Comparison of validated high-performance liquid chromatography methods using two derivatizing agents for gamma-aminobutyric acid quantification

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ABSTRACT

Objective: The purpose of this study was to validate and compare two HPLC analyses of GABA after derivatization with 2-hydroxynaphthaldehyde (HN) or with o-phthaladehyde (OPA) and 3-mercaptopropionic acid (MPA) in terms of reproducibility, sensitivity and stability of two derivatives. **Materials and Methods:** GABA was derivatized with either 2-hydroxynaphthaldehyde (HN) or with o-phthaladehyde (OPA) and 3-mercaptopropionic acid (MPA) and was analyzed and validated using two different HPLC methods. **Results:** Reliability resulted in satisfaction for the HPLC methods in each derivatizing agent. Linear correlation was obtained in the concentration ranges of 40-600 μg/ml and 0.2-0.9 μg/ml for GABA-HN and GABA-OPA/MPA, respectively. The low limit of detection (LOD) and the limit of quantification (LOQ) of GABA-HN were 1 μg/ml and 5 μg/ml while LOD and LOQ of GABA-OPA/MPA were 0.004 μg/ml and 0.02 μg/ml. Validation results of two methods with chosen derivatizing agents showed that the methods were reproducible and precise on quantification of GABA. The method with GABA-OPA/MPA derivatization revealed higher sensitivity compared to one with GABA-HN derivatization. However, GABA-OPA/MPA derivative was less stable. **Conclusion:** Both validated methods could be used for sensitive and accurate analysis of GABA.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a nonprotein amino acid and a neurotransmission agent which can inhibit overstimulation of the brain and reduce hypertension from stress [1-3]. GABA is generally found in human such as in the cortex, hippocampus, hypothalamus, and central nervous system [4], in animals [5] and in plants [6-8]. In terms of physiological functions, GABA has been used to relieve insomnia and anxiety [9,10]. Moreover, GABA can decrease proliferation of cancer cells [11] and increase upregulation of cholesterol in bloodstream [12]. In addition, GABA can reduce glucose levels in diabetic rats [13,14] and decrease blood pressure in Wistar-Kyoto rats [1]. In terms of skin benefits, GABA stimulates dermal fibroblast, especially hyaluronic acid (HA) production, in mouse skin and human dermal fibroblasts [15] and promotes upregulated synthesis human beta-defensin-2 and filaggrin [16]. Furthermore, GABA can inhibit some expressions of pro-inflammatory mediators and speed up the healing process of wound [17]. In addition, these advantages of GABA are known to relate with its concentration. Thereby, quantification of GABA has become an essential part to facilitate the uses of GABA.

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[1]GABA - gamma-aminobutyric acid
HN - 2-hydroxynaphthaldehyde
OPA - o-phthaladehyde
MPA - 3-mercaptopropionic acid

Several techniques have been used to determine amount of GABA, for example, liquid chromatography tandem mass spectrometry (LC/MS) [18,19], electrochemical detection [20], capillary electrophoresis-MS (CE-MS) [21], and high-performance LC (HPLC). HPLC is considered as a standard analytical method because of simplicity, sensitivity, reproducibility, and economics. However, small amino acid, like GABA, is shortly eluted together with solvent front when using the standard method in HPLC. In addition, GABA does not have fluorophore or strong ultraviolet (UV) absorbance characteristic [22]. Thus, derivatization using derivatizing agents may be required to prolong GABA retention time and to provide strong UV or fluorescent absorbance in the analysis of GABA.

In general, derivatizing agents have their own efficiency and affinity which differently interact with amino acids such as 2-hydroxynaphthaldehyde (HN) [23,24] and o-phthalaldehyde (OPA) binding primary amine [25,26], 4-dimethylaminoazobenzene-4'-sulphonyl chloride binding primary and secondary amines [27], and 2-naphthyl chloroformate binding tertiary amine [28]. In addition, derivatization complexity and derivative stability could be affected by using different derivatizing agents. For this reason, several analytical methods have been developed to improve a complete derivatization between amino acid and derivatizing agent with reproducibility and to provide a considerably stable derivative which is feasible to be used for GABA analysis. For an example, the derivative of GABA-OPA was unstable in an acidic medium (half-life ≅ 8 min) [29]. Using thiol co-reagent was reported to improve the stability of OPA derivative [30]. Thiol reagents such as 3-mercaptoethanol, ethanethiol (ET), and 3-mercaptopropionic acid (MPA) have been used with OPA. However, OPA with MPA produced more stable derivative than OPA with ME or ET derivative [31] and provided good

fluorescent absorbency [32]. Therefore, different derivatizing agents showed differences in reaction sensitivity and some derivatives were quickly degraded which may cause some difficulty in analysis.

In this work, two derivatizing agents; HN and OPA with MPA, were used in GABA analysis by HPLC and comparatively validated as per International Conference on Harmonization guideline (Q2 R1) [33] with respect to specificity, linearity, accuracy, precision, low limit of detection (LOD), and limit of quantitation (LOQ). The derivatizations between GABA and the studied derivatizing agents (i.e., HN and OPA with MPA) are presented in Figure 1. Furthermore, stabilities of two derivatives were also studied to provide more information for derivatizing agent selection.

MATERIALS AND METHODS

Chemicals and Instruments

GABA, boric acid, OPA, and MPA were purchased from Merck (Darmstadt, Germany). HPLC grade of methanol and tetrahydrofuran (THF) were obtained from Honey Well (NJ, USA). Sodium acetate and potassium chloride were purchased from Carlo Erba (Milano, Italy). HN was obtained from Sigma-Aldrich (MO, USA). Sodium tetraborate was purchased from Ajax Finechem (NSW, Australian). Deionized water and ultrapure water were used in this study.

The HPLC system consisted of a Shimadzu chromatograph (LC-20AD, Tokyo, Japan) equipped with an autosampler, a degasser (DGU-20A₃), a binary pump (LC-20AD), a photodiode array detection (DAD) model SPD-M20A, a fluorescence detector (FD) model RX-10AXL, and a security guard column (KJO-4282, Phenomenex, CA, USA).
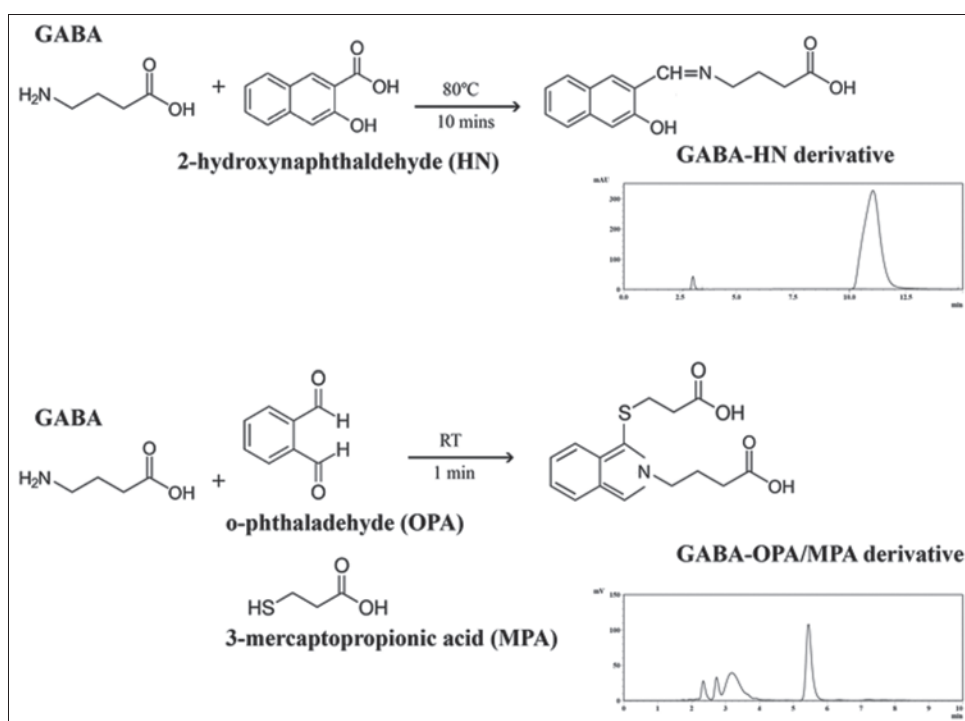

Figure 1: Derivatizations between gamma-aminobutyric acid and 2-hydroynaphthaldehyde and o-phthalaldehyde/3-mercaptopropionic acid

GABA Derivatization

GABA-HN derivatization

Derivatization of GABA with HN was performed based on a protocol modified from Khuhawar and Rajper [34]. GABA solutions were prepared in methanol:water (1:1) and 0.3% (w/v) HN was prepared in methanol. 1 ml GABA solution was mixed with 0.6 ml borate buffer pH 8 and 1 ml of derivatizing reagent. The mixture was heated in a water bath at 80°C for 10 min. Then, it was allowed to cool down and protected from light. The final volume was adjusted to 5 ml with methanol before HPLC analysis.

GABA-OPA/MPA derivatization

Derivatization of GABA with OPA/MPA was performed based on a protocol modified from de Freitas Silva *et al.* [35]. GABA solutions were prepared in ultrapure water and OPA was freshly prepared in methanol. 500 μl of GABA solution was derivatized by 100 μl of 25 mg/ml OPA, 375 μl borate buffer (pH 9.9), and 25 μl MPA. The resulting solution was vortexed. Next, it was stored in the dark at room temperature for 1 min before analysis.

Chromatographic System and Condition

GABA-HN derivative

The system was equipped with a 5 μm Hypersil® BDS C$_{18}$ analytical column (4.6 mm × 250 mm id, Thermo Fisher Scientific Inc., USA). The mobile phase was consisted of methanol and water (66:34, v/v). The mobile phase was filtered through 0.45 μ membrane filters and vacuum degassed before use. The 10 μl of derivatized sample was injected and isocratically eluted at a flow rate of 0.8 ml/min. The UV detector was set at detection wavelength of 330 nm.

GABA-OPA/MPA derivative

The system was equipped with a 3.5 μm Zorbax 300SB C$_{18}$ analytical column (4.6 mm × 150 mm id, Agilent Technology Inc., USA). The mobile phase was consisted of 0.05 M sodium acetate, tetrahydrofuran and methanol (50:1:49, v/v) and adjusted to pH 4.0. The mobile phase was filtered through 0.45 μ membrane filters and vacuum degassed before use. Chromatographic analysis was performed at 25 ± 2°C. The 10 μl of derivatized sample was injected and isocratically eluted at a flow rate of 1 ml/min. The fluorescent detector was set at an excitation wavelength of 337 nm and an emission wavelength of 454 nm.

HPLC Method Validation

The specificity of two methods was tested by comparing the HPLC chromatograms of derivatized GABA and other compounds. Five or six GABA concentrations were prepared for the construction of calibration curve plotting between the peak area and concentration. Each concentration of the GABA solutions was injected in triplicate. The low LOD and the limit of quantitation (LOQ) of GABA under the stated experimental conditions were defined at signal-to-noise (S/N) ratios of 3 and 10, respectively. The

intraday precision was determined by analyzing three concentrations of the standard solution on the same day and the interday precision was determined by analyzing on three consecutive days. Relative standard deviation (RSD) was used to evaluate the precision of the method. Accuracy was performed at three different concentrations covering the range of the method. The percent of recovered analyte was used to measure the accuracy of the method. In precision and accuracy tests, each concentration was done in triplicate.

Stability of Derivative

GABA derivatives (GABA-HN and GABA-OPA/MPA) were analyzed at different time intervals after derivatization to study the stabilities of two studied derivatives.

RESULTS AND DISCUSSION

In comparison, the preparation of GABA-HN derivative required energy and time to form, whereas the preparation of GABA-OPA/MPA derivative did not require energy and the derivative was formed in a minute. Both derivatives were successfully prepared.
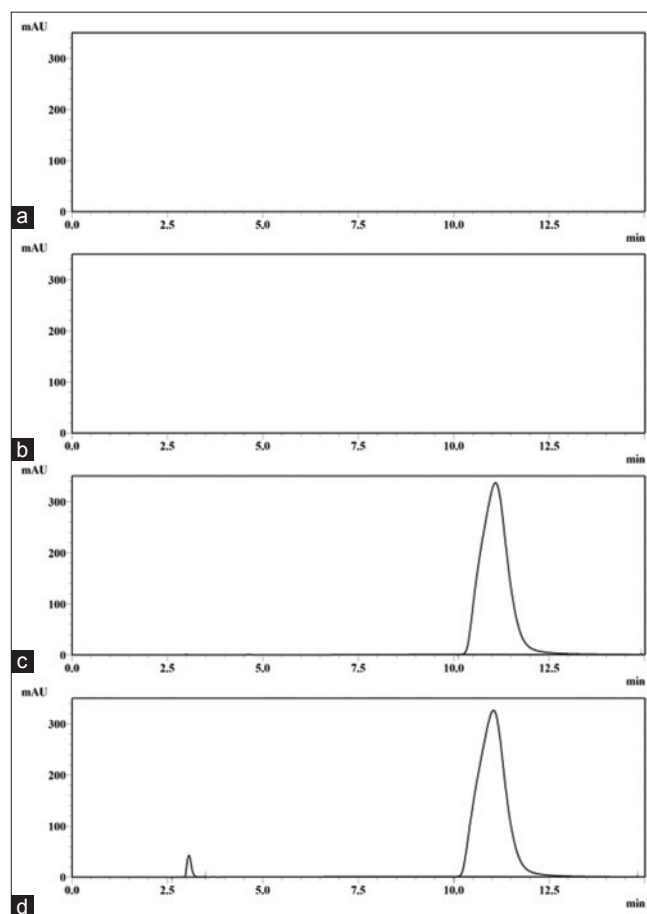

Figure 2: Chromatograms depicting the specificity of gamma-aminobutyric acid-2-hydroxynaphthaldehyde (GABA-HN) derivative. (a) Buffer system, (b) 0.8 mg/ml GABA, (c) HN and, (d) 0.04 mg/ml GABA-HN derivative

HPLC Method Validation

Specificity

The aim of the specificity study is to ensure that there is no interference from other components in the sample. The HPLC chromatograms of buffer, standard GABA solution, derivatizing agent and GABA before and after derivatization with HN or OPA/MPA are presented in Figures 2 and 3, respectively. All chroma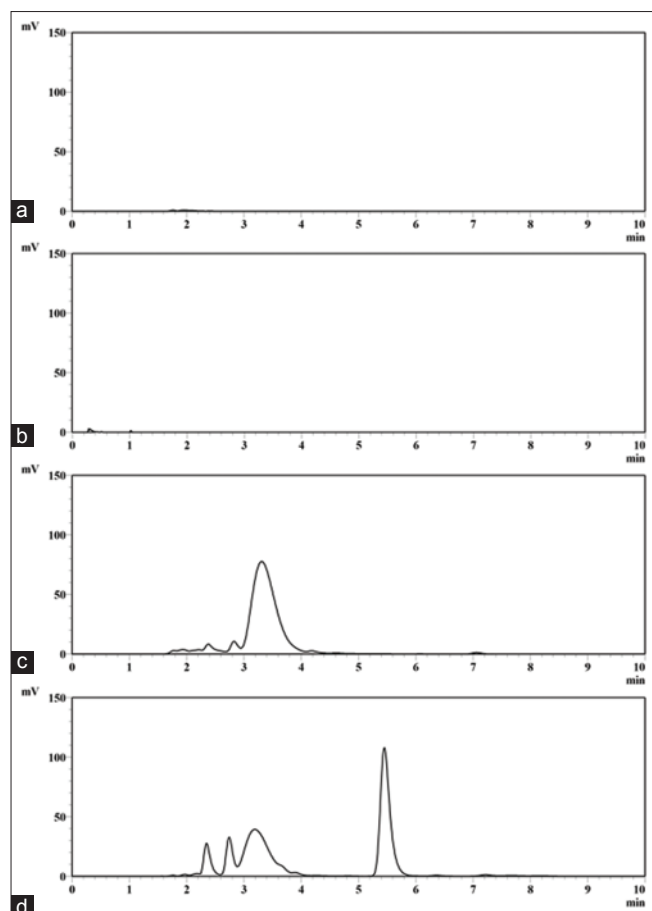tograms are presented under the same attenuation and scale. Derivatives' peaks were clearly separated from their derivatizing agent and there was no interference.

Linearity

The comparative regression analyses of the linearity of HN and OPA/MPA derivatizations are presented in Table 1. Linear correlation was obtained between peak areas and concentrations of GABA-HN and GABA-OPA/MPA derivatives in the range of 40-600 μg/ml ($r^2 > 0.999$) and 0.2-0.9 μg/ml ($r^2 > 0.998$), respectively. These results indicated that both methods could be used to quantify GABA with different concentration ranges.

LOD and LOQ

The LOD is the lowest concentration of the sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The LOQ is the lowest concentration in a sample that might be measured with an acceptable level of accuracy and precision. The LOD and LOQ for GABA-HN derivative were found to be 1 μg/ml and 5 μg/ml, respectively. Whereas, the LOD and LOQ for GABA-OPA/MPA derivative were much lower (i.e., 0.004 μg/ml and 0.02 μg/ml, respectively) as presented in Table 1. More sensitive method could be attained with OPA/MPA derivatization when compared with HN regarding to significantly lower concentration detected.

Precision

Three concentrations of 0.05, 0.1 and 0.5 mg/ml for GABA-HN and 0.3, 0.6 and 0.8 μg/ml for GABA-OPA/MPA were chosen for both precision and accuracy studies. The results of precision study are presented in Table 2. Both RSD values for precision were acceptable (<6% RSD) which could reveal that both methods were reproducible.

Accuracy

The recoveries from the accuracy study were close to 100% and are presented in Table 2. These results informed that HPLC analyses using these two derivatizing agents were accurate on quantitation of GABA.

Stability of derivative

Remaining GABA concentrations after derivatization were monitored over time intervals. Derivatized GABA concentration versus time after derivatization plots of both derivatizing agents are shown in Figure 4. The results revealed that about 99% of GABA+HN derivative remained over 4 h after derivatization, whereas GABA+OPA/MPA derivative was fallen below 80% (slope = −0.3039) in less than an hour. In comparison, more stable derivatives could be obtained with HN derivatizing agent. On the other hand, analysis of GABA using HN would be less time constrained.

CONCLUSION

Two derivatizing agents; HN and OPA/MPA, were used to quantify GABA by HPLC. Following ICH guideline of method validation, these derivatizing agents possessed significant specificity, linearity, precision, accuracy, high sensitivity, and good specificity for analysis of GABA. With OPA/MPA, much lower
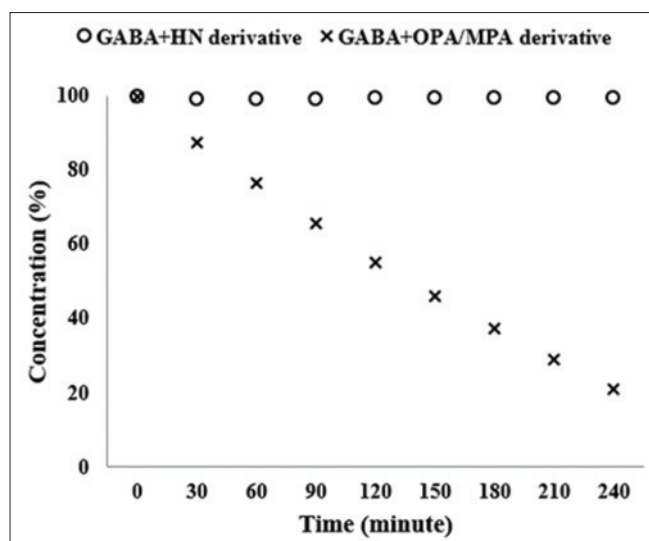

Figure 3: Chromatograms depicting the specificity of gamma-aminobutyric acid-o-phthalaldehyde/3-mercaptopropionic acid (GABA-OPA/MPA) derivative. (a) Buffer system, (b) 1 μg/ml GABA, (c) OPA/MPA and, (d) 0.1 μg/ml GABA-OPA/MPA derivative

Table 1: Comparative regression analyses of the linearity data of GABA-HN and GABA-OPA/MPA derivatives

Validation parameter	GABA-HN derivative	GABA-OPA/MPA derivative
Concentration range	40-600 μg/ml	0.2-0.9 μg/ml
Slope	8534668.10	14103544.79
Intercept	16349.17	222294.59
Coefficient of determination (r^2)	0.999	0.998
LOD (μg/ml)	1	0.004
LOQ (μg/ml)	5	0.02

Gamma-aminobutyric acid, HN: 2-hydroxynaphthaldehyde, OPA: O-phthalaldehyde, MPA: 3-mercaptopropionic acid

Figure 4: Stability profiles of gamma-aminobutyric acid (GABA)-2-hydroxynaphthaldehyde and GABA-o-phthalaldehyde/3-mercaptopro pionic acid derivatives after derivatization

Table 2: Intra- and inter-day precision and accuracy of GABA-HN and GABA-OPA/MPA derivatives

Validation parameter	GABA-HN derivative	GABA-OPA/MPA derivative
Precision (RSD, %)		
Intraday	1.01-2.10	0.68-2.74
Interday	1.35-4.93	3.37-5.45
Accuracy, %	92.04-104.41	90.58-104.74

GABA: Gamma-aminobutyric acid, HN: 2-hydroxynaphthaldehyde, OPA: O-phthalaldehyde, MPA: 3-mercaptopropionic acid, RSD: Relative standard deviation

LOD and LOQ values were obtained when compared to HN or it can be considered as a more sensitive method. The preparation of OPA/MPA was simple and no energy required but the half-life of GABA+OPA/MPA derivative was shorter than GABA+HN derivative. Therefore, time limitation may be needed to take into account when using OPA/MPA as derivatizing agent. However, both validated methods could be chosen for variety purpose in analysis of GABA.

ACKNOWLEDGMENTS

REFERENCES

1. Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y. Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. Br J Nutr 2004;92:411-7.
2. Mody I, De Koninck Y, Otis TS, Soltesz I. Bridging the cleft at GABA synapses in the brain. Trends Neurosci 1994;17:517-25.
3. Takahashi H, Sumi M, Koshino F. Effect of gamma-aminobutyric acid (GABA) on normotensive or hypertensive rats and men. Jpn J Physiol 1961;11:89-95.
4. Roberts E, Frankel S. Gamma-aminobutyric acid in brain: Its formation from glutamic acid. J Biol Chem 1950;187:55-63.
5. Awapara J, Landua AJ, Fuerst R, Seale B. Free gamma-aminobutyric acid in brain. J Biol Chem 1950;187:35-9.
6. Diana M, Quílez J, Rafecas M. Gamma-aminobutyric acid as a bioactive compound in foods: A review. J Funct Foods 2014;10:407-20.
7. Ishikawa A, Oka H, Hiemori M, Yamashita H, Kimoto M, Kawasaki H, *et al.* Development of a method for the determination of gamma-aminobutyric acid in foodstuffs. J Nutr Sci Vitaminol (Tokyo) 2009;55:292-5.
8. Varanyanond W, Tungtrakul P, Surojanametakul V, Watanasiritham L, Luxiang W. Effect of water soaking on gamma-aminobutyric acid (GABA) in germ of different Thai rice varieties. Kasetsart J Nat Sci 2005;39:411-5.
9. Abdou AM, Higashiguchi S, Horie K, Kim M, Hatta H, Yokogoshi H. Relaxation and immunity enhancement effects of gamma-aminobutyric acid (GABA) administration in humans. Biofactors 2006;26:201-8.
10. Amihaesei IC, Mungiu OC. Main neuroendocrine features and therapy in primary sleep troubles. Rev Med Chir Soc Med Nat Iasi 2012;116:862-6.
11. Oh CH, Oh SH. Effects of germinated brown rice extracts with enhanced levels of GABA on cancer cell proliferation and apoptosis. J Med Food 2004;7:19-23.
12. Miura D, Ito Y, Mizukuchi A, Kise M, Aoto H, Yagasaki K. Hypocholesterolemic action of pre-germinated brown rice in hepatoma-bearing rats. Life Sci 2006;79:259-64.
13. Hagiwara H, Seki T, Ariga T. The effect of pre-germinated brown rice intake on blood glucose and PAI-1 levels in streptozotocin-induced diabetic rats. Biosci Biotechnol Biochem 2004;68:444-7.
14. Adeghate E, Ponery AS. GABA in the endocrine pancreas: Cellular localization and function in normal and diabetic rats. Tissue Cell 2002;34:1-6.
15. Ito K, Tanaka K, Nishibe Y, Hasegawa J, Ueno H. GABA-synthesizing enzyme, GAD67, from dermal fibroblasts: Evidence for a new skin function. Biochim Biophys Acta 2007;1770:291-6.
16. Di Cagno R, Mazzacane F, Rizzello CG, De Angelis M, Giuliani G, Meloni M, *et al.* Synthesis of gamma-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: Functional grape must beverage and dermatological applications. Appl Microbiol Biotechnol 2010;86:731-41.
17. Han D, Kim HY, Lee HJ, Shim I, Hahm DH. Wound healing activity of gamma-aminobutyric Acid (GABA) in rats. J Microbiol Biotechnol 2007;17:1661-9.
18. Buck K, Voehringer P, Ferger B. Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry. J Neurosci Methods 2009;182:78-84.
19. Eckstein JA, Ammerman GM, Reveles JM, Ackermann BL. Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. J Neurosci Methods 2008;171:190-6.
20. Sekioka N, Kato D, Kurita R, Hirono S, Niwa Ol. Improved detection limit for an electrochemical γ-aminobutyric acid sensor based on stable NADPH detection using an electron cyclotron resonance sputtered carbon film electrode. Sens Actuators B Chem 2008;129:442-9.
21. Takeda S, Yamano N, Kawasaki N, Ando H, Nakayama A. Rapid determination of 4-aminobutyric acid and L-glutamic acid in biological decarboxylation process by capillary electrophoresis-mass spectrometry. J Sep Sci 2012;35:286-91.
22. Shah AJ, Crespi F, Heidbreder C. Amino acid neurotransmitters: Separation approaches and diagnostic value. J Chromatogr B Analyt Technol Biomed Life Sci 2002;781:151-63.
23. Mañes J, Gimeno MJ, Moltó JC, Font G. Fluorimetric determination of hydrazine in isoniazid formulations with 2-hydroxy-1-naphthaldehyde. J Pharm Biomed Anal 1988;6:1023-7.

24. El-Brashy AM, Al-Ghannam SM. High-performance liquid chromatographic determination of some amino acids after derivatization with 2-hydroxy-1-naphthaldehyde. Analyst 1997;122:147-50.
25. Cronin JR, Pizzarello S, Gandy WE. Amino acid analysis with o-phthalaldehyde detection: Effects of reaction temperature and thiol on fluorescence yields. Anal Biochem 1979;93:174-9.
26. Lee KS, Drescher DG. Fluorometric amino-acid analysis with o-phthaldialdehyde (OPA). Int J Biochem 1978;9:457-67.
27. Handley MK, Hirth WW, Phillips JG, Ali SM, Khan A, Fadnis L, *et al.* Development of a sensitive and quantitative analytical method for 1H-4-substituted imidazole histamine H3-receptor antagonists utilizing high-performance liquid chromatography and dabsyl derivatization. J Chromatogr B Biomed Sci Appl 1998;716:239-49.
28. Gübitz G, Wintersteiger R, Hartinger A. Fluorescence derivatization of tertiary amines with 2-naphthyl chloroformate. J Chromatogr A 1981;218:51-6.
29. Suñol C, Artigas F, Tusell JM, Gelpí E. High-performance liquid chromatography-fluorescence detection method for endogenous gamma-aminobutyric acid validated by mass spectrometric and gas chromatographic techniques. Anal Chem 1988;60:649-51.
30. Jacobs WA. o-Phthalaldehyde-sulfite derivatization of primary amines for liquid chromatography-electrochemistry. J Chromatogr 1987;392:435-41.
31. Godel H, Graser T, Földi P, Pfaender P, Fürst P. Measurement of free amino acids in human biological fluids by high-performance liquid chromatography. J Chromatogr 1984;297:49-61.
32. Kucera P, Umagat H. Design of a post-column fluorescence derivatization system for use with microbore columns. J Chromatogr A 1983;255:563-79.
33. International Conference on Harmonisation of Technical Requirement for Registration of Pharmaceuticals for Human Use, Q2(R1): Validation of Analytical Procedures: Text and Methodology; 2005.
34. Khuhawar MY, Rajper AD. Liquid chromatographic determination of gamma-aminobutyric acid in cerebrospinal fluid using 2-hydroxynaphthaldehyde as derivatizing reagent. J Chromatogr B Analyt Technol Biomed Life Sci 2003;788:413-8.
35. de Freitas Silva DM, Ferraz VP, Ribeiro AM. Improved high-performance liquid chromatographic method for GABA and glutamate determination in regions of the rodent brain. J Neurosci Methods 2009;177:289-93.