Development and Validation of Assay and Stability-Indicating of gamma-Aminobutyric Acid in Tablets by Micellar Thin-Layer Chromatography

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A micellar thin-layer chromatography (TLC) method has been developed and validated for the determination of gamma-aminobutyric acid (GABA) in pharmaceutical formulation "Aminalon" (coated tablets). Aqueous micellar mobile phase contained 0.005 mol·L^{-1} of non-ionic surfactant Brij-35, adjusted to pH 2.0 with hydrochloric acid was used for GABA separation. The reaction with ninhydrin was applied for visualization of chromatographic spots. The developed micellar TLC method allows assaying GABA in 20 min. The method was validated with respect to linearity, specificity, accuracy, precision and robustness. GABA response showed good linearity in the range of 240–360 μg·mL^{-1}, LOD and LOQ were 8.3 μg·mL^{-1} and 25 μg·mL^{-1}, respectively. The average %RSD value did not exceed 1.0%. The method is accurate, reproducible, cost-effective, and can be used in routine quality control analysis and for stability-indicating analysis.

Keywords: gamma-aminobutyric acid, aminalon coated tablets, micellar thin-layer chromatography, method validation

Разработка и валидация методики количественного определения и определения стабильности гамма-аминобутановой кислоты в таблетках методом мицеллярной тонкослойной хроматографии

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Метод мицеллярной тонкослойной хроматографии (ТСХ) был использован при разработке и валидации методики определения гамма-аминобутановой кислоты (ГАБА) в готовом лекарственном средстве «Аминалон» (таблетки, покрытые оболочкой). Для определения ГАБА была использована водная мицеллярная подвижная фаза состава 0.005 моль/л неионогенного ПАВ Бридж-35, доведенная до pH 2.0 концентрированной хлористоводородной кислотой. Для визуализации определяемого аналита был использован нингидрин. Разработанная методика позволяет провести определение ГАБА за 20 минут. Методика была валидирована в соответствии с современными требованиями: диапазон линейности находится в пределах 240-360 мкг/мл, предел детектирования и предел количественного определения составили 8.3 мкг/мл и 25 мкг/мл, соответственно. Величина RSD при определении точности, правильности и воспроизводимости методики не превышала 1%. Методика воспроизводима, экономична и может быть рекомендована для рутинного анализа и определения стабильности препарата в течение его срока годности.

Ключевые слова: гамма-аминобутановая кислота; аминалон, таблетки, покрытые оболочкой; мицеллярная тонкослойная хроматография; валидация

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Gamma-aminobutyric acid (GABA) is one of the most prominent amino acid neurotransmitters in the central nervous system [1]. GABA arises through decarboxylation of glutamate via the enzyme glutamic acid decarboxylase and regulates many neuronal processes [2], and is involved in many aspects of normal brain functioning.

GABA is often quantified with a standard analytical technique reversed-phase HPLC coupled with the fluorescence [3], electrochemical [4] or MS detection [5, 6]. Since GABA as amino acid has low UV/Vis light absorption, provides insufficient MS sensitivity and does not show prominent reduction/oxidation properties, the pre- or post-column derivatization is usually required for its HPLC determination. Derivatization of amino acids has been carried out with o-phthalaldehyde [7], dabsyl chloride [8], phenylisothiocyanate [9], 9-fluorenylmethylchloroformate [10], 2,4-dinitrofluorobenzene [11, 12], etc. However, all those derivatization methods have limitations that are discussed elsewhere.

TLC as a simple, rapid, inexpensive and high effective technique for separation of complex mixtures can also be used for amino acids determination [13-15], especially after combining with densitometric detection. In the last years aqueous micellar solutions of surfactants [16-21] were widely used as mobile phases for TLC method development as a green alternative to organic and aqueous-organic mobile phases. Unfortunately, application of pure micellar solutions usually results in poor efficiency and asymmetric chromatographic spots. Thus, small amounts of short chain alcohols are added to improve the efficiency in micellar TLC [20, 21].

In this work we developed a simple, accurate, faster and cost-effective method for determination of GABA as an active ingredient in the pharmaceutical preparation. We completed an extensive validation of green micellar TLC method for GABA quantification during in-process control, assay in tablets, and stability-indicating investigation during its storage.

Experimental method and instrumentation

Gamma-aminobutyric acid (purity ≥99%) standard was from Sigma-Aldrich Chemie (Taufkirchen, Germany). Nonionic surfactant Brij-35 (purity ≥99%), methanol, n-butanol, formic acid, glacial acetic acid, acetonitrile, formic acid, ninhydrine and lactose were of analytical grade and purchased from Fluka Chemie (Buchs, Switzerland) and Sigma-Aldrich Chemie (Taufkirchen, Germany). Double-distilled water was used in all experiments.

The pharmaceutical formulations ("Aminalon" tablets) are commercially available on the Ukrainian medical market.

Sample were spotted on aluminum-backed plates Sorbfil-PTLC-UV-A (15x15 cm, 100-μm thickness, IMID Ltd, Krasnodar, Russian Federation) using Linomat 5 automatic sample applicator with a Hamilton syringe of 100 μL capacity. A rectangular flat bottomed development tank and CAMAG TLC Sprayer (CAMAG, Muttenz, Switzerland) was used for thin-layer chromatograms development. The obtained chromatograms were scanned with CAMAG TLC Visualizer.

ESI mass spectra were obtained using quadruple time-of-flight mass spectrometer (qTOF) Agilent 6510 (Agilent Technologies, Waldbronn, Germany) using following settings: capillary voltage 1750 V in negative mode and 1850 V in positive mode; gas (nitrogen) temperature: 325 ºC; gas flow: 5 $L \cdot min^{-1}$; fragmentor: 175 V, skimmer 1:65 V; octopole RF peak: 750 V; mass range 50-2200 m/z; MS/MS range 20-2000 m/z; MS scan rate: 1; MS/MS scan rate 2; flow rate infusion: 2 $\mu L \cdot min^{-1}$. Samples for ESI-MS analysis were the same as were used for TLC analysis, but 50 times diluted. After the reaction of GABA with ninhydrin the spot was extracted with 25% acetonitrile, 0.1% formic acid in water and used for ESI-MS/MS analysis.

Solution preparation, chromatographic development and detection

GABA stock solution was prepared by dissolving 150.0 mg of GABA standard in 100-mL of methanol. The prepared standard was stored at +4 ºC. Working standard solutions were freshly prepared each day by dilution of GABA stock solution with methanol to obtain five different concentrations within the range of interest: 240.0, 270.0, 300.0, 330.0 and 360.0 $\mu g\ mL^{-1}$.

Samples were prepared by accurate weighing of powdered tablets that contained approximately 75 mg of GABA. Powder was dissolved in 25-mL methanol and sonicated for 10 minutes. Afterwards the suspension was cooled to room temperature, diluted to 50 mL with methanol and mixed thoroughly. 20 mL of the obtained suspension was centrifuged at 10000 x g for 10 min. 5.0 mL of supernatant was diluted to 25.0 ml with methanol and used for micellar TLC analysis.

TLC plates were activated for 60 min at 105 ^{O}C before chromatographic development. Plates were placed between two sheets of glass to prevent deformation of the aluminum back during the heating process. 10 μL of samples and standard solutions were spotted in bands of 5 mm width. Ascending development of the plate with a migration distance of 100 mm, was performed using an aqueous 5 $mmol \cdot L^{-1}$ Brij-35 solution adjusted to pH 2.0 with hydrochloric acid, as a mobile phase. Chromatographic chamber was not saturated before TLC analysis due to low volatility of micellar mobile phase. The average development time was 20 min. The plate was dried in a stream of air, sprayed with ninhydrin solution (0.3 g ninhydrin in 100 mL of n-butanol with 3 mL of glacial acetic acid), warm air dried for 10-20 sec, and heated at 100 ºC during 5 min to detect GABA as a pink-violet spot.

Plates were then scanned at white light with the

CAMAG TLC Visualizer; collected pictures were saved as unpacked file in .bmp format. TLC Manager ver. 4.0.2.3 D planar chromatographic software (in-house written program) was used for data acquisition and peaks integration.

Results and Discussion

Method Development. In order to separate GABA from excipients of tablet pharmaceutical preparation the composition of micellar mobile phase and sample preparation procedure was optimized. It was found, that a significant amount of lactose (non-active excipients in tablets) exerted the influence on the form of the GABA spot and prevented GABA migration on TLC plate. To eliminate this problem we applied alcohols (methanol, ethanol) for sample preparation of tablet, because lactose is practically insoluble in pure alcohol. The presence of small amounts of water in ethanol resulted in partial dissolving of lactose that prevented GABA separation. Methanol showed reproducible results on GABA recovery and was used for sample and standard preparation in further experiments. Micellar mobile phases on the basis of pure non-ionic (Brij 35) or cationic (cetylpyridinium chloride, CPC) aqueous solutions without pH adjustment did not provide ideal form of GABA chromatographic spot due to tailing. In order to reduce the tailing of GABA spot we systematically tested different micellar mobile phase on the basis of CPC and Brij 35 with and without small additives of alcohols (methanol, propanol, butanol) and aliphatic carboxylic acid (acetic, propionic, butyric). Micellar mobile phases on the basis of Brij-35 with addition of aliphatic carboxylic acids or with pH adjusted to 2.0 with hydrochloric acid allowed to obtain symmetrical chromatographic spot of GABA. The aqueous micellar mobile phase contained 0.005 mol·L^{-1} of Brij-35 adjusted to pH 2.0 with HCl was selected for GABA separation and quantitative method development. The separation was completed in 20 min, R_F of GABA was around 0.7.

The influence of chromatographic chamber saturation on GABA chromatographic behavior was investigated under the selected separation conditions. The separation of GABA was performed after chamber saturation for 0, 30, 60 and 120 min. The obtained R_F did not depend on the time of chamber saturation and were in the range 0.73±0.03. It was demonstrated, that there is no need for the preliminary chamber saturation with using micellar mobile phase. This significantly simplified analytical procedure in comparison with traditional normal phase TLC, where volatile organic solvents are used as mobile phase components. All further investigations were carried out without preliminary saturation of TLC chamber.

Method validation. The procedures and parameters used to validate the chromatographic developed method are those described in the International Conference of Harmonization (ICH) Guidelines [22] and recently published works on TLC methods validation [22-27].

TLC method selectivity was demonstrated during the optimization of the micellar mobile phase composition. The chromatograms obtained during the method development showed that method is specific for the GABA assay and selective for the active pharmaceutical ingredient, since there were no blank interferences from the excipients (Fig. 1).

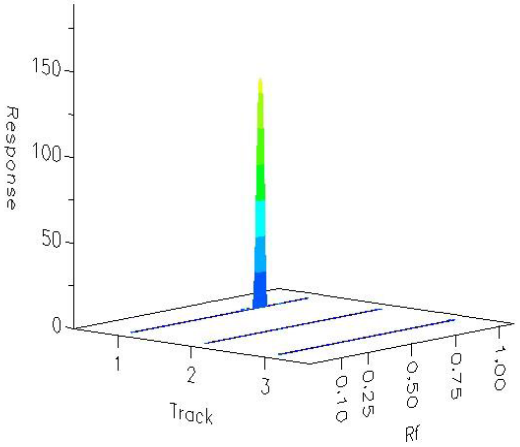

Fig. 1. Typical 3D densitogram of the solution containing 3 μg.spot^{-1} of GABA using micellar mobile phase with 0.005 mol·L^{-1} Brij-35 adjusted to pH 2.0 by HCl, chamber without saturation, TLC plate 7 cm x 15 cm, sample volume 10 μL. Track 1: gamma-aminobutyric acid standard solution; track 2: placebo solution (contained only excipients); track 3: methanol (solvent).

The ninhydrin reaction with primary amino groups is used to detect amino acids, peptides and proteins since 1910. After more than a century of ninhydrin application it is generally accepted that during the reaction with alpha-amino acids, amines, cysteine etc. Ruhemann's purple dye is formed [28]. However, the reaction mechanism is very complex and involve formation of a set of intermediates [29]. In this work we conducted an attempt to reveal the reaction products between GABA and ninhydrin using ESI MS/MS analysis. GABA has intense peaks at 104, 87 and 86 m/z in positive mode, which correspond to $[GABA+H^+]^+$, $[GABA-NH_3+H]^+$ and $[GABA-H_2O+H]^+$ (Fig. 2A). Ninhydrin ($C_9H_6O_4$) in negative mode has high abundant signals at 177 $[Ninhydrin-H]^-$ and 160 m/z $[Ninhydrin-OH]^-$. The spectra of reaction products in positive mode shows the presence of non-reacted GABA (m/z 104) and excess of $[Ninhydrin-OH+H^+]$ with m/z 162. This indicate that the linearity range for could be limited by the number of accessible sites of GABA on the surface of TLC plate. Among the products we observed Ruhemann's purple (m/z 304), and most probably adduct of Ruhemann's purple with a molecule of GABA (m/z 407) (Figure 2C).

Linearity was evaluated across the range of analytical assay. Preliminary experiment for linearity investigation (diapason from 1 to 10 μg·spot^{-1} with step 1 μg·spot^{-1}) showed, that dependence of coloring spot area *vs* GABA concentration has the significant deviation from linearity at GABA concentration above 5 μg·spot^{-1}. The good linearity response of GABA determination after reaction with ninhydrin was in the range of 1-5 μg·spot^{-1}.

To validate assay for GABA determination in tablet we selected the range from 240 μg·mL^{-1} to 360 μg·mL^{-1} (2.4–3.6 μg·spot^{-1}) that corresponds to 80-120% from nominal concentration of 300 μg·mL^{-1} (3 μg.spot^{-1}) of GABA in analyzed sample. The regression line was calculated as Y=A+BX, where X is GABA concentration in μg·spot^{-1} and *Y* is the spot area. Parameters of the calibration curve $Y=(7 \pm 4)\cdot10^2 + (3.3 \pm 0.1)\cdot10^3\cdot X$ were obtained using the linear least-squares regression. The correlation coefficient (*r*) was close to unity (0.9968).

Limits of detection (LOD) and quantitation (LOQ) were calculated in accordance with the 3.3N/B and 10N/B criteria, respectively, where N is a standard deviation of the spot area of the estimate, and B is the slope of the calibration curve. The calculated LOD and LOQ were 8.3 μg·mL^{-1} and 25 μg·mL^{-1}, respectively.

During the validation study it was observed that parameters of the calibration curve are strongly affected by the batch of chromatographic plate and derivatization conditions. Thus, we decided to add standard solutions on each chromatographic plate and estimate the parameters of the corresponding linear equation during the data analysis (Table 1).

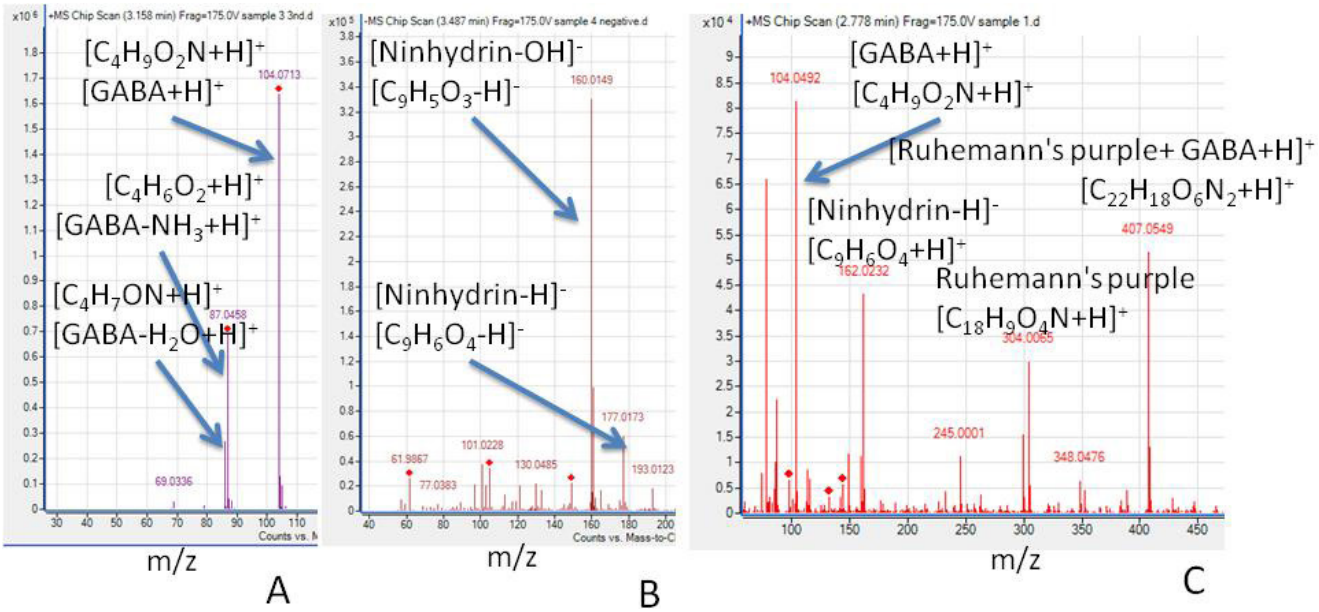

Fig. 2. ESI-MS spectra of gamma-aminobutyric acid solution (A) measured in positive ionization mode; ninhydrin solution (B) measured in negative ionization mode; products of reaction between GABA and ninhydrin (C) measured in positive mode.

Table 1. Recovery of GABA studied during 4 days at 3 different concentrations.

Linearity equation and correlation coefficient, obtained in different plates	Added GABA, μg·spot^{-1}	Founded GABA using corresponding linearity equation, μg·spot^{-1}	Recovery,%
$Y=(6 \pm 3)\cdot10^2 + (3.7 \pm 0.2)\cdot10^3\cdot X$, $r = 0.9973$	240.0	240.4	100.2
	300.0	299.8	99.9
	360.0	361.1	100.3
$Y=(3 \pm 4)\cdot10 + (5.1 \pm 0.4)\cdot10^3\cdot X$, $r = 0.9969$	240.0	238.9	99.5
	300.0	301.2	100.4
	360.0	358.8	99.7
$Y=(1 \pm 2)\cdot10^3 + (3.2 \pm 0.3)\cdot10^3\cdot X$, $r = 0.9990$	240.0	240.9	100.4
	300.0	300.3	100.1
	360.0	361.7	100.5
$Y=(5 \pm 5)\cdot10^2 + (3.7 \pm 0.5)\cdot10^3\cdot X$, $r = 0.9988$	240.0	239.4	99.8
	300.0	301.1	100.1
	360.0	363.0	100.8
		mean value	**100.1**

This allowed avoiding biases in GABA determination that is proved by study of GABA recovery (Table 2). GABA recovery on average was equal to 100.1 ± 0.2.

Accuracy was estimated by analyzing a sample of known concentration and comparing the measured value with the true value. Results obtained during accuracy investigations are presented in Table 3. The method showed a good accuracy.

Table 2. Accuracy, repeatability (intra-day) and reproducibility (inter-day) precision data for GABA determination by MLC.

Accuracy			
Concentration of working standard solutions, μg·mL^{-1}	**Experimental concentration founded*, μg·mL^{-1}**	**Accuracy, %**	Δ
240.0	238.3	99.3	-0.7
300.0	301.5	100.5	+0.5
360.0	358.9	99.7	-0.3
	Mean, RSD, %	99.4, 0.3	

Repeatability and reproducibility				
Concentration in working standard solutions, μg.mL^{-1}	**Intra-day* Mean, % + RSD, %**	**Inter-day * Relative amount, % + RSD, %**		
		Day 1	**Day 2**	**Day 3**
240.0 (100%)	99.9 ± 0.7	100.1 ± 0.4	100.4 ± 0.5	99.8 ± 0.7
300.0 (100%)	101.1 ± 0.8	99.3 ± 0.9	100.1 ± 0.5	101.2 ± 0.7
360.0 (100%)	99.9 ± 0.4	100.8 ± 0.8	99.6 ± 0.7	100.2 ± 0.5

* mean value of the three determinations

Table 3. Results of GABA assays in different batches of “Aminalon” tablets using RP-HPLC and micellar TLC methods.

Sample	**MLC*, mg**	**RP-HPLC*, mg**	Δ	**Calculated F-values**	**Calculated t-values**
Aminalon, coated tablets 250 mg. Sample 1	258.7 ± 0.7	257.9 ± 0.3	+0.8	2.55	1.88
Aminalon, coated tablets 250 mg. Sample 2	260.3 ± 0.7	260.8 ± 0.3	-0.5	1.36	1.04
Aminalon, coated tablets 250 mg. Sample 3	247.5 ± 0.8	247.9 ± 0.2	-0.4	2.02	1.03
labeled drug content 237.5– 262.5 mg per tablet				F**=6.39	t**=2.31

* mean value of the three determinations; ** tabulated t and F values are given for P=95%

Also accuracy of micellar TLC method was estimated by comparing the obtained results with results of reversed-phase HPLC [8]. Results are summarizes in Table 3. Student t-test and the F-test did not show statistically significant difference between micellar TLC and RP-HPLC results.

The repeatability (intra-day) and intermediate (inter-day) precision of the method were demonstrated by analyzing GABA working standard solutions with concentration 240, 300 and 360 μg·mL^{-1} during one day and on each of three days under the same conditions. The obtained results are summarized in Table 3 as mean relative amount (%). We did not observe significant difference between assay results either within-day or between days, implying that the precision of the proposed method is satisfactory (%RSD less than 0.9 %).

The stability of GABA solutions during the storage was also investigated. The GABA working standard solution with concentration 300 μg·mL^{-1} were measured in 0, 1, 3, 5, 7, 9, 24 and 36 h after preparation. The obtained results were compared with the results from the freshly prepared solution. The obtained data indicated that decreasing of GABA

concentration was negligible during 36 h. Correlation of GABA concentration in freshly prepared working standard solutions and working standard solution stored 24 h at 4 ºC was about 98%.

To measure robustness, the experimental conditions were deliberately changed slightly, and the effect on the results was examined. The studied parameters were saturation time, mobile phase composition and its pH value, development chamber and reagent manufacturers. Except for chemicals and development tank, other parameters were varied in a range of ±10%. Changing concentration of Brij-35, pH value and time of chromatographic chamber saturation had no significant effect on R_F value of GABA (R_F=0.73±0.03).

Three different analysts carried out the GABA analysis in triplicates, and the %RSD of peak area was calculated. The %RSD value for GABA was 0.95% for analyst 1, 1.55% for analyst 2 and 1.42% for analyst 3. The observed %RSD was less than 2% in all cases that indicated that developed method is robust [22-27].

Aminalon Assay and Stability Investigation. The validated micellar TLC method was applied to the determination of GABA in three different batches of Aminalon coated tablets. Table 3 shows satisfactory results for GABA content in tablets that were in good agreement with the labeled drug content (237.5 mg to 262.5 mg per tablet). Typical densitogram obtained during GABA assay is presented in Fig. 3.

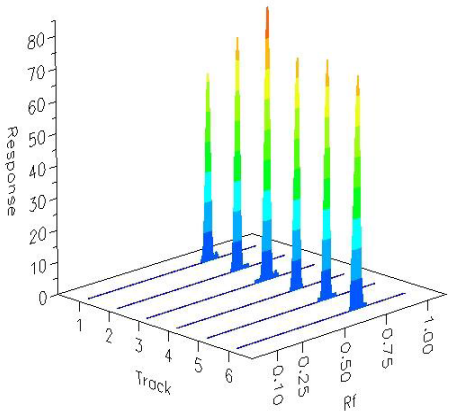

Fig. 3. 3D densitogram obtained during GABA assay. Micellar mobile phase: 0.005 mol·L^{-1} Brij-35 adjusted to pH 2.0 by HCl; chromatographic chamber without saturation; sample volume 10 µL. Tracks 1–3: gamma-aminobutyric acid standard solutions (concentration 2.7, 3.0, 3.3 µg·spot^{-1}, respectively); tracks 4–6: sample solution from different Aminalon batches.

Table 4. Stability data obtained under accelerate (40 ^{O}C/75% relative humidity) ICH stability conditions over a 12-month storage period.

GABA assay, % relative to start value	0 months	3 months	6 months	9 months	12 months
Sample 1	100.0	99.5 ± 0.8	99.8 ± 0.6	100.4 ± 0.8	99.9 ± 0.7
Sample 2	100.0	99.1 ± 0.9	100.3 ± 0.9	99.2 ± 0.9	99.5 ± 0.9
Sample 3	100.0	100.1 ± 0.7	99.5 ± 0.5	100.6 ± 0.8	100.1 ± 0.8

Conclusions

A new micellar TLC method for gamma-aminobutyric acid assay was developed and validated according to ICH guidelines. The proposed method is simple, fast, accurate and cost-effective for routine analysis and stability investigation of GABA in coated tablets. Additionally, the micellar TLC method is “green”, has a very simple sample preparation step. Moreover, the analysis of multiple samples can be completed in 30 min.

References

1. Gaetz W., Bloy L., Wang D.J., Port R.G., Blaskey L., Levy S.E., Roberts T.P.L. GABA estimation in the brains of children on the autism spectrum: Measurement precision and regional cortical variation. *J. NeuroImage,* 2014, 86, 1-9.

2. Bourcier S., Benoist J.F., Clerc F., Rigal O., Taghi M., Hoppilliard Y. Detection of 28 neurotransmitters and related compounds in biological fluids by liquid chromatography/tandem mass spectrometry. *Rapid Comm. Mass Spectrom.* 2006, 20, 1405-1421.

3. Ballini C., Corte L.D., Pazzagli M., Colivicchi M.A., Pepeu G., Tipton K.F. Extracellular levels of brain aspartate, glutamate and GABA during an inhibitory avoidance response in the rat. *J. Neurochem*. 2008, 106, 1035-1043.

4. Macinnes N., Duty S. Group III metabotropic glutamate receptors act as hetero-receptors modulating evoked GABA release in the globus pallidus in vivo. *Eur. J. Pharmacol*., 2008, 580, 95-99.

5. Song Y., Shenwu M., Dhossche D.M., Liu Y.M. A capillary liquid chromatographic/tandem mass spectrometric method for the quantification of aminobutyric acid in human plasma and cerebrospinal fluid *J. Chromatogr. B.* 814 (2005) 295-302.

6. 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. Meth.,* 2009, 182, 78-84.

7. D.M. de Freitas Silva, Ferraz V.P., Ribeiro Â.M. Improved high-performance liquid chromatographic method for GABA and glutamate determination in regions of the rodent brain. *J. Neurosci. Meth.,* 2009, 177, 289–293.

8. Syu K.Y., Lin C.L., Huang H.C., Lin J.K. Determination of Theanine, GABA, and Other Amino Acids in Green, Oolong, Black, and Pu-erh Teas with Dabsylation and High-Performance Liquid Chromatography. *J. Agric. Food. Chem.,* 2008, 56, 7637–7643.

9. Bidlingmeyer B.A., Cohen S.A., Tarvin T.L.. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr*., 1984, 336, 93–104.

10. Einarsson S., Josefsson B., Lagerkvist S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr.,* 1983, 282, 609–618.

11. Boichenko A.P., Kulikov A.U., Loginova L.P., Iwashchenko A.L. Aliphatic carboxylic acids as new modifiers for separation of 2,4-dinitrophenyl amino acids by micellar liquid chromatography. *J. Chromatogr. A.,* 2007, 1157, 252-259.

12. Boichenko A.P., Chernyshova O.S., Kulikov A.U., Loginova L.P. Properties of 2,4-Dinitrophenyl Derivatives of Amino Acids as Analytical Forms for High-Performance Liquid Chromatography *Rus. J. Appl. Chem.* 84 (2011) 957-963.

13. Kazmierczak D., Ciesielski W., Zakrezewski R. Separation of amino acids as phenyl thiocarbamyl derivatives by normal and reversed-phase thin-layer chromatography. *J. Planar Chromatogr. – Mod. TLC*., 2005, 18, 427-431.

14. Sharma S.D., Sharma H., Sharma S.C. Normal- and reversed-phase thin-layer chromatography of amino acids on unconventional supports with mixed mobile phases containing dimethyl sulfoxide. *J. Planar Chromatogr. – Mod. TLC.,* 15 (2002) 371-376.

15. Babu C.S., Kesavanarayanan K.S., Kalaivani P., Ranju V., Ramanathan M. A Simple Densitometric Method for the Quantification of Inhibitory Neurotransmitter Gamma-Aminobutyric Acid (GABA) in Rat Brain Tissue. *Chromatogr. Res. Intern.,* 2011, Article ID 732409, 5 pages

16. Mohammad, A.; Hena, S.; Moheman, A. Micellar TLC of Inorganic Ions: Simultaneous Separation of Lead(II), Zinc(II), and Cobalt(II) and Spectrophotometric Estimation of Zinc(II). *J. Planar Chromatogr - Mod. TLC.,* 2010, 23, 162–165.

17. Mohammad, A.; Sirwal, Y. H. Micellar Thin-Layer Chromatography of Coinage Metal Cations. *J. Planar Chromatogr - Mod. TLC*., 2002, 15, 107–115.

18. Mohammad, A.; Agrawal, V. Use of Cationic Micellar Mobile Phases in Normal-Phase TLC for Enhanced Selectivity in the Separation of Transition Metal Ions: Simultaneous Separation of Mixtures of Zinc, Nickel, Mercury, and Cadmium or Manganese Cations. *Acta Chromatogr.,* 2002, 12, 177–188.

19. Mohammad, A.; Sirwal, Y. H. Chromatography of Heavy Metal Cations with Formic Acid Containing Mobile Phases: Effect of Added Organic Solvents and Surfactants on the Mobility of Cations. *Indian J. Chem. Tech.,* 2004, 11, 726–731.

20. Shtykov, S. N.; Sumina, E. G.; Tyurina, N. V. Micellar Thin-Layer Chromatography: Particularities and Analytic Potentialities. *Rus. J. Chem.,* 2003, 47, 119–127.

21. Shtykov, S. N.; Sumina, E. G.; Smushkina, E. V.; Tyurina, N. V. Normal-and Reversed-Phase Thin Layer Chromatography of Fluorescein Dyes with Aqueous Micellar Solutions as Mobile Phases. *J. Planar Chromatogr - Mod. TLC.,* 1999, 12, 129–134.

22. Validation of Analytical procedures: Text and Methodology Q2 (R1). In: www.ich.org

23. Ermer J., Ploss H.J. Validation in pharmaceutical analysis: Part II: central importance of precision to establish acceptance criteria and for verifying and improving the quality of analytical data. *J.Pharm. Biomed.Anal.,* 2005, 37, 859-870

24. Coran S.A., Mulas S., Mulinacci N. Crucial aspects of high performance thin layer chromatography quantitative validation. The case of determination of rosmarinic acid in different matrices. *J.Chromatogr.A.,* 2012, 1220, 156–161.

25. Shewiyo D.H., Kaale E., Risha P.G., Dejaegher B., De Beer J., Smeyers-Verbeke J., Vander Heyden Y. Accuracy profiles assessing the validity for routine use of high-performance thin-layer chromatographic assays for drug formulations. *J. Chromatogr.A.,* 2013, 1293, 159–169.

26. Shewiyo D.H., Kaale E., Risha P.G., Dejaegher B., Smeyers-Verbeke J., Vander Heyden Y. HPTLC methods to assay active ingredients in pharmaceutical formulations: a review of the method development and validation steps. *J.Pharm.Biomed. Anal.,* 2012, 66, 11-23.

27. Renger B., Végh Z., Ferenczi-Fodor K. Validation of thin layer and high performance thin layer chromatographic methods. J.Chromatogr.A., 2011, 1218(19), 2712–2721.

28. Friedman M. Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences. *J. Agr. Food Chem.,* 2004, 52, 385-406.

29. D. Tyler, R.C. Reiter, C.D. Stevenson. Zwitterion Radicals and Anion Radicals from Electron Transfer and Solvent Condensation with the Fingerprint Developing Agent Ninhydrin. *J. Org. Chem.,* 2001, 66, 7596-7603.