



Full Length Research Article

DETERMINATION OF GAMMA-AMINOBUTYRIC ACID BY HPLC: ITS APPLICATION TO POLYMERIC NANOPARTICLES AND STABILITY STUDIES

*Gülsel Yurtdaş Kırımlioğlu and Yasemin Yazan

Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Technology,
Eskişehir, Turkey, 26470

ARTICLE INFO

Article History:

Received 26th March, 2016
Received in revised form
08th April, 2016
Accepted 11th May, 2016
Published online 30th June, 2016

Key Words:

Gamma-aminobutyric acid (GABA),
HPLC, Orthophthalaldehyde,
Polymeric nanoparticle,
Stability, Drug loading,
Derivatization.

ABSTRACT

HPLC analysis of gamma-aminobutyric acid (GABA) and its relevant application to the unique polymeric nanoparticles (PNP) is described in this study. GABA is a major inhibitor neurotransmitter which is widely distributed in the central nervous system. Changes in GABA metabolism may play an important role in the origin and spread of seizure activity in epilepsy. Therefore, determination of GABA level is very significant in epilepsy treatment. In this study, determination of GABA in formulations was performed by HPLC using a developed validated method based on ICH Q2(R1). The best parameters were determined for HPLC analysis: optimized mobile phase of methanol:disodium hydrogen phosphate buffer (Na_2HPO_4) (40:60, v/v) (pH 6.7) at a flow rate of $0.8 \text{ mL} \cdot \text{dk}^{-1}$, injection volume $27 \mu\text{L}$. Fluorescence detection was performed at an excitation wavelength of 280 nm and an emission wavelength of 450 nm and the column temperature was set to 30°C . Orthophthalaldehyde/ β -mercaptoethanol (OPA/BME) was used as the derivatization agent. Specified working range was derived from linearity studies and was kept in the range of $0.6\text{-}1.4 \mu\text{g} \cdot \text{mL}^{-1}$. Good correlation, accuracy and precision values were obtained. Limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 0.0107 and $0.3239 \mu\text{g} \cdot \text{mL}^{-1}$, respectively. In stability studies GABA recovery from the PNPs stored at $4 \pm 1^\circ\text{C}$, $25 \pm 1^\circ\text{C}$, $40 \pm 1^\circ\text{C}$ and $40 \pm 1^\circ\text{C}$ -60 % relative humidity (RH) for 3 months were investigated and compared to the freshly prepared samples statistically. The HPLC method developed in this study is rapid, simple and suitable for routine analysis of GABA in PNPs.

Copyright©2016, Gülsel Yurtdaş Kırımlioğlu and Yasemin Yazan. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the central nervous system and essential for brain metabolism and function. GABA plays a fundamental role in controlling neuronal excitability and information processing, neuronal plasticity and network synchronization (Conti *et al.*, 2004). Changes in GABA metabolism may play an important role in the origin and spread of seizure activity in epilepsy (Petroff, 2002). GABA is not electroactive naturally and does not possess fluorescent or strong UV-vis absorbance characteristics rendering its direct analysis by high performance liquid chromatography (HPLC). Precolumn derivatization almost overcomes this drawback and a number of different reagents have been used for this purpose including 2,4,6-trinitrobenzenesulphonic acid, dansyl

chloride (Kang *et al.*, 2006), o-phthalaldehyde (OPA) (Zuo *et al.*, 2007, Reisi *et al.*, 2009), dansyl chloride (Krause *et al.*, 1995), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Salazar *et al.*, 2012) and naphthalene-2,3-dioxaldehyde (Clarke *et al.*, 2007). One of the most commonly used derivatization agent is OPA which reacts with primary amines in the presence of a thiol to give 2,2-disubstituted isoindole derivatives (Clarke *et al.*, 2007). The second advantage of using OPA over other reagents is that it itself is not fluorescent or electroactive whereas the derivatives have suitable fluorescent and electrochemical characteristics (Shah *et al.*, 2002). Derivatization of OPA and suitable nucleophile (usually a thiol) forms a derivative can be measured both spectrophotometrically and electrometrically (Figure 1). Depending on the most recent theory regarding reduced GABA levels in the epileptic brain, one of the most important neurotransmitter, GABA, was incorporated into a unique polymeric nanoparticle system to provide the entrance of GABA into the brain for epilepsy treatment. The present study

*Corresponding author: Gülsel Yurtdaş Kırımlioğlu

Anadolu University, Faculty of Pharmacy, Department of
Pharmaceutical Technology, Eskişehir, Turkey, 26470

is based on two different aims. The first one is to develop a HPLC method for quantify OPA derivative of GABA based on its fluorescent property and to validate it by employing common analytical and statistical tests depending on the suggestions of ICH guidelines (ICH Q2B, 1996; ICH Q2R1, 2005).

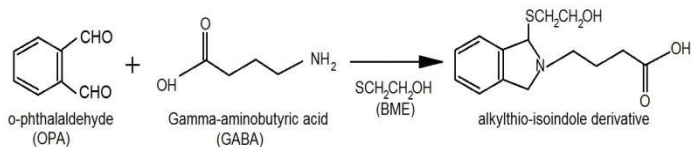


Figure 1. Derivatization procedure of GABA for determination

The second part of the study is to test the application of the mentioned method to the polymeric nanoparticle GABA content test and to investigate the effect of the storage stability conditions on GABA incorporated into polymeric nanoparticles using HPLC.

MATERIALS AND METHODS

GABA was purchased from Sigma (China). OPA, disodium hydrogen phosphate, sodium tetraborate, maleic anhydride (MA), n-hexane and polyethylene glycol (PEG) 1000 were the products of Merck (Germany). 2-mercapto ethanol (BME), calcium carbonate, vinylbenzyl chloride and dimethyl cocoamine were purchased from Sigma Aldrich (Germany), N,N-methylenebisacrylamide (NMBAc) from Fluka (Switzerland) and orthophosphoric acid from Alfa Aesar (Germany). Methanol, N,N-dimethylacrylamide (DMAc) and azoisobutyronitrile (AIBN) were obtained from Sigma Aldrich (France).

Preparation of polymeric nanoparticles

Polymeric surfactant (PSUR) with a quat group was synthesized uniquely using a confidential method. Synthesis was performed using vinyl benzyl chloride (Sigma, Germany) and dimethyl cocoamine. Incorporation of GABA into PNPs was achieved by inverse emulsion polymerization method. GABA loaded PNPs were synthesized by mixing aqueous solution (2 mL) containing NMBAc, DMAc and GABA with n-hexane solution containing the polymeric surfactant. In order to prepare nanoparticles with PEG 1000 MA, as it had been synthesized with PEG 1000 and MA, were added to the mixture. Pre-emulsion was obtained by stirring with ultraturrax (Janke and Kurtel® IKA, Labortechnik, Germany) at 8000 rpm for 30 min. Nanoparticle synthesis was initiated by AIBN. Emulsion was then stirred under nitrogen for 30 min with a magnetic stirrer (IKA®- Werke, Germany) and the reaction was continued for 3 hours at 60°C. Following completion of polymerization, the solvent was removed and the particles were washed twice with n-hexane. After filtration, nanoparticles were dried at an oven of 50°C. Formulation components and ratios are summarized in Table 1. Placebo nanoparticle formulations with no GABA were also prepared under the same conditions.

Optimization of the HPLC method

Experiments were performed to determine optimum conditions. Different parameters including mobile phase

composition, flow rate, derivatization reaction, and injection volume were used and suitable conditions were determined for reliability of HPLC validation and quantification of GABA in formulations.

Methanol: water: acetonitrile (20:3.5:76.5, 30:3.5:66.5, v/v), methanol: water (30:70, 40:60, 50:50, v/v) and methanol: Na₂HPO₄(40:60, 50:50, v/v) buffer were investigated in various ratios as the mobile phase. Flow rates were tested in the range of 0.5 and 1 mL.min⁻¹ in accordance with column properties. Injection volume were analyzed within the range of 20-30 µL. Different dilutions prior to the derivatization reaction (perchloric acid, phosphate buffer saline or methanol: water (50:50, v/v)), different ratios of sample: derivatization reagent (400 µL:300 µL or 400 µL:400 µL) and different reaction times (1-2 min) were also tested.

Table 1. Components and molar ratios of GABA loaded polymeric nanoparticles (Numerical values represent molar ratios)

Code	F1	F2	F3
PSUR	1	1	1
Cross linker (NMBAc)	0.2	0.2	0.2
Active agent (GABA)	1	1	1
Monomer (DMAc)	1	-	1
PEG 1000 MA	-	0.2	0.2

Validation of the HPLC method

Analytical process validation method of the International Harmonization Committee was used in this study. Parameters, namely, linearity, accuracy, precision, sensitivity, robustness and specificity were calculated (ICH Q2B, 1996; ICH Q2R1, 2005). HPLC conditions applied during the validation process are given in Table 2. Stock solutions of GABA were prepared in methanol: bidistilled water (50:50, v/v). OPA/BME was selected as the derivatization agent. Stock solution of OPA/BME was prepared as follows: 27 mg OPA was dissolved in 1mL methanol; 5 µL of BME and 9 mL tetraborate buffer (pH 9.3) were added subsequently and mixed thoroughly.

Table 2. Operating conditions of HPLC

Apparatus	Shimadzu-20 A
Column	4.6 x 100 mm, 3 µm C ₁₈ Inertsil CDS-3
Oven Temperature	30°C
Mobil phase	Methanol: disodium hydrogen phosphate buffer (Na ₂ HPO ₄) (40:60, v/v), (pH 6.7, adjusted by o-phosphoric acid)
Detector	Fluorescence Detector
Detection Wavelength	280 nm-450 nm
Flow Rate	0.8 mL.min ⁻¹
Injection volume	27 µL

The solution was stored at 4°C for 5 days and protected from light. Daily fresh OPA/BME derivatizing reagent was prepared by diluting OPA/BME with 7.5 mL tetraborate buffer. Derivatization procedure was optimized by mixing 400 µL of sample with 300 µL of derivatizing reagent at 2000 rpm using a vortex stirrer for 2 min. Derivatization procedure was protected from light. Calibration curve was prepared using samples containing OPA derivatives of GABA. Specified working range was derived from the linearity studies and kept

in the concentration range of 0.6-1.4 $\mu\text{g.mL}^{-1}$. For the accuracy test, three different concentrations within the working range of the analytical study were determined (0.75, 1 and 1.25 $\mu\text{g.mL}^{-1}$). Recovery values of those three concentrations were evaluated for the accuracy parameter. Specificity of the HPLC method was determined using empty formulation analysis. Sample preparation was the same as the steps taken for quantification. Specificity was investigated using HPLC chromatograms. Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the ICH guideline using the chromatograms of the calibration set prepared with GABA. Stability of the active substance under the operating conditions was evaluated by HPLC. Operating conditions used were for polymeric nanoparticle preparation (60°C in hexane, 3hr) and sampling for determining GABA in polymeric nanoparticles (50°C in methanol:bidistilled water (50:50, v/v), 30 min).

Preparation of samples for determination of GABA content in PNPs

Accurately weighed formulation (50 mg) was dispersed in methanol:bidistilled water (50:50, v/v) at 50°C using an ultrasonic bath to dissolve the active material. Dispersion was filtered through a 0.22 μm polyamide filter and derivatization procedure was performed on transparent filtrate for HPLC analysis. The experiment was repeated in triplicate.

Stability study of GABA in PNPs

GABA loading capacity of PNPs stored at $4 \pm 1^\circ\text{C}$, $25 \pm 1^\circ\text{C}$, $40 \pm 1^\circ\text{C}$ -60 % relative humidity (RH) and $40 \pm 1^\circ\text{C}$ for 3 months were investigated and compared with the freshly prepared samples. HPLC was used to determine GABA quantity in the PNPs. Student-t test were conducted for all statistical analysis.

RESULTS AND DISCUSSION

Optimization of the method

A variety of system suitability parameters such as mobile phase, flow rate, injection volume, oven temperature, *etc* was established to ensure the validity of the analytical procedure (Berkman and Yazan, 2011). Na_2HPO_4 buffer was selected for GABA determination. Initially Na_2HPO_4 buffer was investigated in the concentration range of 50-100 mM and 50mM buffer provided the optimum results. Methanol was preferred for the modifier of the background electrolyte. It was found that better retention times and good peak shapes were obtained with the use of 40 % (v/v) methanol. pH were adjusted to pH 6.7 with o-phosphoric acid. Considering the separation of GABA peak, peak morphology and suitable retention time, it was decided to use methanol: Na_2HPO_4 buffer (pH 6.7) (40:60, v/v). The effect of the flow rate on the peak area was investigated in the range of 0.5 and 1 mL.dk^{-1} . Although all the flow rates are equivalently usable, a flow rate of 0.8 mL. dk^{-1} was selected for the method because it was practical and gave an appropriate peak with consecutive injections. Injection volume of 27 μL was selected within the range of 20-30 μL used in all validation studies. Column temperature of 30°C was selected.

For the derivatization reaction, ratio of 400 μL :300 μL (sample:derivatization reagent) and methanol:water (50:50) (v/v) for diluting were selected. Reaction was carried out at 2000 rpm on a vortex mixer for 2 min by protecting from light. The optimum conditions determined for the method were shown at Table 2. Under optimum conditions chromatogram of GABA were shown in Figure 2.

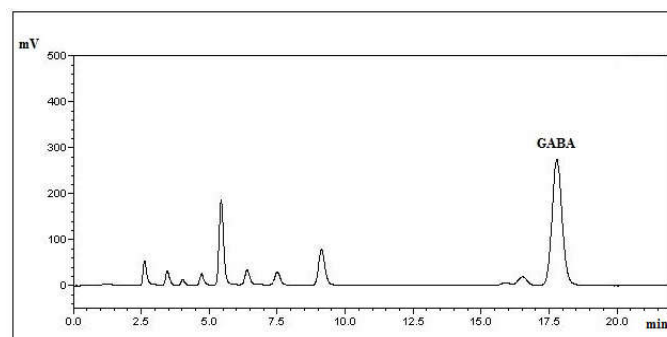


Figure 2. Chromatogram of GABA

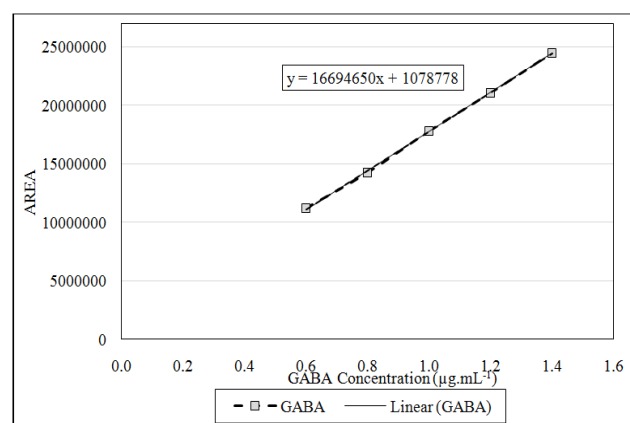


Figure 3. GABA linearity in methanol: bidistilled water (50:50, v/v) (n=9)

HPLC method validation

The calibration curve was constructed by plotting the peak area ratio (y) of the OPA derivative of GABA. The linearity of the method was examined in the range of 0.6-1.4 $\mu\text{g.mL}^{-1}$ and the calibration curve were chosen in this range (Figure 3). The statistical data according to linearity results were presented in Table 3. Good correlation fit the equation as $y=16694650.33x + 1078777.667$ with correlation coefficient of 0.9997. Accuracy is the similarity of the test results obtained by the analytical method to the true values. It was reported that recovery values must be in the range of 98-102 % to decide on the accuracy of the method used⁽¹³⁾. HPLC method used in this study yielded high recovery values (99-101 %) (Table 4) which indicated the accuracy of the method used. Measurements performed on three different concentrations (low, medium and high) evaluating repeatability and reproducibility of the analytical method used seem to verify the precision of the method since the percentage of relative standard deviation (RSD) was found to be below 2 % (Shabir, 2003) which is within the targeted interval (Table 5).

Table 3. Linearity results of GABA solution in the concentration of 0.6-1.4 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=6)

	Intraday (n=3)			Inter-day (n=9) whole days
	Day 1	Day 2	Day 3	
Slope, a	1.633×10^7	1.713×10^7	1.662×10^7	1.669×10^7
Intercept, b	1.412×10^6	7.421×10^5	1.082×10^6	1.079×10^6
Correlation Coefficient, r	0.9978	0.9997	0.9998	0.9997
SD of the slope (S_e)	5.909×10^5	5.843×10^5	5.715×10^5	5.797×10^5
SD of the intercept (S_i)	6.978×10^5	2.483×10^5	6.816×10^5	5.408×10^5
CI of the slope	1.621×10^7 - 1.718×10^7			

SD: Standard Deviation, CI: Confidence Interval

Table 4. Accuracy and recovery results (n=6)

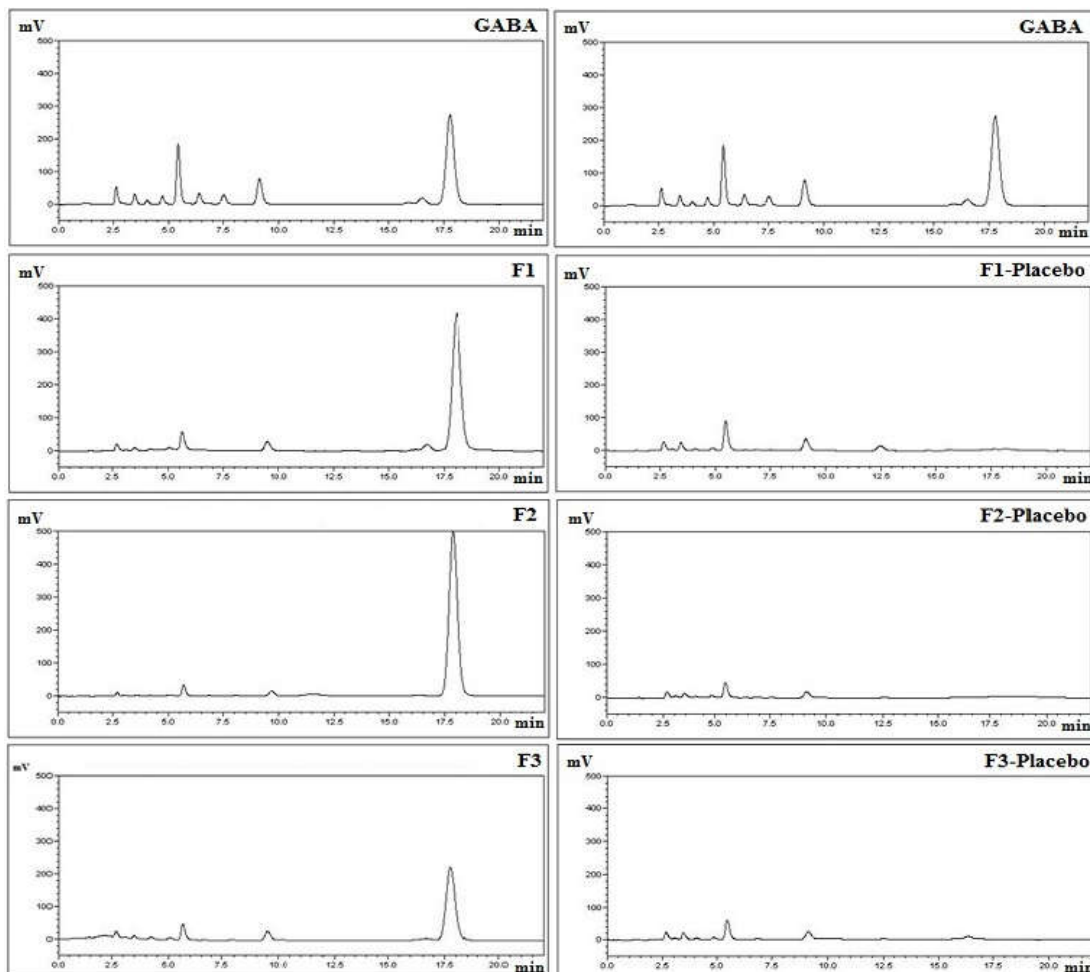
GABA ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery %	SE	RSD
0.75	0.747	99.596	0.487	0.012
1	1.005	100.530	0.699	0.017
1.25	1.261	100.870	0.561	0.014

SE: Standard Error; RSD: Relative Standard Deviation

Table 5. Inter-day and intra-day precision results

GABA ($\mu\text{g}\cdot\text{mL}^{-1}$)		Intra-day (n=3)			Inter-day (n=9)
		Day 1	Day 2	Day 3	
0.6	Mean area	1.114×10^7	1.126×10^7	1.103×10^7	1.114×10^7
	SE	1.789×10^5	2.807×10^5	1.551×10^5	2.091×10^5
	RSD	0.016	0.025	0.014	0.019
1	Mean area	1.794×10^7	1.795×10^7	1.821×10^7	1.803×10^7
	SE	4.766×10^5	3.269×10^5	2.241×10^5	3.377×10^5
	RSD	0.027	0.018	0.012	0.019
1.4	Mean area	2.485×10^7	2.520×10^7	2.488×10^7	2.498×10^7
	SE	2.517×10^5	6.577×10^4	6.127×10^5	3.737×10^5
	RSD	0.010	0.003	0.025	0.015

SE: Standard Error; RSD: Relative Standard Deviation

**Figure 4. Chromatograms of GABA in methanol solution and polymeric Nanoparticle formulations**

It was determined that overlapping effect of other components in the formulation did not affect the GABA peak. The chromatograms obtained showed that GABA peak is distinctly separated from other components in the formulations (Figure 4). It was therefore concluded that the method used is specific to GABA. LOD and LOQ values were found to be $0.0107 \mu\text{g.mL}^{-1}$ and $0.3239 \mu\text{g.mL}^{-1}$, respectively. The lowest concentration level used in this method was $0.6 \mu\text{g.mL}^{-1}$. Since LOD and LOQ values are less than this concentration, it can be concluded that the method used is sensitive for GABA quantification. Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. System suitability testing is an integral part of mainly analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system to be evaluated. Equipment, model, calibration and operation information for system suitability testing are given in Table 6.

Stability study of GABA in PNPs

Determination of GABA loading in PNPs were prepared as described in experimental. These solutions were injected and the analysis were carried out in optimum conditions. The results were evaluated statistically and were presented in Table 7. The values of RSD were below 2 % and the percent recoveries were almost 100 %. GABA loaded in freshly prepared PNP samples and in samples stored at 4°C , 25°C , 40°C -60 % RH and 40°C for 3 months is demonstrated in Table 8. GABA quantity loaded in PNPs was found to be 16.62 %, 15.07 % and 15.18 % for freshly prepared F1, F2 and F3, respectively. GABA amount in F1 after 3 months changed to 15.87 % (4°C), 15.56 % (25°C), 15.60 % (40°C -60 % RH) and 15.55 % (40°C). The amounts for F2 were found to be 14.02 % (4°C), 13.85 % (25°C), 12.54 % (40°C -60 % RH) and 12.01 % (40°C) while amounts were 14.30 % (4°C), 14.21 % (25°C), 14.00 % (40°C -60 % RH) and 14.24 % (40°C) for F3. When variation of GABA content in PNP formulations was evaluated by student-t test statistically, no significant

Table 6. Operation information for system suitability testing of HPLC

Equipment	Brand	Model
Degasser	Shimadzu	DGU-20 A5
Pump	Shimadzu	LC-20 AT
System control	Shimadzu	CBM-20 A
Auto-injector	Shimadzu	SIL-20 A
Fluorescence Detector	Shimadzu	RF-100 A XL
Column Oven	Shimadzu	CTO-10 AS VP

Table 7. Recovery % of GABA from polymeric Nanoparticles (n=3)

PNP	GABA	
	% Recovery	100.324
	SD	1.123
	RSD	1.046

SD: Standard error; RSD: Relative standard deviation

Table 8. Drug loaded in PNP formulations kept at different stability conditions (n=3)

Code	Condition	Drug Loading % \pm SE			
		T0	T30	T60	T90
F1	4°C	16.622 \pm 0.012	16.893 \pm 0.541	16.012 \pm 0.705	15.886 \pm 1.832
	25°C	16.622 \pm 0.012	16.045 \pm 0.732	16.012 \pm 0.065	15.564 \pm 1.743
	40°C -60 % RH	16.622 \pm 0.012	16.425 \pm 0.203	16.034 \pm 1.230	15.601 \pm 1.342
	40°C	16.622 \pm 0.012	16.073 \pm 0.828	15.834 \pm 1.103	15.554 \pm 2.457
F2	4°C	15.074 \pm 0.007	14.534 \pm 0.549	14.630 \pm 0.721	14.023 \pm 1.004
	25°C	15.074 \pm 0.007	14.620 \pm 0.312	14.047 \pm 0.884	13.850 \pm 1.238
	40°C -60 % RH	15.074 \pm 0.007	14.053 \pm 0.605	13.526 \pm 0.927	12.543 \pm 1.919
	40°C	15.074 \pm 0.007	14.270 \pm 0.407	13.481 \pm 1.264	12.008 \pm 1.076
F3	4°C	15.180 \pm 0.019	15.004 \pm 0.661	14.726 \pm 1.176	14.301 \pm 1.904
	25°C	15.180 \pm 0.019	14.886 \pm 0.286	15.328 \pm 1.509	14.206 \pm 1.553
	40°C -60 % RH	15.180 \pm 0.019	14.874 \pm 0.773	14.701 \pm 1.690	14.003 \pm 1.744
	40°C	15.180 \pm 0.019	14.698 \pm 0.865	14.665 \pm 1.703	14.236 \pm 0.972

RH: Relative humidity, SE: Standard error, T0: 0 month, T30: 1 month, T60: 2 months, T90: 3 months

As a conclusion, the method used in this study was found to be reliable after testing several parameters. It was found that 95.5 ± 1.235 % (mean \pm SE) of GABA remained stable in preparation conditions of PNPs (60°C in hexane, 3hr). 99.5 ± 0.724 % (mean \pm SE) of GABA was found to remain stable in PNPs at the end of 15 min under sample preparation conditions. Therefore, it was decided that the conditions used were suitable.

($p > 0.05$) changes in GABA content were found for PNP formulations kept at different stability conditions. There was also no degradation peak observed in the chromatograms of samples stored when compared with the chromatogram of freshly prepared formulation. In this study, GABA loaded unique polymeric nanoparticle formulations were prepared with inverse emulsion polymerization method. Determination of GABA in polymeric nanoparticle formulations was performed by HPLC using a developed validated method

which allows separation of OPA/BME derivatives of GABA with fluorometric detection. Method validation conducted proved accuracy, reliability and specificity of the method. It was found that all the results of present coefficient of variation are below 2 % showing the method was valid. No significant difference ($p > 0.05$) in GABA loading of PNPs stored at 4°C, 25°C, 40°C-60 % RH and 40°C was found after 3 months. The method developed in this study for determining GABA in PNP formulations was found to be rapid, reproducible and sensitive to GABA. Suggested method can be used as routine analysis of GABA in PNP formulations. Moreover, a simple and suitable HPLC method was developed for the analysis of GABA in PNPs for stability studies.

Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgements

The authors would like to thank Assoc. Prof. Dr. ErolŞener and Prof. Dr. Yusuf Menceloğlu for their consultancy in the validation studies and polymeric nanoparticle synthesis. This research was supported by Anadolu University Scientific Research Project Committee (No: 1206S106).

REFERENCES

- Berkman, M.S., Yazan Y. 2011. A validated HPLC method for the determination of octocrylene in solid lipid nanoparticle systems. *Pharmazie*. 66, pp. 105-110.
- Clarke, G., O'Mahony, S., Malone, G., Dinan, T.G. 2007. An isocratic high performance liquid chromatography method for the determination of GABA and glutamate in discrete regions of rodent brain. *J. Neurosci. Meth.*, 160, pp. 223-230.
- Conti, F., Minelli, A., Melone, M. 2004. GABA transporters in the mammalian cortex: localization, development and pathological implications. *Brain Res. Rev.* 45, pp. 196-212.
- ICH, Q2B 1996. Harmonised Tripartite Guideline, Validation of Analytical Procedure: Methodology, The European Agency for the evaluation of medicinal products; CPMP/ICH/281/95, in: Proceedings of the International Conference on Harmonization, Geneva.
- ICH, Q2R1 2005. Harmonised Tripartite Guideline, Validation of Analytical Procedure: Text and methodology, Complementary Guideline on Methodology, in: Proceedings of the International Conference on Harmonization, Canada.
- Kang, X., Xiao, J., Huang, X., Gu, Z. 2006. Optimization of dansyl derivatization and chromatographic conditions in the determination of neuroactive amino acids of biological sample. *Clin.Chim.Acta*. 366, pp. 352-356.
- Krause, I., Bockhardt, A., Neckermann, H., Henle, T., Klostermeyer, H. 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high performance liquid chromatography of the dansyl derivatives. *J.Chromatogr. A.*, 715(1), pp. 67-79.
- Petroff, O.A.C. 2002. GABA and glutamate in the human brain. *The Neuroscientist*. 8(6), pp. 562-573.
- Reisi, P., Alaei, H., Babri, S., Sharifi, M. R., Mohaddes, G., Soleimannejad, E. 2009. Determination of the extracellular basal levels of glutamate and GABA at dentate gyrus of streptozotocin-induced diabetic rats. *Pathophys.*, 16, pp. 63-66.
- Salazar, C., Armenta, J. M., Shulaev, V. 2012. An UPLC-ESI-MS/MS assay using 6-Aminoquinolyl-N-Hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: Application to screening of *Arabidopsis thaliana* mutants. *Metabolites*, pp. 398-428.
- Shabir, G.A. 2003. Validation of high-performance liquid chromatography methods for pharmaceutical analysis. Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *J.Chromatogr. A.*, 2003, 987, pp. 57-66.
- Shah, A. J., Crespi, F., Heidbreder, C. 2002. Amino acid neurotransmitters: separation approaches and diagnostic value. *J. Chromatogr. B*. 781, pp. 151-163.
- Zuo, G.C., Yang, J.Y., Hao, Y., Dong, Y.X., Wu, C.F. 2007. Ethanol and acetaldehyde induce similar changes in extracellular levels of glutamate and taurine and GABA in rat anterior cingulate cortex. *Toxicol. Lett.*, 169, pp. 253-258.
