatographic run time for analysis was 10 min.

System control, data acquisition, and processing were performed with Waters Empower Version 2002 chromatography software with the system suitability options installed. Peak spectra and peak purity parameters at a bandwidth of 4.8 nm were obtained from this software. Calibration data were obtained by linear regression analysis of the individual peak area of GABA versus its nominal concentrations of the standard solutions. The zero concentration sample (blank) was used to verify the purity of the reagents and the absence of the other potentially interfering substances.

Derivatization

Synthesis of n-Benzoyl-γ-Aminobutyric Acid (GABA derivative)

Derivatization reaction scheme is shown in Fig. 1. Standard GABA sample was taken in a small round-bottomed flask and dissolved in excess of 4 M NaOH solution at room temperature with slight shaking in vortex. To this, equimolar benzoyl chloride was added in 3–5 portions, and the solution was vigorously vortexed after each addition of benzoyl chloride until no oil droplets or no smell of benzoyl chloride was perceived. The solution was kept aside for 15 min, and later, saturated NaCl solution was added to quench the benzoylation reaction. The reaction mixture was poured onto crushed ice. The pH of the solution was first neutralized using 0.6 M HCl solution, added slowly with constant stirring. The melted ice was timely re-
placed with small quantities of crushed ice. The pH of the solution was checked in regular intervals after each addition of HCl solution. The solution was acidified to obtain a white product. The addition of acid was continued until the precipitate seemed to become no thicker. The pH of the solution at this point was found to be nearly 2. The solution was kept aside in ice until a thick precipitate of the benzoyl derivative settled down. The solution was filtered; the residue was air-dried and washed 3–5 times with CCl₄ to remove excess benzoic acid, formed as a byproduct. Each washing was decanted on a filter paper. The n-benzoyl GABA was recrystallized with hot water and kept aside for the formation of pure crystals. The derivative was weighed and dissolved in the solvent with same composition as that of the mobile phase and injected into the HPLC system for further analysis.

The formation of the n-benzoyl GABA (GABA derivative) was confirmed based on the physical (melting point) and spectral data (IR and NMR spectroscopy). This GABA derivative obtained in large quantity was reserved as a standard to determine the retention time and for comparisons in subsequent biological sample analysis.

**Preparation of Calibration standards and Quality Control Samples**

Standard stock solutions of GABA were prepared by dissolving 2.07 μg of n-benzoyl GABA in 10 mL of mobile phase composition, i.e., 0.025 M disodium hydrogen phosphate buffer:methanol (65:35, v/v; pH 6) to yield a final concentration of 1.00 μmol L⁻¹ standard solution. Serial dilution (500.00, 250.00, 100.00, 50.00, 10.00, 5.00, 2.50, and 1.00 nmol L⁻¹) from 1.00 μmol L⁻¹ of working standard was carried out. These calibration standards were prepared freshly in triplicate for the analytical run. Quality control samples were prepared similarly in mobile phase in the concentrations of 1.00 (lower limit of quantification (LLOQ)), 2.50, 5.00, 25.00, and 100.00 nmol L⁻¹, as de-
scribed above for the calibration standards. The samples were prepared from a stock solution different from that used to generate calibration curve samples. These quality control samples were used to investigate intra- and inter-run variations.

**Animals and Extraction of GABA from Brain Tissue Preparation**

Adult male Wistar rats (250–300 g) bred in Central Animal House facility of Panjab University, Chandigarh were used. All the animal protocols were approved by the Institutional Animal Ethics Committee. Under slight ether anesthesia, the animals were sacrificed by decapitation. The brains were rapidly dissected in ice-cold conditions. Three brain regions including the cerebral cortex, striatum, and globus pallidus were separated and frozen in cold 0.9% \( w/v \) saline at \(-80 °C \) until taken for GABA analysis. The tissue was homogenized (20% \( w/v \)) in cold 0.5 N chloric (VII) acid solution. The homogenate was centrifuged (15,000 \( \times g \)) at 4 °C for 15 min, and the resultant supernatant was filtered through 0.22 µm filter. Five hundred milliliters of this filtrate was used for derivatization, and 20 µL of the final derivative was injected (Hamilton Syringe, USA) into the HPLC system.

For recovery studies, whole brain was dissected out and was incised into two uniform cerebral hemispheres that weighed almost equal. To one part of the brain tissue, a known amount of working standard solution containing GABA was added. To the other equal hemisphere, equal quantity of the blank solution was added. The percentage recovery of GABA was determined from the following equation:

\[
\text{Recovery (\%)} = \left( \frac{C_1}{C_2 + N} \right) \times 100
\]

where \( C_1 \) is the calculated concentration from the brain tissue treated with standard GABA sample, \( C_2 \) is the calculated concentration from the brain tissue treated with blank (both concentrations were derived by relating the respective peak areas to the regression line of the standard calibration), and \( N \) is the nominal concentration added to the brain tissue.

**Derivatization of Rodent Brain Sample**

To the 500 µL of the standard GABA solution (10 nmol L\(^{-1}\)) or the biological sample with unknown concentration of GABA, 1 mL of 4 M NaOH solution was added followed by 10 µL benzoyl chloride:acetonitrile (1:1, \( v/v \)). The solution was vortex mixed and kept aside for 15 min at room temperature.
Later, 2 mL of saturated NaCl solution was added to stop the benzylation reaction. The solution was filtered into a beaker with ice. The reaction mixture was acidified with 6 M HCl solution until the pH was ~2. The reaction mixture was washed with CCl₄ for 3–4 times. Following this, the aqueous layer was extracted with CHCl₃. The chloroform layer was evaporated. Subsequently, the residue was dissolved in 500 μL of mobile phase mixture and analyzed by HPLC.

**Statistical Analysis**

Data was analyzed with SigmaStat software version 2. The results were expressed in the form of mean ± standard deviation (SD). Linearity was determined with the linear regression method. Slopes were compared by means of ANOVA of the regression. Coefficient of variance (CV) and average relative percentage deviation (% DEV) were calculated by eqs. (1) and (2).

\[
CV(\%) = \left( \frac{\text{Standard deviation}}{\text{Average calculated concentration}} \right) \times 100
\]

\[
DEV(\%) = \left(1 - \frac{\text{Average calculated concentration}}{\text{Nominal concentration}}\right) \times 100
\]

**Results and Discussion**

**Melting Point and UV Spectroscopy of n-Benzoyl GABA (GABA Derivative)**

The melting point of n-benzoyl GABA was found to be ~81 °C. The \( \lambda_{\text{max}} \) was found to be 230 nm in buffer–methanol mobile phase composition.

**Structural Confirmation of n-Benzoyl GABA by Infrared and Nuclear Magnetic Resonance Spectra**

IR (KBr): 3426 (N–H); 1738 (O=C-OH); 1628 (O=C-NH).

\(^1\)H NMR (CDCl₃) \( \delta \) ppm: 1.95 (p, 2H, –CH₂–CH₂–CH₂–); 2.45 (t, 2H, –CH₂–CH₂–COOH–); 3.5 (q, 2H, –NH–CH₂–CH₂–); 6.87 (t, 1H, –NH–CH₂–); 7.38 (m, 2H, –Ar–H); 7.45 (t, 1H, –Ar–H); 7.75 (d, 2H, –Ar–H).
Optimization of Derivatization Conditions

Major factors affecting derivatization process were the quantity of benzoyl chloride, pH of the reaction mixture, reaction time, and temperature. Different types of basic media were tested including weak bases like sodium bicarbonate, potassium bicarbonate, buffers, etc.; however, the maximum yield of the benzoylated product (GABA derivative) was favored only with excess quantity of strong base like NaOH and that too in higher molar concentrations. Moreover, the use of sodium hydroxide was also reported to be more convenient and it gives consistently good yields of benzoyl derivatives, and also does not lead to racemization [17]. The amount of the benzoyl chloride added in this reaction was critical for the formation of n-benzoyl GABA and, at the same time, determines the extent of byproducts. In the preparation of the derivative in bulk, the derivatization reaction was started with the equal molar ratio of both GABA and benzoyl chloride since the by-product (benzoic acid) formed can be completely removed from the product by washing with CCl₄. During optimizing the conditions to simulate for the preparation of the GABA derivatives for the brain samples, the quantity of the benzoyl chloride was reduced. The derivatization reaction required a critical control of the pH of the reaction medium in order to ensure the maximum yield. Derivatization reaction did not terminate in the range of pH >2. In the reaction between GABA and benzoyl chloride, it was found that the final formation of n-benzoyl GABA (GABA derivative) was favored only in the acidic conditions, at a pH ~2, and therefore, the basic reaction mixture was acidified with HCl to obtain an acidic pH, wherein a thick, white product of n-benzoyl GABA was precipitated; moreover, this acidic pH prevents the degradation of GABA derivative. The use of 0.6 M HCl was earmarked following several preliminary trials. Following subsequent recrystallization with hot water, pure crystals of n-benzoyl GABA were collected, which gave a very specific chromatographic peak at around 6 min in the mobile phase under study (Fig. 2).

In an unknown concentration of the biological sample, i.e., in case of the extracted rodent brain tissue, derivatization was critically carried out by optimizing every step such as using varied concentrations and/or volume of reagents (benzoyl chloride, NaOH and HCl) optimizing the reaction time, reaction temperature, and final extraction of the derivative for injection into column. All these steps were performed until a constant peak area for the detector response of n-benzoyl GABA and the minimized peak of benzoic acid (by-product) was reached.
Fig. 2. (a) Chromatogram of n-benzoyl GABA standard (5.00 nmol L\(^{-1}\)) prepared in bulk. Analytical conditions: column: C-18, Inertsil ODS-3, 250 × 4.6 mm I.D., 5 µm particle size; mobile phase: isocratic 0.025 M disodium hydrogen phosphate buffer:methanol (65:35, \(v/v\); pH 6); flow rate of 1 mL min\(^{-1}\) isocratic water:methanol:acetonitrile (88:5:7, \(v/v/v\)); flow rate: 1.00 mL min\(^{-1}\); temperature: 30 °C. Injection volume: 20 µL and detection wavelength: 210–400 nm. (b) Blank injection without GABA
The effects of reaction temperature and time on the derivatization yields were evaluated from 20 to 40 °C and 5–25 min, respectively (Table I). Maximal yield and constant peak areas (less coefficient of variance (CV)) were achieved when GABA was derivatized at a temperature of 30 °C for 15 min. Increase or decrease in temperature or reaction times resulted in inconsistent results and, moreover, resulted in several unknown interference peaks. Similar reaction conditions were followed in optimizing the derivatization conditions both for the known concentration of standard GABA solutions and for the brain tissue samples with unknown concentration of GABA. As the benzoylation is a rapid reaction, the optimized reaction temperature and time provide mild reaction conditions.

Table I. Effect of temperature and time of benzoylation on the sensitivity of HPLC analysis of GABA

<table>
<thead>
<tr>
<th>Time of benzoylation</th>
<th>Peak area of n-benzoyl GABA (absorbance at 230 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>20 °C</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>13349 ± 540</td>
</tr>
<tr>
<td>10 min</td>
<td>12618 ± 450</td>
</tr>
<tr>
<td>15 min</td>
<td>13349 ± 306</td>
</tr>
<tr>
<td>25 min</td>
<td>12672 ± 902</td>
</tr>
<tr>
<td>30 °C</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>16343 ± 547</td>
</tr>
<tr>
<td>10 min</td>
<td>16474 ± 405</td>
</tr>
<tr>
<td>15 min</td>
<td>17409 ± 275</td>
</tr>
<tr>
<td>25 min</td>
<td>16551 ± 470</td>
</tr>
<tr>
<td>40 °C</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>18333 ± 1060</td>
</tr>
<tr>
<td>10 min</td>
<td>19742 ± 1150</td>
</tr>
<tr>
<td>15 min</td>
<td>19528 ± 2238</td>
</tr>
<tr>
<td>25 min</td>
<td>16849 ± 1527</td>
</tr>
</tbody>
</table>

CV (%) is the coefficient of variance calculated from eq. (1) mentioned in text.

In addition, the effect of benzoyl chloride and the NaOH concentration on derivatization yields was also investigated. The peak areas of benzoyl-derivative increased along with the increasing amounts of benzoyl chloride, but at the same time, the amount of benzoic acid formed was also increased. In case of tissue samples which contain lower quantity of GABA, the concentration of benzoyl chloride had to be decreased by diluting it with the acetonitrile in order to obtain the maximum yield of the derivative and minimize the formation of the benzoic acid. In case of small quantity of standard GABA solutions, a 20-μL benzoyl chloride was standardized to be
sufficient to yield good peak of GABA derivative. On the other hand, as the brain tissue sample contains smaller amount of the GABA, the benzoyl chloride has to be diluted in 1:1 ratio with a neutral solvent such as acetonitrile and a volume of 10 μL was optimized to be the quantity essential for the formation of n-benzoyl GABA. Further, after 15 min of derivatization reaction, 1–2 g of NaCl was added to the reaction mixture to terminate the benzoylation reaction. Based on the preliminary studies, CCl₄ was selected to wash the reaction mixture for 3–4 times. CCl₄ removes the unwanted benzoic acid from the final GABA derivative.

Since an entire solidified precipitate cannot be obtained with GABA in brain sample, the reaction mixture after acidification was subjected to washing with CCl₄ as mentioned above. The organic phase containing benzoic acid derivatives was removed, and the aqueous portions were collected. These aqueous portions containing GABA derivative were again extracted with 3–4 volumes of CHCl₃. The CHCl₃ fractions were collected and evaporated under reduced pressure. The resulting residue was processed for HPLC analysis after dissolving in appropriate amount of mobile phase.

**Chromatographic Estimation**

A sensitive, specific, and reproducible method for the detection of GABA in biological sample was obtained using silica RP column. The retention time of GABA in the analytical conditions of the present study was around 6 min. Selectivity for GABA is indicated by the sharp and symmetrical resolution of the peak, as well as by the lack of any other interfering peaks from the derivatizing agents or from the biological matrix.

Most of the existing literature on the estimation of the GABA and related aminoacids in the brain use OPA with β-mercaptoethanol or sodium sulphite as the derivatizing agent for the detection and/or quantification of this aminoacid. However, the products formed using these derivatizing agents are not stable for longer duration and require faster online estimation [7]. The half-lives of various GABA derivatives are compared in Table II. One of the preliminary reports of GABA estimations by Lasley et al. (1984) showed a derivative with a half-life of 4.1 min and a run time of 35 min using OPA–β-mercaptoethanol [18]. Since then, several modifications of this method have been tried in subsequent literature emanating for GABA estimation. Recent literature has shown the use of modified derivatizing agents like 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate, substitution of β-mercaptoethanol with β-mercaptpropionic acid, sodium sulphite, etc. which could slightly increase the stability of the derivatives until the analysis time such as for few hours, ensuing from various precautionary
steps [4, 8, 14]. However, in some studies the run time remained the same [4, 14]. In addition, the chromatograms of the GABA derivatives using OPA or other derivatizing agents showed prominent peaks of these derivatizing agents which hindered the resolution of the amino acids under study [11, 12, 19].

Table II. Comparison of benzoyl derivatives with reported derivatization reagents for GABA analysis including peculiar chromatographic conditions

<table>
<thead>
<tr>
<th>Derivatizing agent</th>
<th>Stability of GABA derivative</th>
<th>Mobile phase composition</th>
<th>Flow rate (mL/min)</th>
<th>Detection</th>
<th>Elution time of GABA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA/β-mercaptoethanol</td>
<td>41 min</td>
<td>Gradient elution</td>
<td>0.1 M NaH2PO4 at pH 5.50 and 33% methanol</td>
<td>1.60</td>
<td>HPLC-BCD</td>
<td>&lt;35 min</td>
</tr>
<tr>
<td>OPA/sodium sulphite</td>
<td>30 min</td>
<td>Isoelectric elution</td>
<td>0.1 M Na3P04 and 0.5 mM EDTA with 25% methanol (v/v), water, pH 4.5</td>
<td>0.70</td>
<td>HPLC-BCD</td>
<td>&lt;25 min</td>
</tr>
<tr>
<td>OPA/sodium sulphite</td>
<td>3 hr</td>
<td>Isoelectric elution</td>
<td>25% methanol, pH 5.5, 0.1 M NaH2PO4 and 0.5 mM EDTA</td>
<td>0.30</td>
<td>HPLC-BCD</td>
<td>&lt;35 min</td>
</tr>
<tr>
<td>1,2-benzo-3,4- dihydroxycarbazole-5- ethyl chloroformate</td>
<td>48 hr</td>
<td>Gradient elution</td>
<td>Eluent A: 30% of acetonitrile consisting of 30mM ammonium formate buffer (pH 3.7) Eluent B: 100% of acetonitrile</td>
<td>1.00</td>
<td>HPLC-FLD</td>
<td>&lt;30 min</td>
</tr>
<tr>
<td>OPA/3-mercaptopyruvic acid</td>
<td>&lt;30 min</td>
<td>Isoelectric elution</td>
<td>0.1 M CH3COONa, acetic acid, and methanol (50:49:1, v/v)</td>
<td>1.00</td>
<td>HPLC-FLD</td>
<td>&lt;3 min</td>
</tr>
<tr>
<td>Naphthyl-2,3-dicarbboxylhydride</td>
<td>16 hr</td>
<td>Isoelectric elution</td>
<td>0.1 M NaH2PO4, 50μM EDTA (pH 5.6) and methanol (55:65)</td>
<td>0.55</td>
<td>HPLC-BCD and HPLC-FLD</td>
<td>&lt;20 min</td>
</tr>
<tr>
<td>OPA/β-mercaptoethanol</td>
<td>&lt;10 min</td>
<td>Gradient elution</td>
<td>0.05 M NaH2PO4, acetonitrile, tetrahydrofuran, water, pH 4.0</td>
<td>1.00</td>
<td>HPLC-FLD</td>
<td>&lt;5 min</td>
</tr>
<tr>
<td>OPA/β-mercaptoethanol</td>
<td>&lt;10 min</td>
<td>Gradient elution</td>
<td>Methanol and 0.01 M CH3COONa, pH 5.35</td>
<td>0.50</td>
<td>HPLC-FLD</td>
<td>&lt;14 min</td>
</tr>
<tr>
<td>OPA/β-mercaptoethanol</td>
<td>&lt;5 min</td>
<td>Isoelectric elution</td>
<td>0.05 M NaH2PO4, 45% methanol, 5% tetrahydrofuran, pH 6.14</td>
<td>2.50</td>
<td>HPLC-BCD</td>
<td>&lt;3 min</td>
</tr>
<tr>
<td>Benzoyl chloride</td>
<td>3 months</td>
<td>Isoelectric elution</td>
<td>0.05 M NaH2PO4 and methanol (95:5, v/v), pH 6</td>
<td>1.00</td>
<td>HPLC-PDA</td>
<td>&lt;7 min</td>
</tr>
</tbody>
</table>

The recent methods documented on the estimation of GABA by several modifications of the existing methods through modification of mobile phase composition, pH adjustments, changing the flow rate, column temperature, or the substitution of the conventional derivatizing agents also resulted in an increase in the runtime for the analysis of GABA which ranged from 10 to 60 min [3, 4, 12]. Based on the hippuric acid (n-benzoyl glycine) synthesis, we have conceived to synthesize the n-benzoyl GABA derivative since the benzoyl derivatives are quite stable for nearly 2 months and showed characteristic UV absorbance. The UV-PDA detection has other advantages like
wide availability because of comparative lower cost, free from the probability of coulometric or amperometric electrode degradation usually present in electrochemical detectors, and therefore, good reproducibility can be obtained with derivatives detectable by UV–PDA detectors [14, 16].

The GABA derivative was synthesized in bulk using large quantities of the reactants and at each step for the derivatization reaction was monitored by TLC. After several attempts, we optimized the proper molar quantifications of the reactants and specific steps were drawn out to recover the pure GABA derivative outlined above.

In order to circumvent the complexities in the use of gradient mobile phases, we have used a modified isocratic mobile phase consisting of 0.025 M disodium hydrogen phosphate buffer:methanol (65:35, v/v) at a pH 6. Initial experiments with several combinations of water and acetonitrile and/or methanol produced several impurity peaks which interfered with the peak of interest. Therefore, a normalization of the pH was found essential and a buffer was selected with low concentration where the impurity peaks were reduced. The substitution of acetonitrile with methanol also resulted in a more economical means of mobile phase utilization since it has reduced the higher costs of procuring acetonitrile in times of sheer acetonitrile shortage. The mobile phase flow rate was adjusted at 1 mL min\(^{-1}\) which gave the sharp peak of GABA at ~6 min. Although the isocratic mobile phase has been used in some of the recent reports, the run times ranging from 20 to 35 min and very low flow rate of 0.1 to 0.3 mL min\(^{-1}\) show a decrease in the efficiency of chromatographic analysis of amino acids [4, 11].

Since the brain contains lesser quantity of GABA, a micromolar range solution of standard GABA was also subjected to the same method of preparation of the derivative. In this, the amount of the benzoyl chloride added to the reaction mixture was modified accordingly. In this process, we arrived at an optimized composition of all the reactants which formed minimum quantity of the benzoic acid as observed in the ultimate chromatogram. For example, we have optimized the amount of benzoyl chloride to 10 \(\mu\)L and 3 M NaOH solution to 1 mL, for derivatizing 500 \(\mu\)L of 10 nmol L\(^{-1}\) standard GABA solution (Figs. 3a and 3b).

Standard calibration curve of GABA derivative did not show the presence of the benzoic acid. However, in the case of brain sample, the supernatant contains very little amount of GABA, and therefore, the same amount of benzoyl chloride resulted in the formation of the benzoic acid. Therefore, the chromatograms obtained from the brain samples showed a peak of benzoic acid along with the \(n\)-benzoyl GABA, which, however, did not hinder the specificity of the method for GABA estimation (Fig. 4a and 4b).
Fig. 3. (a) Chromatogram of standard GABA solution (10 nmol L$^{-1}$) prepared in accordance to the optimized method for brain sample estimation. (b) Blank injection without GABA. Analytical conditions are described in the legend of Fig. 2a

The identity of the GABA peak was confirmed by the retention time of the standard n-benzoyl GABA and also by spiking with per se GABA standard in the biological sample. Moreover, the ice cold condition maintained during the neutralization of the highly basic benzoylation product with 0.6 M HCl did not result in the formation of the precipitates corresponding
to glutamate, aspartate, and other biological amino acids tested in our preliminary experiments. In addition, spectral identity between the standard stored in the Empower software with that of the GABA present in the analyte sample also emphasizes the identification of GABA derivative in biological sample.

**Fig. 4.** (a) Representative chromatogram of n-benzoyl GABA in rat striatal tissue. (b) Blank injection without brain tissue sample. Analytical conditions are described in the legend of Fig. 2a.
Overall, the two most attractive advantages of this method are higher stability of benzoyl derivative and convenience in analysis. The utility of isocratic mobile phase of disodium hydrogen phosphate buffer:methanol (65:35, v/v) and simple derivatization process reduced the chromatographic runtime below 7 min, indicating the higher efficiency of the present method as compared to the existing chromatographic methods for the detection of GABA, which yielded the run times up to 60 min [3, 4] and with less stability of derivatives [7, 3, 12].

**Method Validation**

**Assay Linearity**

Working standard of GABA in mobile phase composition ranging from 1.00 to 250.00 nmol L\(^{-1}\) was used to build the calibration curves. The peak areas obtained from these linearity studies were plotted against the concentration, and the plots were subjected to linear regression analysis. The data for the triplicates of the standard GABA samples gave an \(r^2\) value of 0.9996 (regression equation, \(y = 3093x - 2955.8\)) in the concentration range studied. The data from the linearity curve show that the response of the GABA dilutions was absolutely linear in the concentration ranges studied. A very high correlation was obtained for all the components in the mixture with an RSD of <0.70%.

**Accuracy and Precision**

The accuracy and the precision of the results for intra-run quality control samples were within an acceptable level with DEV and CV values <5.00% (Table III). The reproducibility of the method was evaluated by repeatability studies of these quality control samples of 1.00, 2.50, 5.00, 25.00, and 100.00 nmol L\(^{-1}\). The accuracy and precision of the assay results in case inter-run samples (Table III) are shown by DEV and CV values <6.00%, which is acceptable according to established validation protocols [20, 21].
Table III. Intra- and inter-run precision and accuracy of GABA from quality control samples. The data are shown as averages ± standard deviation, precision (coefficient of variance, % CV), and accuracy (percent deviation, % DEV)

<table>
<thead>
<tr>
<th>Nominal concentration (nmol L(^{-1}))</th>
<th>Calculated concentration (μmol L(^{-1}))</th>
<th>CV (%)</th>
<th>DEV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-run ((n = 6))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (LLOQ)</td>
<td>1.00 ± 0.04</td>
<td>4.14</td>
<td>-0.33</td>
</tr>
<tr>
<td>2.50</td>
<td>2.54 ± 0.10</td>
<td>4.11</td>
<td>-1.60</td>
</tr>
<tr>
<td>5.00</td>
<td>5.05 ± 0.07</td>
<td>1.53</td>
<td>-1.13</td>
</tr>
<tr>
<td>25.00</td>
<td>24.96 ± 0.14</td>
<td>0.56</td>
<td>0.16</td>
</tr>
<tr>
<td>100.00</td>
<td>99.53 ± 0.73</td>
<td>0.73</td>
<td>0.46</td>
</tr>
<tr>
<td>Inter-run ((n = 24))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (LLOQ)</td>
<td>0.99 ± 0.05</td>
<td>5.62</td>
<td>1.00</td>
</tr>
<tr>
<td>2.50</td>
<td>2.39 ± 0.07</td>
<td>3.01</td>
<td>4.40</td>
</tr>
<tr>
<td>5.00</td>
<td>4.98 ± 0.10</td>
<td>2.14</td>
<td>0.33</td>
</tr>
<tr>
<td>25.00</td>
<td>24.13 ± 1.00</td>
<td>4.17</td>
<td>3.16</td>
</tr>
<tr>
<td>100.00</td>
<td>99.00 ± 1.51</td>
<td>1.52</td>
<td>1.00</td>
</tr>
</tbody>
</table>

LLOQ and LLOD

The lower LOQ and LOD of GABA were corresponded to 1.00 and 0.50 nmol L\(^{-1}\), wherein the accuracy values (% DEV) ranged within ±14.00% of the nominal concentration.

Specificity

In the current method, the resolution obtained was fine for the estimation of GABA in benzoylation reaction, which indicates good specificity of the method (Figs. 2 and 3). The online purity values obtained from the Empower PDA software showed that the apex purity (absorbance) of the analyte in the tissue sample was spectrally homogenous to that in the standard n-benzoyl GABA stored in reference library.

Stability

There was no evidence of degradation of n-benzoyl GABA at various time periods and storage temperatures tested. GABA derivative was found to be stable during three freeze–thaw cycles (at 25 °C) with inter-run accuracy and precision limits within ±4.00% (http://www.fda.gov/cber/guidelines.html, Shah et al., 2000). The GABA derivative was also found to be stable after 6 h and 24 h bench-top standing (30 °C). Moreover, the stability of the derivative measured after 2 months of storing at 4 °C showed an in-
ter-run accuracy and precision within ±6.00% (data not shown). This data proves the excellent stability of the benzoyl derivative compared with the widely used OPA derivatives.

### Sensitivity and Recovery

PDA detection of GABA in the range of 3.39 nmol mg\(^{-1}\) tissue indicates the good sensitivity of this method. The experimental recovery of the analyte extracted from brain tissues by this method was assessed by relating the peak area to the regression line of the standard calibration curve. The analytical recovery of GABA derivative was found to be 96.31%. The absolute recoveries of this analyte eliminated the need of an internal standard. The CV (%) of the peak areas and the retention times were within ±5.00% and ±0.50%, respectively. The results demonstrated that the pretreatment and derivatization with benzoyl chloride are satisfactory for the quantification of GABA in biological tissue samples.

### Application of the HPLC Method to Study GABA Levels in Rat Brain

Determination of GABA in animal models has been important to understand the normal function and clinical aspects of some neuronal diseases. Several methods have been attempted to estimate GABA levels using HPLC. To demonstrate the utility of the present method, a quantitative determination of GABA was done in discrete rat brain tissue preparations treated with either saline or pentylenetetrazol. Fig. 4a shows the typical chromatogram of an extract in one of the rat brain tissues. The GABA peak shows utmost resolution, stable baseline, and decreased runtime compared to the chromatograms of such amino acids in biological samples reported in the literature [8, 11, 12, 22]. The quantification of GABA in these brain samples is given in Table IV. The concentration of GABA observed in this method is comparable with those reported in literature [8, 11, 18, 23]. The animals treated with acute pentylenetetrazol (80 mg kg\(^{-1}\) i.p.), a potent convulsant, showed a slight increase in the GABA levels in postmortem brain tissues, which may be due to the blockade of presynaptic GABA receptors mediating an inhibition of GABA release on GABAergic nerve terminals [24, 25]. Though the sensitivity of the present method is comparable to or lower than some of the existing reports, the use of electrochemical or fluorescence detector, a high-pressure six-way switching valve, gradient elution, microbore column, and column oven in the reported methods leads to the intricate and costlier analyte estimation which are [12, 22].
runtimes for GABA, increased stability of GABA derivative, and increased efficiency of analysis make this method competent for the comparison of changes in GABA levels in both diseased and control conditions.

Table IV. GABA levels in discrete regions of rat brains. The amounts are expressed as nmol mg\(^{-1}\) wet tissue. Pentylenetetrazol (PTZ) was administered at a dose of 80 mg kg\(^{-1}\) i.p. Values are mean ± SD of six animals with three technical replicates

<table>
<thead>
<tr>
<th></th>
<th>Frontal cortex</th>
<th>Striatum</th>
<th>Globus pallidus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.39 ± 1.91</td>
<td>10.44 ± 3.67</td>
<td>9.37 ± 2.95</td>
</tr>
<tr>
<td>PTZ</td>
<td>5.56 ± 1.22</td>
<td>11.34 ± 2.49</td>
<td>8.88 ± 1.78</td>
</tr>
</tbody>
</table>

Conclusion

The RP-HPLC chromatographic method described over here is simple, specific, and reproducible for the determination of GABA. It was demonstrated that this method could be applicable for quantifying this foremost inhibitory neurotransmitter in the rat brain tissue samples. In particular, this method selectively allows for the determination of this analyte without any interference from the peaks of varied matrix components including several other similar aminoacids and biological amines in the brain samples. The benzoyl derivative of GABA formed by this method was stable for 2 months compared to those formed by conventional derivatizing agents. Moreover, the method utilizes the widely available benzoyl chloride, which is more economical compared to the OPA used in most of the existing methods for the estimation of the amino acids and biological amines. The use of the intricate gradient methods of elusion for the estimation of these aminoacids has been overcome by the isocratic mixture of phosphate buffer and methanol (65:35, \(v/v\)) at lower strengths. This isocratic mobile phase led to the rapid estimation of GABA within 7 min. Further refinement of this derivatization procedure to completely eliminate the chromatographic peak of benzoic acid, formed as byproduct, will still improve this method in revealing the role of GABA in normal and pathological processes.

The better specificity of the method allows for its application to study the possible correlation of the GABA in the diagnosis of several neurological and psychiatric disorders and their modification by drugs.
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Conflict of Interest

The authors do not have any conflict of interest.

References

HPLC Method for the Estimation of GABA


Accepted by DA