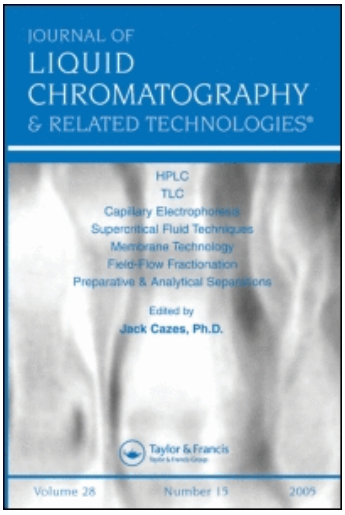

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of γ-Aminobutyric Acid and Glutamate in Human Gastric Mucosa by HPLC, as their Phenylisothiocyanate Derivatives

Li Tan[a]; Fangnan Liu[a]; Nan Luo[a]; Jieshou Li[a]
[a] Department of General Surgery, Jinling Hospital, Nanjing University, Nanjing, P. R. China

To cite this Article Tan, Li , Liu, Fangnan , Luo, Nan and Li, Jieshou(2006) 'Simultaneous Determination of γ-Aminobutyric Acid and Glutamate in Human Gastric Mucosa by HPLC, as their Phenylisothiocyanate Derivatives', Journal of Liquid Chromatography & Related Technologies, 29: 1, 45 — 53
To link to this Article: DOI: 10.1080/10826070500362144
URL: http://dx.doi.org/10.1080/10826070500362144

Journal of Liquid Chromatography & Related Technologies®, 29: 45–53, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500362144

Simultaneous Determination of γ-Aminobutyric Acid and Glutamate in Human Gastric Mucosa by HPLC, as their Phenylisothiocyanate Derivatives

Li Tan, Fangnan Liu, Nan Luo, and Jieshou Li
Department of General Surgery, Jinling Hospital, Nanjing University, Nanjing, P. R. China

Abstract: A high performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of γ-aminobutyric acid (GABA) and glutamate (Glu) in human gastric mucosa. After gastric mucosa tissues were dried under liquid nitrogen, ground, and ultrafiltered, the amino acids in these tissues were derivatized with phenylisothiocyanate. The phenythiocyanates of amino acids (PTC-amino acid) were then separated on Pico·Tag™ column, eluted with gradient mobile phases, and detected at a wavelength of 254 nm. The linear responses observed were 0.125–6.25 μM for GABA and 0.025–2.5 mM for Glu with the correlation coefficients of 0.9988 and 0.9998, respectively. The detection limits for GABA was 0.05 μM. The recoveries for GABA and Glu determinations were in the ranges of 90.4–104% and 88.1–105.5%, respectively. The intra- and inter-day RSDs were less than 10%. The method was sensitive, specific, and accurate for clinical application of GABA and Glu in human gastric mucosa tissues. It was found that GABA and Glu concentrations in gastric cancer tissues were higher than those in normal gastric mucosa tissues.

Keywords: γ-Aminobutyric acid, Glutamate, HPLC, Gastric mucosa

INTRODUCTION

γ-Aminobutyric acid (GABA) is found in both the central and peripheral nervous systems. In the central nervous system it functions as an inhibitory

Address correspondence to Li Tan, Department of General Surgery, Jinling Hospital, Nanjing University, Nanjing 210002, P. R. China. E-mail: tanli_1@yahoo.com.cn

neurotransmitter and modulates energetic changes in GABA-ergic neurons. It is synthesized principally from glutamate (Glu) (the major excitatory amino acid) via single enzymatic catalysis of glutamic acid decarboxylase (GAD).[1] Studies have also revealed that GABA, like other transmitting amino acids (glutamic acid), takes part in the regulation of cell division and affects the differentiation and maturation of mucous membrane cell in the digestive system. Several recent reports have suggested that the GABA content and GAD activity are significantly higher in neoplastic gastric tissue than in the surrounding, macroscopically unchanged tissues.[2] So, it is of clinical importance to determine GABA and Glu in human gastric mucosa.

Amino acids have been analyzed through the derivative first,[3] because they are not fluorescent, ultraviolet, or electroactive. Automated amino acid analysis based on separation with sulphonated cation exchange resins, post-column derivatization with ninhydrin, and spectrophotometric or colorimetric detection has been widely used for over 20 years.[4] However, resolution and sensitivity of analysis of GABA and Glu were not satisfactory. GABA and Glu have been determined usually using either fluorescent or electrochemical detection after derivatization by *o*-phthaldialdehyde in the presence of an alkythiol reagent,[5] mercaptoethanol,[6–8] sulphite,[9] or tert-butylthiol.[10,11] Several problems exist with these methods: the relative instability of the derivatives and their pungent odor with the use of thiols; for electrochemical detection, the reagent may shorten the life-time of the glassy carbon working electrode and may destroy the chromatogram. Gunaway S. et al.[4] reported quantitative analysis of aspartate, glutamate, serine, glutamine, glycine, and γ-aminobutyric acid in rat brain using precolumn derivatization with phenylisothiocyanate (PITC) and separation by HPLC. This method overcame the drawback of the unstable above methods. Struys et al.[12] described a method for the determination of GABA in cerebrospinal fluid (CSF) using a single GC/MS combined with the use of a stable isotope labeled internal standard.

Several of the above cited methods were described for the detection of GABA in CSF and brain tissue as well as plasma, but there were few reports on detection of GABA in gastric mucosa. This paper describes an HPLC method for the quantitative determination of GABA and Glu in gastric mucosa using precolumn derivatization with phenylisothiocyanate. The method validation studies were performed. The proposed method has been applied to observe the changes of GABA and Glu amounts in gastric mucosa of carcinoma ventriculi patients.

EXPERIMENTAL

Reagents

The standards of γ-aminobutyric acid and glutamate were obtained from Sigma (St. Louis, MO, USA). PITC was purchased from Pierce (Rockford,

IL, USA). Triethylamine was obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and Sodium acetate trihydrate from BDH Chemical (Ltd Poole, England). Water used to prepare aqueous solutions was thrice distilled. All other reagents used were of analytical grade.

Stock solutions of GABA (10 mM) and Glu (10 mM) were prepared in 0.1 M HCl, respectively, and kept at 4°C for one month. Working solutions were prepared daily by dilutions of the stock solutions. The derivatizing agent was prepared as follows: PITC-methanol-ethanol-triethylamine-water (1 : 6 : 1 : 1 : 1, v/v/v/v/v); this should be freshly prepared.

Apparatus

The Waters (Millipore, USA) liquid chromatographic system, consisted of two Model 510 pumps connected with an automated gradient controller and a 486 Tunable absorbance detector, as well as a water temperature control module. A 7725i injector with 20 μL loop (Rheodyne, USA) was used with Pico·Tag™ Work Station (Waters, USA).

General Procedure

Normal, cancerous, and peri-cancerous tissue of gastric mucosa were obtained from surgery patients. They were removed and put into liquid nitrogen immediately, then kept in an −80°C freezer until analysis. Tissue samples were thawed, then about 100 mg was weighed and put into a small mortar. Pass 5 mL liquid nitrogen into the tissue; grind it when the tissue becomes frozen. Replicate this three times and transfer the ground material into 400 μL of 5 mM Na_2HPO_4 solution (pH 7.4). The resultant solution was centrifuged for 10 min at 1600 g, and the supernatants were then ultrafiltered (Mr 10000, Millpore, USA) at 15000 g for centrifugation 30 min.

The 20 μL ultrafiltered aliquots were added into a 5 mm × 5 cm borosilicate test tube and dried under vacuum. The residue was dissolved in 10 μL of methanol −1.0 M NaAc - triethylamine (2 : 2 : 1, v : v : v) and evaporated to dryness under vacuum. A 50 μL of derivatizing agent was added to the residue and allowed to react for 20 min at room temperature to form PTC derivatives of the amino acids. Excess reagent was then removed under vacuum. For injection into the chromatograph, the PTC-amino acid derivatives were dissolved in 50 μL of 5 mM Na_2HPO_4 (pH 7.4).

The chromatographic separation was carried out in a reversed-phase system with a Pico·Tag™ column for free amino acids (3.9 mm, i.d. × 30 cm). The column was maintained at 40°C. A total running time of 30 min is needed between sample injections to allow the system to equilibrate adequately. The detection wavelength is 254 nm. Mobile phase A: 70 mM

sodium acetate buffer (pH 6.5, adjusted with 10% acetate acid) and acetonitrile (975 : 25, v/v) mixture containing 0.025% 10 mM EDTA. Mobile phase B: acetonitrile-water-methanol (450 : 400 : 150, v/v/v). These are filtered under vacuum through a 0.45 μm membrane filter before use. The flow rate of mobile phase was 1.0 mL/min and the gradient system is described in Table 1.

RESULTS AND DISCUSSION

Chromatographic Separation

PITC as a derivatization agent can react not only with primary amino acids but also with secondary amino acids, which is also a significant drawback of *o*-phthaldialdehyde. This method should be quantitative, simple, rapid, sensitive, stable, and reproducible. Although the derivative reagent was excessive, it could be removed under vacuum due to its volatility. Interference from reagent peaks was minimized. In addition, the peaks of GABA and reagent were separated completely by adjusting the gradient elution program of the analysis. Increasing the column temperature can shorten analysis time, and lower column pressure, but can reduce resolution. If raising the column temperature (just at 46°C) the GABA would overlap the cittrulline and reagent peaks following it, because GABA is sensitive to temperature. In fact, the optimal column temperature was kept at 40.0 ± 0.5°C.

Figure 1A presents the chromatogram of a blank of derivative reagent. The peaks at the retention time, approximately 2.63 min and 18.3 min, are coupling reagent peaks. Figure 1B illustrates the chromatographic separation of the standards. The retention time is 3.97 min for Glu and 16.2 min for GABA, respectively. Figure 1C represents the chromatogram of a sample

Table 1. Gradient elution program for PTC-GABA and PTC-Glu analysis

Time (min)	Mobile phase A	Mobile phase B	Curve
Initiate	100	0	*
13.50	97	3	11
20.00	96	4	8
20.50	20	80	5
22.50	0	100	6
23.00	100	0	6
30.00	100	0	6

Curve shows the exchange form of mobile phase A and B. Number 11 means according to right angle folding line; 8 is concave line; 5 is protruding form; and 6 means diagonal.

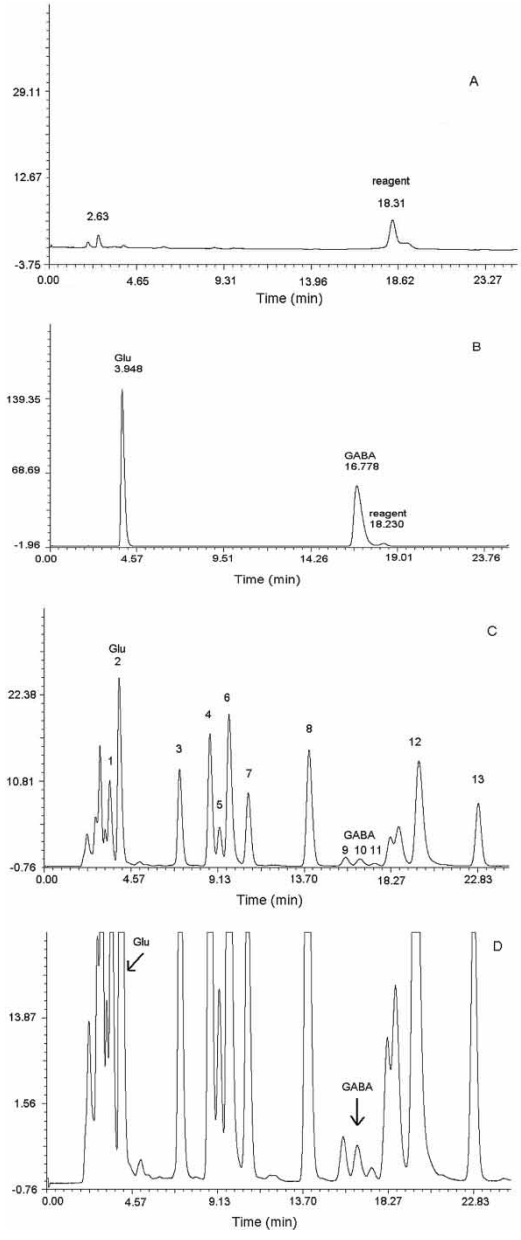

Figure 1. Chromatograms of determination of Glu and GABA. (A) The blank of derivative reagent, (B) standard of 10 μM Glu and GABA, (C) a tissue sample, (D) the amplification chromatogram of the above tissue sample. Peak 1: aspartic acid, peak 2: glutamate, peak 3: hydroxyproline, peak 4: serine, peak 5: asparagine, peak 6: glycine, peak 7: β-alanine, peak 8: taurine, peak 9: histidine, peak 10: γ-aminobutyric acid, peak 11: citrulline, peak 12: alanine, and peak 13: arginine.

from a patient. Figure 1D is the amplification chromatogram of Figure 1C and indicates that GABA and other peaks have good resolution. As can be seen from the chromatograms, not only GABA and Glu are simultaneously separated, but also other amino acids can be separated with good resolution in a analysis of less than 25 min. Compared with standards, these amino acids were aspartic acid, hydroxyproline, serine, asparagine, glycine, β-alanine, taurine, histidine, citrulline, alanine, and arginine, respectively.

The Pico·Tag^{TM} column for free amino acids is a patented product of Waters Company. It has the high efficiency needed for the reverse phase separation of PTC-amino acids. But Liang and Chang[13] accounted that it only analyzed no more than 150 PTC samples. In fact, we injected about 500 samples through the evaporating derivative reagent and then rinsing the column with acetonitrile.

Linearity and Limit of Quantification

The stock standard solution was diluted carefully with pure water, the concentrations of GABA were 0.125, 0.25, 0.625, 1.25, 2.5, and 6.25 μM, respectively, and of Glu were 0.025, 0.05, 0.10, 0.25, 0.50, 1.00, and 2.50 mM, respectively. Then, a 20 μL standard solution was moved to a test tube to be derivatized and detected according to sample precolumn derivatization and chromatographic condition. A linear relationship between the concentration of working solution and the peak area was observed. Thus, the range of quantitation for tissue was determined based on these results. The calibration curves of Glu and GABA resulted in correlation coefficients of 0.9998 for Glu and 0.9988 for GABA. Linear regression was found to be the most suitable at the concentration ranges. The limit of detection (LOD) of GABA was calculated as the peak height at the signal-to-noise ratio of 3 on the column. But, only the amount of 0.01 mM of Glu as limit determination concentration was observed. Glu concentration in gastric mucosa was far above its detection limit. The limit for GABA was 0.05 μM on the column.

Validation of the Method

Validation of the method was performed by assaying Glu and GABA standards, determined in a day ($n = 5$) and from replicate analysis on three separate days, in order to achieve the intra-day and inter-day relative standard deviations (RSD), respectively. The mixed ultrafiltered liquid was spiked with GABA 0.5, 0.75 μM and Glu 0.5, 1, 5 mM, respectively. The recoveries were determined by adding GABA and Glu. The recoveries of the methods are shown in Table 2. The intra-day RSD was 3.56% for GABA and 1.12% for Glu, respectively. The inter-day RSD was 7.47% for GABA and 5.98% for Glu, respectively.

Table 2. Recoveries of GABA and PTC-Glu determinations

	Initiate	Spiked	Found	Recovery, ($n = 3$, % $\pm$SD)
GABA (μM)	0.318 ± 0.013	0.50	0.852 ± 0.014	106.8 ± 3.0
		0.75	1.057 ± 0.082	95.3 ± 5.3
Glu (mM)	0.310 ± 0.005	0.50	0.837 ± 0.049	105.5 ± 8.1
		1.00	1.234 ± 0.036	92.5 ± 4.4
		5.00	4.825 ± 0.134	92.1 ± 1.4

Stability of PTC-GABA and PTC-Glu

We investigated the stability of PITC derivatives of GABA and Glu. A solution of ultrafiltered liquid spiked with 2.5 μM of GABA and 0.50 mM of Glu and treated according with the process of sample precolumn derivatization. Then, the reaction materials were stocked in refrigerator at 4°C. The resultant samples were detected at first day, third day, and fifth day, respectively. It is not a statistical change at the peak area of PTC-GABA and PTC-Glu.

Pretreatment of Gastric Mucosa Tissue

There are several methods to cause the tissue to rupture before further sample pre-treatment can be done. Homogenization is often done in an equal volume of aqueous buffer, such as saline or phosphate-buffered saline, or it can be performed directly in an organic solvent. Furthermore, sonications with a probe and ultrasound bath for soft tissues are often sufficient. A more rigorous rupture of the tissue is obtained with manual or motorized glass or Teflon homogenisers of the potter type.[14] The gastric mucosa tissue is first congealed in liquid nitrogen so that it can be ground easier and form smaller residue. The resultant was ultrafiltered to remove the protein and the filtered liquid was directly derived with PITC. Deproteinization by ultrafiltration has been proven to be an effective way to get rid of protein from a sample, and this method is especially suitable to the analysis of lower amounts of amino acids in tissues because the sample is not diluted. The slight imperfection of this method is higher cost.

Clinical Application

GABA and Glu in normal, cancerous, and peri-cancerous gastric tissue in 30 operative patients with gastric carcinoma were determined and the results are presented in Figure 2. The present results indicate that the concentration of

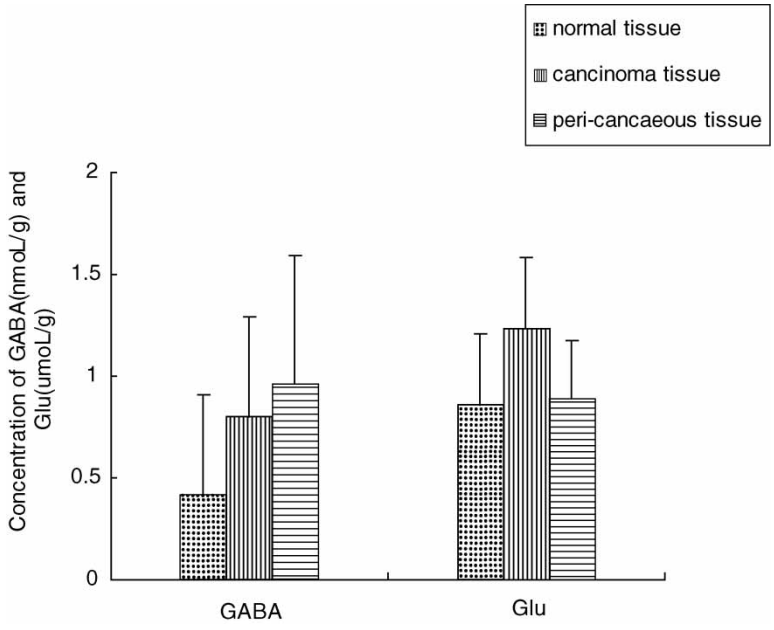

Figure 2. The changes in concentration of GABA and Glu in normal, cancerous, and peri-cancerous tissue in 30 operative patients with gastric carcinoma.

Glu in normal tissue is significantly lower than in the cancerous tissue, but there is not a significant change in the peri-cancerous tissue. On the other hand, the concentration of GABA in normal tissue is significantly lower than in both of the cancerous and peri-cancerous tissue. The results are worthy of consideration in clinical practice, which may be helpful for the diagnosis of gastric carcinoma.

REFERENCES

1. Martin, D.L.; Rimval, K. Regulation of GABA synthesis in the brain. J. Neurochem. **1993**, *60* (2), 395–407.
2. Matuszek, M.; Jesipowicz, M.; Kleinrok, Z. GABA content and GAD activity in gastric cancer. Med. Sci. Monit. **2001**, *7* (3), 377–381.
3. Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. B. **1984**, *336* (1), 93–104.
4. Gunawan, S.; Walton, N.Y.; Treiman, D.M. High performance liquid chromatographic determination of selected amino acids in rat brain by precolumn derivatization with phenylisothiocyanate. J. Chromatogr. A **1990**, *503* (1), 177–187.
5. Smith, S.; Sharp, T. Measurement of GABA in rat brain microdialysates using *o*-phthaldialdehyde-sulphite derivatization and high performance liquid chromatography with electrochemical detection. J. Chromatogr. Bio. Sci. Appl. **1994**, *652* (2), 228–233.

6. Kamisaki, Y.; Takao, Y.; Itoh, T.; Shimomura, T.; Takahashi, K.; Uehara, N.; Yoshino, N. Determination of γ-aminobutyric acid in human cerebrospinal fluid by isocratic high performance liquid chromatography. J. Chromatogr. Bio. Sci. Appl. **1990**, *529*, 417–423.
7. Kehr, J. Determination of γ-aminobutyric acid in microdialysis samples by microbore column liquid chromatography and fluorescence detection. J Chromatogr. Bio. Sci. Appl. **1998**, *708* (1,2), 49–54.
8. Piepponen, T.P.; Skujins, A. Rapid and sensitive step gradient assays of glutamate, glycine, taurine, and γ-Aminobutyric acid by high performance liquid chromatography-fluorescence detection with *o*-phthaladehyde-mercaptoethanol derivatization with an emphasis on microdialysis samples. J. Chromatogr. Bio. Sci. Appl. **2001**, *757* (2), 277–283.
9. Rowley, H.L.; Martin, K.F.; Marsden, C.A. Determination of in vivo amino acid neurotransmitters by high performance liquid chromatography with *o*-phthalaldehyde-sulphite derivatisation. J. Neur. Methods **1995**, *57* (1), 93–99.
10. Naini, A.B.; Vontzalidou, E.; Côté, L.J. Isocratic HPLC assay with electrochemical detection of free γ-aminobutyric acid in cerebrospinal fluid. Clin. Chem. **1993**, *39* (2), 247–250.
11. Qu, Y.; Arckens, L.; Vandenbussche, E.; Geeraerts, S.; Vandesande, F. Simultaneous determination of total and extracellular concentrations of the amino acid neurotransmitters in cat visual cortex by microbore liquid chromatography and electrochemical detection. J. Chromatogr. A **1998**, *798* (1,2), 19–26.
12. Struys, E.A.; Guérand, W.S.; Brink, H.J.T.; Jakobs, C. Combined method for the determination of γ-aminobutyric and β-alanine in cerebrospinal fluid by stable isotope dilution mass spectrometry. J. Chromatogr. Bio. Sci. Appl. **1999**, *732* (1), 245–249.
13. Liang, D.S.; Chang, N.M. Precolumn derivatization methods for the high performance liquid chromatographic determination of amino acids. Se Pu. **1993**, *11* (3), 140–143.
14. Gundersen, T.E.; Blomhoff, R. Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples. J. Chromatogr. A **2001**, *93* (1,2), 13–43.

Received February 27, 2005
Accepted July 28, 2005
Manuscript 6604