DETERMINATION OF GLUTAMATE AND GABA FROM RAT CENTRAL NERVOUS SYSTEM SAMPLES WITH HPLC UTILIZING FLUORESCENT DETECTION

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Abstract

The research of neurological diseases has great importance and the determination of neurotransmitter concentrations is essential during these investigations. The levels of main excitatory (glutamate) and inhibitory (γ-amino-butyric acid (GABA)) neurotransmitters often change as a result of pathological alterations. For example, in case of migraine, elevated glutamate release is suggested to be a part of the pathomechanism, causing hypersensitivity to pain. Our goal in this study was to optimize a liquid chromatography method to measure glutamate and GABA from the trigeminal nucleus pars caudalis (TNC) of rats, which is responsible for pain processing. As a result, we were able to validate our method according to international guidelines where the investigated parameters were LOD, LOQ, precision and recovery. Furthermore, we applied a new internal standard which has not been published so far. This method will be utilized in the investigation of migraine animal models to evaluate potential new therapeutic approaches.

Introduction

Headache, one of the most common disorders of the nervous system, is a major health problem worldwide. The global prevalence of active headache disorders for the adult population is 46% for headache in general, 11% for migraine, 42% for tension-type headache and 3% for chronic daily headache [1]. The treatment of primary headache disorders is challenging and requires both acute and preventive therapeutic strategies [2]. The efficacy of these treatments is not always satisfactory and the contraindications and side-effects often limit the options of the physician [3, 4]. There is, therefore, a constant need for the study and development of new therapeutic approaches.

Animal and human studies suggest that glutamate receptors are present in various parts of the trigeminal system [5] which is the system responsible for processing most of the pain originating from the head area [6]. The stimulation of the trigeminal nerve results in elevated glutamate levels in the spinal trigeminal nucleus pars caudalis (TNC, [7]). The peripheral application of glutamate to deep craniofacial tissue proved to activate and sensitize nociceptive afferents and neurons in the upper cervical cord [8]. These findings suggest that excitatory amino acid receptors (particularly N-methyl-D-aspartate receptors (NMDAR)) play an important role in pain processing and the sensitization process as well [9].

Based on these findings, our aim in this study was to optimize an HPLC method to measure the excitatory amino acid glutamate and its inhibitory counterpart γ-amino-butyric acid

(GABA) in rat TNC samples for the future evaluation of these neurotransmitters in preclinical investigations of migraine.

Sample acquisition and preparation

For this study we used male Sprague-Dawley rats weighing 250-300 g. The animals were housed in cages under standard conditions with a 12-12-h light-dark cycle and free access to food and water. To acquire the TNC, rats were anesthetized with 4 w/w% chloral hydrate intraperitoneally then they were transcardially perfused with artificial cerebrospinal fluid (pH = 7.4, concentrations in mM: 122 NaCl, 3 KCl, 1 Na_2SO_4, 1,25 KH_2PO_4, 10 D-glucose monohydrate, 1 $MgCl_2$ x 6 H_2O, 2 $CaCl_2$ x 2 H_2O, 6 $NaHCO_3$). The samples containing the medullary segment of TNC were then removed and stored at -80°C until measurement.
Before measurement, the tissue samples were sonicated in ice cold 85% methanol (10 µl/mg tissue) then they were centrifuged (12 000 RPM, 10 min, 4°C) and the supernatants were collected. Then 100 µl sample was derivatized with 100 µl freshly prepared solution (2 ml o-phthaldialdehyde, 7.94 ml 0.2 M borate puffer (pH = 9.9), 60 µl 3-mercaptopropionic acid) and 50 µl distilled water containing the internal standards homoserine and (1R,2S)-2-amino cyclopent-3-ene-1-carboxylic acid (ACK).

HPLC method

For the determination of glutamate and GABA, we used an Agilent 1100/1260 system (Agilent Technologies, Santa Clara, CA, USA) equipped with a fluorescence detector (FLD). Chromatographic separations were performed on a Kinetex C18 150x4.6 i.d. 5 µm particle size column (Phenomenex Inc., Torrance, CA, USA) after passage through a SecurityGuard pre-column C18, 4x3 mm i.d., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) applying gradient elution. Mobile phase A was 95:5 V/V% 0.05 M sodium acetate (pH = 5.5):methanol, while mobile phase B was 45:45:10 V/V% methanol:acetonitrile:water. The elution started with 95% A decreasing linearly to 50% then staying there for 2 min and reequilibrating to 95% in 1 min for a total 16 min runtime. The flow rate was 1 ml/min, injection volume was 10 µl and the fluorescent detector was set to 230/440 nm for excitation/emission wavelengths.

Results and discussion

The first step of our method optimization was to determine the optimal excitation and emission wavelengths for the fluorescent detector. The acquired spectrum can be seen in Figure 1. and 2. and based on these spectrums the wavelengths were set to 230/440 nm for excitation/emission.

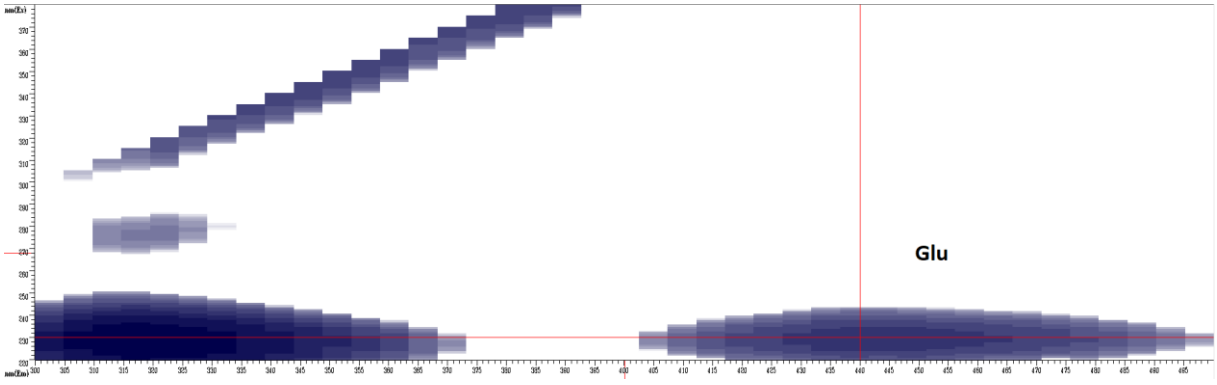

Figure 1. The fluorescent spectum of glutamate

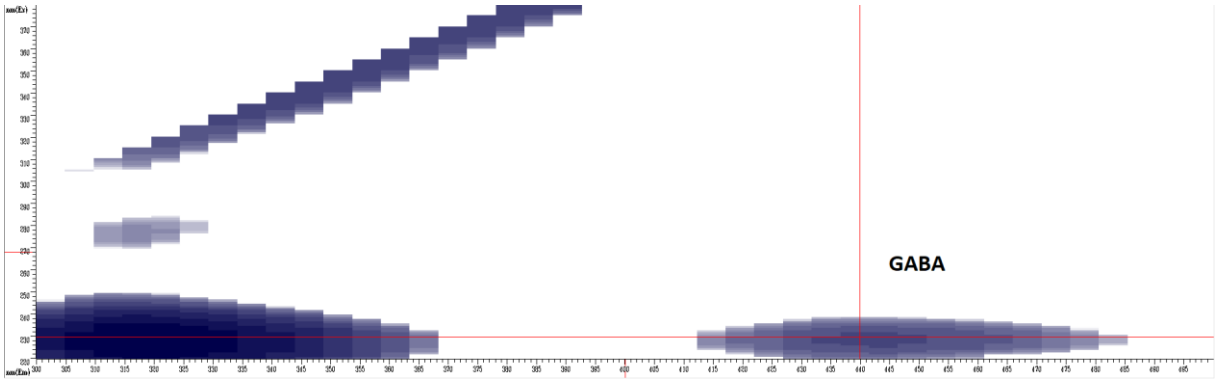

Figure 2. The fluorescent spectrum of GABA

During our measurements we used standard solutions in 6 different concentrations for external calibration. The calculated calibration curve was linear in the investigated concentration range and its details can be seen in Table 1.

Table 1. Main characteristics of calibration

	Glu	**GABA**	**Homoserine**	**ACK**
Calibration range (µG/ML)	0.08–4	0.008–0.4	0.4	0.4
Calibration line slope	0.591	3.581	-	-
Calibration line	0.0013	0.0081	-	-
Intercept linearity (R^2)	0.999	0.999	-	-
Retention time (min)	5.348	10.713	7.036	12.627

The selectivity of the method was checked by comparing the chromatograms of glutamate, GABA, homoserine and ACK for a blank TNC sample and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference. A sample chromatogram of a TNC sample can be seen on Figure 3.

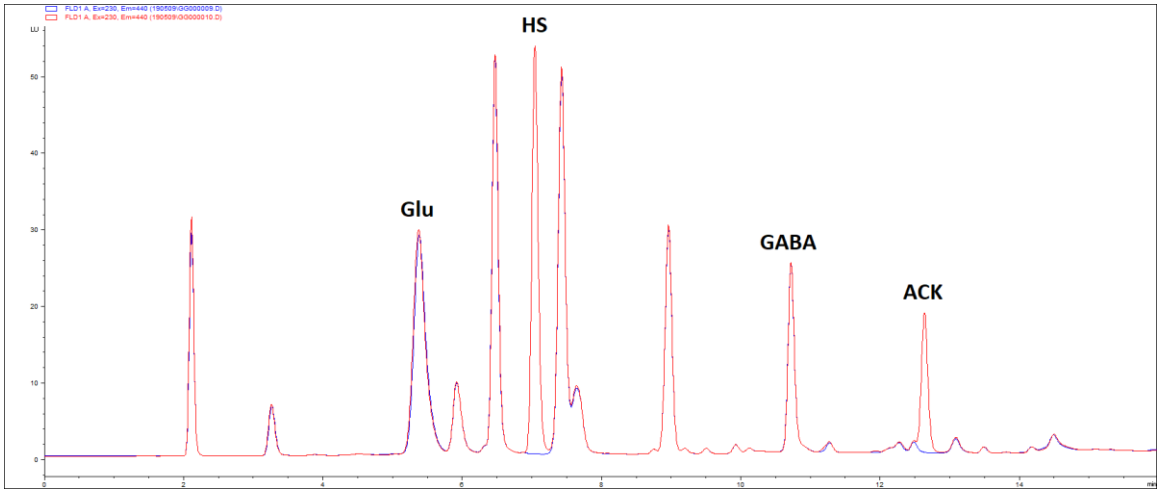

Figure 3. Representative chromatogram of a TNC sample with (red) and without (blue) internal standards. *Glu* glutamate; *HS* homoserine; *GABA* γ-amino-butyric acid; *ACK* (1R,2S)-2-amino cyclopent-3-ene-1-carboxylic acid

In the current study, LOD and LOQ were calculated as shown in Equation 1. The LOD values for glutamate and GABA were 0.03 and 0.006 μg/ml, respectively. The LOQ values for glutamate and GABA were 0.09 és 0.019 μg/ml, respectively

$$\mathrm{LOD} = 3.3 * \frac{\mathrm{Df}}{S'} \text{ and } \mathrm{LOQ} = 10 * \frac{\mathrm{Df}}{S'}$$

Equation 1.
The calculation of LOD and LOQ values by formula, where σ is the standard error of the intercept and S' is the slope of the calibration curve of the analyte. *LOD* limit of detection; *LOQ* limit of quantification.

With regard to the within-run precision, after 6 consecutive injections the coefficients of variation of the concentrations were 1.3% and 0.35% for glutamate and GABA, respectively. The relative recoveries were estimated by measuring spiked samples of glutamate and GABA at two different concentration levels with three replicates of each. No significant differences were observed for the lower and higher concentrations. The recoveries for the TNC samples ranged from 88 to 100% for glutamate and 104 to 122% for GABA. These results are in line with Food and Drug Administration recommendations [10].

Conclusion

As a result of our method optimization, we were able to measure glutamate and GABA from rat TNC samples with a robust, reliable method. This region of the central nervous system was not investigated before with similar applications. Furthermore, we also applied a new internal standard for amino acid determination, which was not described before. In the future, this method will be applied in animal models of migraine to investigate potential protective therapies and we plan to extend this method to other biological matrices too, especially for cerebrospinal fluid and microdialysate samples.

Acknowledgements

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