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

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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.

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

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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

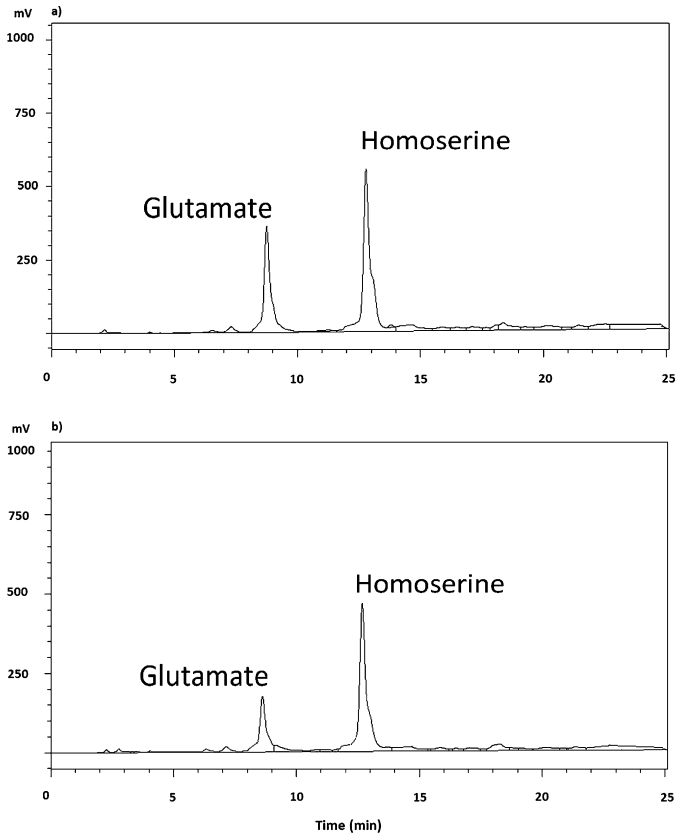

Fig. 1. Retention time of glutamate and homoserine in fluorescence HPLC detection. Upper and lower chromatograms demonstrated the glutamate and homoserine peak before and after retinal tissue glutamate incubation, respectively.

37 °C, 8 °C and 0 °C during 10 min. Protein content in the retinal tissues was determined with the assay described by Bradford [27].

2.8. Statistical analyses

All experiments were conducted in triplicates and repeated 3–4 times. Results were expressed as means ± SD of micromolar concentration of glutamate. ANOVAs (followed by Tukey–Kramer post-test, when appropriate) were performed with BIOESTAT 5.0. Kinetic parameters were determined using Graph Pad Prism 4.0 with non-linear fitting of the data to a Michaelis–Menten curve.

3. Results

For validation of chromatographic approach for glutamate detection we used the parameters determined by BANVISA and USA FDA. As demonstrated in Fig. 1 glutamate and homoserine (internal standard) were identified by their retention times [8,9,12,5] [8.9 and 12.5 min, respectively] and selectivity (data not shown). As demonstrated in Table 1, we demonstrated a significant pattern of linearity for glutamate concentration (correlation coefficient = 0.998). The LOD and LOQ were also determined and the values were 0.025 μg/mL and 0.1 μg/mL, respectively. For the LOQ the relative standard deviation was about 7 ± 0.0099%. Precision intra- and inter assays were determined by the relative standard deviation (RSD) and the values are described in Table 2. The recuperation was also determined and the values observed were 99.2%; 99.8% and 99.6% for the low, intermediate and high glutamate concentration, respectively. Analysis of stability was evaluated and our results showed a range between 90.76% and 110.08%. As

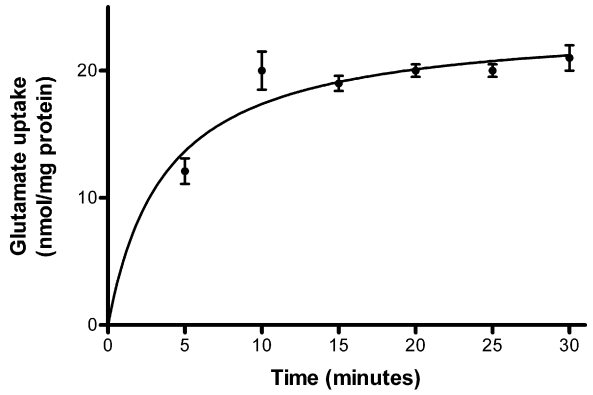

Fig. 2. Time course of L-glutamate uptake in retinal tissue. Explants of chick retina were incubated with 50 μM of L-glutamate by different time intervals as described in Section 2. Until the first 5 min was demonstrated an increase in the glutamate uptake, after this period a saturable phenomenon was observed. Data are expressed in nmol of glutamate uptake/mg of protein/min.

demonstrated in the Table 3, similar results were observed in the freeze–thaw stability (89% and 104%).

After validation procedure, time courses of L-glutamate uptake in retinal tissue were evaluated as described in Section 2. Our results demonstrated that $t_{1/2}$ for glutamate equilibrium at 50 μM concentration was about 10 min (Fig. 2). Based on these results, this incubation time was used in the succeeding experimental procedures.

To verify the kinetics of glutamate uptake, retinal tissue was exposed to different concentrations of glutamate as described in Section 2. Our results demonstrated that incubation with increasing concentrations of glutamate (ranging from 50 μM to 2000 μM) evoked a saturable uptake process (Fig. 3). K_m and V_{max} values were 8.2 ± 0.5 nmol and 9.8 ± 0.18 nmol/mg protein/min, respectively.

We also evaluated the effect of temperature in glutamate uptake. Our data showed a decrease of about 50% in glutamate uptake at 4 °C and a decreased about 95% in retinal tissue exposed to Hank's solution at 0 °C (Fig. 4).

In order to evaluate the specificity of glutamate transport, we performed experiments in sodium-free medium as described in Section 2. Our data showed a significant decrease in glutamate uptake (about 50%) in the absence of sodium (Fig. 5). Similar results were observed in tissue treated with increasing concentrations of zinc chloride, a known inhibitor of the sodium-dependent glutamate/aspartate transporter (GLAST) [28]. As demonstrated in Fig. 6, $ZnCl_2$ induced a concentration-dependent decrease in glutamate

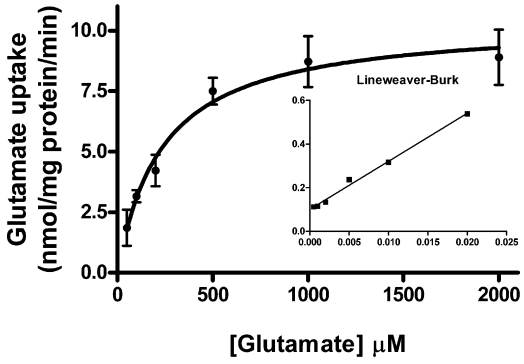

Fig. 3. Kinetics of L-glutamate uptake in retinal tissue. Results demonstrated a saturable curve concentration-dependent in retinal explants exposed to different concentrations of L-glutamate. The insert is a Lineweaver–Burk representation and the values of K_m and V_{max} are 8.2 μM and 9.8 nmol/mg protein/min, respectively.

Table 1
The calibration of glutamate in retinal tissue by HPLC–Fluorescence detector.

Analyte	Internal standard	Equation[a]	(R^2)[b]	Linear range (μg/mL)	LOD[c] (μg/mL)	LOQ[d] (μg/mL)	RT[e] (min)
Glutamate	Homoserine	$Y = 0.125 + 0.136$	0.998	0.1–20	0.025	0.1	8.9

[a] The calibration curves were constructed by the ratio between the area under the peak corresponding to glutamate and the area corresponding to homoserine. Calibration curve was derived from seven data points ($n = 5$).
[b] Correlation coefficient.
[c] LOD referred to the limits of detection in μg/mL.
[d] LOQ referred to the limits of quantification in μg/mL.
[e] Retention time of the glutamate.

Table 2
Determination of glutamate by HPLC–Fluorescence detector: accuracy, precision and recovery in three different concentrations (1, 10 and 20 μg/mL).

Glutamate	Precision (RSD)[a]		Accuracy (%)[b]		Recovery[c]
	Intra assay	Inter assay	Intra assay	Inter assay	
Low	5.2	4.5	102	107	99.2
Mid	8.8	3.2	97.6	98.3	99.8
High	6.1	4.1	98	98.8	99.6

[a] For both precision tests, the values were in percentage of RSD.
[b] Accuracy values were determined as the ratio between the experimentally determined mean concentration and the theoretical corresponding concentration.
[c] The recovery was determined from the control solution and the result was calculated as the ratio of the value obtained and the added value as a percentage.

Table 3
Determination of glutamate by HPLC–Fluorescence detector: validation results on stability.

Analyte[a]	Stability (%)[b]			Freeze–thaw stability (%)[c]		
	Low	Mid	High	Low	Mid	High
Glutamate	89.76	98.56	110.08	87.10	97.44	104.05

[a] Glutamate concentrations of 1, 10 and 20 μg/mL.
[b] Stability evaluated after 24 h at room temperature.
[c] Stability was evaluated after freezing and thawing cycle (48 h storage at −20 °C the analyte kept at ambient temperature.

uptake in retinal tissue, with the maximal inhibition observed at 100 μM.

4. Discussion

The present study describes an alternative methodology utilizing high performance liquid chromatography (HPLC) coupled to fluorescence detection for the determination of kinetic parameters of glutamate uptake in a nervous tissue. HPLC has been extensively used for determination of glutamate concentration with high levels of sensitivity in different tissues including brain. In this context, our methodology showed similar parameters chromatographic after the validation process [29–32]. Added to this our results for glutamate quantification are in agreement with pattern determined by BANVISA and USA FDA. Nonetheless, to date, few methods were developed to evaluate glutamate uptake such as biosensors [23], analytical methods [31] and radiolabeled glutamate [14]. In the present study we propose an innovative protocol for the determination of glutamate uptake parameters in retinal tissue.

In our experimental procedure, the tissue is exposed to a known concentration of glutamate, and the levels of the neurotransmitter in the incubation medium are measured after determined incubation times by HPLC (Fig. 1). Because glutamate is not degraded in the extracellular environment [3], the decrease of glutamate concentration in the medium observed in our results (Fig. 2) was attributed to glutamate influx mediated by glutamate transporters.

In relation to the experimental model used in this work, studies showed that the avian retina is very similar to mammalian retina and represents an extension of central nervous system with physiology regulated by similar neurotransmission systems

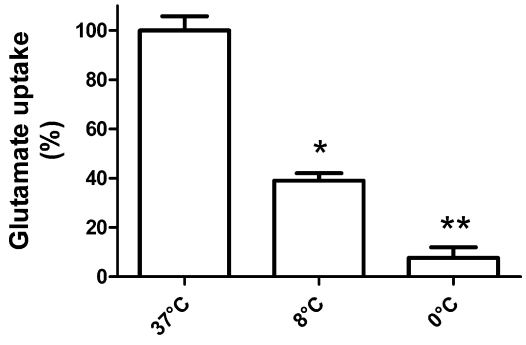

Fig. 4. Effect of temperature on the glutamate uptake in retina. Results showed a decrease of the L-glutamate uptake temperature-dependent in the retinal tissue. Data as expressed in percentage of Control (37 °C) and the statistical analysis was performed using the software BIOESTA 5.0 (*$p < 0.05$, **$p < 0.01$ vs. Control and #$p < 0.05$ vs. 8 °C. ANOVA followed by Tukey test).

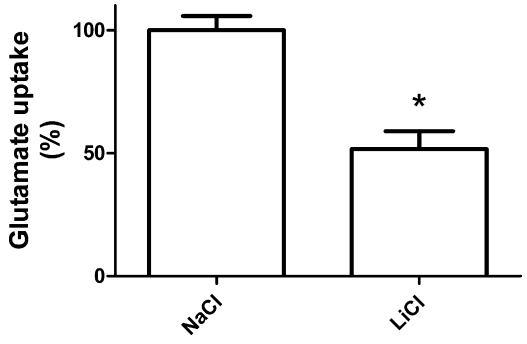

Fig. 5. Effect of sodium medium free on the glutamate uptake in retinal tissue. Data demonstrated that replacement of Na^+ by Li^+ in the incubation medium reduces the L-glutamate uptake in chick retinal tissue. The values are expressed as percentage of control (NaCl) and the statistical analysis was performed using the BIOESTAT 5.0 software. *$p < 0.01$ (Student's t-test).

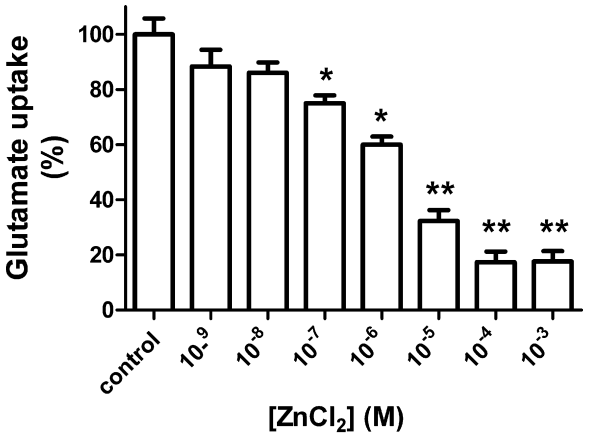

Fig. 6. Effect of zinc chloride on the glutamate uptake in the retinal preparations. Treatment with zinc chloride, a potent blocker of the main glutamate-aspartate transporter (GLAST) in the central nervous system, evokes a significant decrease on the L-glutamate uptake in retinal tissue. Date are expressed as percentage of the control ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control. ANOVA followed by Tukey test).

[13,33]. Added to this, chick retinal cells express the major glutamate transporters and some retinal injuries are closely associated with alterations in glutamate uptake in this tissue [13,15,34,35]. Thus, chick retinal tissue represents an excellent model for studies related with neurochemical events in the central nervous system including glutamate uptake.

With regard to the evaluation of glutamate transport, alternative and sensitive methods, able to characterize few alterations in the normal pattern of glutamate uptake, became essential for neuroscience researches. Numerous reports suggest that glutamate represents an important mediator of neuronal toxicity in the retina and all central nervous system [10,36,37]. Several works describe that the family of high-affinity Na^+-dependent transporters is involved in the maintenance of extracellular glutamate concentrations below toxic levels in the brain tissue [7,38].

Previous work performed by our group have demonstrated that retinal tissue express Na^+-dependent and Na^+-Independent glutamate transporters. In we also showed, utilizing L-[^{3}H]-glutamate, that replacement of NaCl for LiCl induced a decrease 50% of glutamate uptake in retinal cell [39]. In the present study similar results were observed suggesting that our methodology is able to characterize the presence of two main classes of glutamate transporters in the central nervous system. This hypothesis is supported by our results that demonstrated that low temperature induced a significant decrease in the glutamate uptake evaluated by our methodology in retinal tissue. Previous studies have shown that activity of glutamate transporters in the central nervous system is temperature dependent [14]. Our data also showed that pharmacological intervention in glutamate transport can be detected utilizing the present methodology, since we demonstrated that a recognized glutamate transporter inhibitor, zinc chloride [28], evoked a concentration dependent decrease in glutamate uptake evaluated by HPLC.

Alternative and sensible methodologies able to verify alterations in activity of these transporters are important to characterize events that occur in early stages of tissue toxicity. When compared to commonly used techniques, some advantages of our protocol are highlighted. Among them, loss of information associated to radiolysis or quenching phenomena is not observed since radioactive substances are not observed with our method. Previous reports demonstrated that these physical phenomena are duce significantly the sensitivity of experiments that involves radiolabeled compounds stocked for long periods [21,22]. In relation to protocols that measure intracellular glutamate levels in the central nervous system, an important technical limitation is that glutamate is quickly metabolized to glutamine by glial cells [40]. These events do not permit a precise quantification of glutamate transported into the cell influencing the precise determination of glutamate transporter activity. Silva et al. [31] recently demonstrated that the HPLC method coupled to fluorescent detection is an efficient technique for the determination of high concentrations of glutamate in the different central nervous areas. Although this methodology represents an interesting assay for measurements of tissue glutamate levels, alterations in glutamate uptake are firstly identified at the micromolar range, which creates the necessity for an assay that allows exact measurements of tiny differences in the concentrations of this neurotransmitter [23].

The chromatographic conditions demonstrated in the present work have shown an elevated level of recovery in the micromolar range; similar results were described in previous works [30–32]. Added to this, the indirect determination of the glutamate uptake (based on the consumption of extracellular glutamate) is not influenced by disturbances in cellular metabolism or sample loss due to matrix preparation processes.

In relation to kinetic parameters, our results demonstrated a high sensitivity (Figs. 1 and 2), with values K_m and V_{max} equal to 8.2 and 9.8 nmol/mg protein/min, respectively. These results are in agreement with previous works that demonstrated K_m values between 4 ± 2 and 7 ± 2 nmol for glutamate transport in different areas of the central nervous system, including retina. In these studies are also observed that saturation for glutamate uptake in the nervous tissue occurs in 10 min as demonstrated in our results [4,41–43].

5. Conclusion

In conclusion, the present work describes a simple and efficient method for quantification of glutamate uptake in retinal tissue using high performance liquid chromatography. We have shown that our methodology is sensible to effect of the several blockers of glutamate transporters suggesting that our protocol can be used for evaluations of glutamate uptake in other areas of the central nervous system.

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