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Determination of glutamate uptake by high performance liquid chromatography (HPLC) in preparations of retinal tissue

Edinaldo Rogério da Silva Moraes[a], Alan Barroso Araújo Grisolia[a], Karen Renata Matos Oliveira[b], Domingos Luiz Wanderley Picanço-Diniz[c], Maria Elena Crespo-López[d], Caio Maximino[a,*], Evander de Jesus Oliveira Batista[a], Anderson Manoel Herculano[a,*]

[a] Universidade Federal do Pará, Instituto de Ciências Biológicas, Laboratório de Neuroendocrinologia, Belém, Pará State, Brazil
[b] Universidade Federal do Pará, Instituto de Ciências Biológicas, Laboratório de Neuroquímica Molecular e Celular, Belém, Pará State, Brazil
[c] Universidade Federal do Oeste do Pará, Núcleo Oriximiná, Oriximiná, Brazil
[d] Universidade Federal do Pará, Instituto de Ciências Biológicas, Laboratório de Farmacologia Molecular, Belém, Pará State, Brazil

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ABSTRACT

The present study describes a simple and efficient method utilizing high performance liquid chromatography (HPLC) coupled to fluorescence detection for the determination of kinetic parameters of glutamate uptake in nervous tissue. Retinal tissue obtained from 7-day-old chicks was incubated with known concentrations of glutamate (50–2000 μM) for 10 min, and the levels of the *o*-phtaldehyde (OPA)-derivatized neurotransmitter in the incubation medium were measured. By assessing the difference between initial and final concentrations of glutamate in the medium, a saturable uptake mechanism was characterized (K_m = 8.2 and V_{max} = 9.8 nmol/mg protein/min). This measure was largely sodium- and temperature-dependent, strongly supporting that the mechanism for concentration decrements is indeed uptake by high-affinity transporters. Added to this, our results also demonstrated that zinc chloride (an inhibitor of glutamate/aspartate transporters) evoked a concentration-dependent decrease in glutamate uptake, demonstrating the specificity of our methodology. Overall, the present work characterizes an alternative methodology to evaluate glutamate uptake in nervous tissue using HPLC. This approach could be an important tool for studies associated to the characterization of minute alterations in glutamate transport related with central nervous system injury.

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1. Introduction

Glutamatergic neurotransmission in the retinal tissue requires an efficient removal of presynaptically released neurotransmitter [1–4]. This action must be carefully regulated to result in the fine-tuning of excitatory and inhibitory transmission necessary for the proper processing of visual information [5]. Many studies describe that high-affinity glutamate transporters are responsible for neurotransmitter deactivation by removing glutamate from the synaptic cleft during retinal activation [3,5–7].

In fact, since the glutamatergic synapse represents the main excitatory pathway in the brain, several experimental models can be used to evaluate alterations in glutamate uptake in the central nervous system, including brain slices, cell cultures or retinal tissue [4,8–11]. With regard to visual system that is considered as a component of central nervous system, the chick retina is a recognized as an important model for the evaluation of neurochemical alterations in the central nervous system [12,13]. Added to this, previous studies have demonstrated that the embryonic chick retina expresses the main high-affinity sodium-dependent and low-affinity sodium-independent glutamate transporters, being an excellent model for the analysis of glutamate uptake kinetics [14–16].

Data of literature have shown a considerable number of reports that demonstrate alterations in glutamate uptake associated with neurotoxicity events in retinal tissue and brain [5,17]; however, we can observe a limited number of methodologies used to evaluate the pattern of glutamate uptake in the central nervous system, including the retina. In fact, the studies aimed to determine kinetic parameters or alterations in glutamate uptake use mainly radiolabeled glutamate or indirect biochemical determination of intracellular glutamate [14,18–20]. In this context, it is well recognized that quenching phenomena and the rapid metabolism of glutamate in

* Corresponding authors at: Laboratório de Neuroendocrinologia, Instituto de Ciências Biológicas, Universidade Federal do Pará, Campus do Guamá., Av. Augusto Corrêa, 01, 66075-900 Belém, PA, Brazil. Tel.: +55 91 32017742; fax: +55 91 32017742.
E-mail addresses: herculano@ufpa.br, aherculanos@yahoo.com.br (A.M. Herculano).

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the intracellular environment could difficult the precise determination of alterations in glutamate uptake. Therefore, the development of new methodologies becomes important to auxiliary pharmacological studies related to kinetics glutamate uptake in the retinal tissue and in the other areas of the central nervous system [21–23].

In the present work, we propose a new methodology for the evaluation of glutamate uptake in the retinal. Here, we used high performance liquid chromatography (HPLC) with fluorescence detection for the indirect determination of glutamate uptake in retinal tissue that could be applied in other nervous tissues.

2. Materials and methods

2.1. Chemical reagents

HPLC-grade methanol, 2-propanol, sodium acetate, boric acid, zinc chloride ($ZnCl_2$), *o*-phtaldehyde (OPA) and amino acid standards were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of the highest purity available and water was Milli-Q deionized water.

2.2. Equipment and chromatographic conditions

The HPLC system used in the present work was a Shimadzu model (LC-10 AD, Tokyo, Japan) with a 20 μl injection loop and a fluorescent detector (RF-10AXL) coupled to an LC-20AT pump. The system was equipped with a Shimadzu C18 analytical column (Shim-pack VP-ODS 4.6*250LC, internal diameter 4.6 mm) and a pre-packed column holder. The column was heated to 29 °C with a thermostat system (CTO-20A). An integrator was also used to analyze the chromatographic data. The mobile phase was composed of 50 mM sodium acetate, methanol 5% and 2-propanol (pH 5.67) as phase A and methanol 70% as phase B. These phases were eluted in a low-pressure gradient as follows: Initially 100% phase A, after 20 min 50%, and back to 100% at 25 min elution time. Mobile phases were filtered with Millipore 0.22 μm Durapore membrane filters before use. The fluorescent detector was set at 340 nm (excitation wavelength) and 460 nm (emission wavelength).

2.3. Derivatization procedure and glutamate quantification

The derivatization process was performed by mixing 60 μl of sample or glutamate standard solution, 10 μl of freshly prepared methanolic OPA (13 mg), and 40 μl borate buffer (pH 9.5). This final solution was vortexed and analyzed after 5 min.

2.4. Analytical curves and method validation

The stock solutions of glutamate and internal standard (homoserine) were dissolved in ultra pure water at 100 μg/mL. The working solutions for glutamate (0.1, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 μg/mL) and homoserine (6 μg/mL) were used. Retention time, linearity, calibration, selectivity, Limit of Detection (LOD) and Quantification (LOQ), accuracy, precision, recovery and stability were determined in accordance with Guideline for validation methods described by Brazilian National Agency of Sanitary Vigilance (BANVISA) and US Food and Drug administration (US FDA) [24–25]. The selectivity was performed by comparing between matrix with and without glutamate. Working solution and calibration solution were used to determine the linearity and calibration curve, respectively. We used the Hank's buffer after retinal incubations periods to perform calibration curve. The values of the curves were calculated by the ratio between the peak areas corresponding to glutamate and the internal standard, respectively, utilizing the LC solution software. Linear regression curve ($y = ax + b$) was performed to obtain the correlation coefficient and equation of the line.

The selectivity was analyzed by addition of glutamate into our matrix solution and posterior association with the free analyte matrix solution.

The LOD was determined utilizing glutamate concentration below or equal of the last concentration point utilized for the calibration curve. The glutamate peak considered as LOD was tree time superior of baseline. The LOQ was evaluated by the relative standard deviation (RSD) as described in FDA guide. Precision and accuracy were carried out in quintuplicate in three non-consecutive days utilizing low (1 μg/mL), intermediate (10 μg/mL) and high (20 μg/mL) of glutamate. Precision was expressed by the RSD performed intra and inter assay. The recovery method analysis was determined from solutions control in three different concentrations (low, intermediate and high) in the presence of 1% trichloroacetic acid (TCA) during no consecutive days. The stability of analyte was determined by measurement of low, intermediate and high glutamate concentration maintained at room temperature for 24 h. We also preformed analysis of analyte storage at −20 °C for 48 h followed by freeze–thaw process of evaluation being the values determined utilizing calibration curve.

2.5. Animals

Fertilized pathogen-free white leghorn chicken (*Gallus domesticus*) eggs were obtained from Makaru LTDA (Ananindeua, PA). These eggs were incubated at 37.5 °C and 60% of humidity and the stages of the chick embryo were determined according to Hamburger and Hamilton [26]. All experiments were authorized by the Institutional Animal Care Committee at Federal University of Pará.

2.6. Experimental procedures

Eyes from 7-day-old chicks were removed and transferred to a calcium- and magnesium-free salt solution. Retinal tissues were dissected and maintained in 12-well culture plates containing Hank's solution (128 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$, 12 mM glucose and 20 mM HEPES). After that, the tissue was incubated with different concentrations of L-glutamate (50–2000 μM) for 10 min. This incubation time was determined based in previous experiments with retinal tissues exposed to 50 μM for different time periods (t_{end} = 5, 10, 15, 20, 25 and 30 min). All incubation periods were followed by treatment with 1% trichloroacetic acid and centrifugation at 7000 rpm by 5 min. Glutamate quantification in the extracellular environment was performed utilizing a mix solution with homoserine (6 μg/mL) that was used as internal standard. The transported glutamate (ΔGlut) was determined based on the equation,

$$\Delta\text{Glut} = [\text{glut}]t_0 - [\text{glut}]t_{\text{end}}$$

where $[\text{Glut}]t_0$ and $[\text{Glut}]t_{\text{end}}$ represent the glutamate concentrations at the beginning and at the end of the experiment, respectively.

2.7. Applicability of the method and tissue treatments

To verify the applicability of the method, we evaluated the uptake of glutamate in retinal tissue in different situations: in a medium containing sodium or not, different temperatures and medium contain different concentrations of $ZnCl_2$. Retinal tissue was incubated in sodium-free Hank's medium (128 mM LiCl; 4 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$, 12 mM glucose and 20 mM HEPES) and medium containing different concentrations $ZnCl_2$ (10^{-3}–10^{-9} M), an inhibitor of glutamate transporters. We also evaluated the effect of different temperatures on the glutamate uptake, for that retinal tissue was exposed to Hank's solution at

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