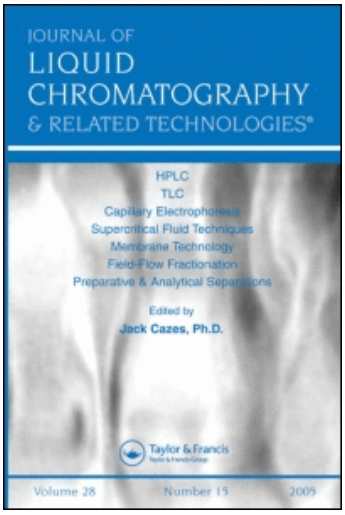

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Development and Validation of an HPTLC Method for Simultaneous Estimation of Excitatory Neurotransmitters in Rat Brain

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Abstract: L-glutamate and L-aspartate are two major excitatory neurotransmitters in the mammalian central nervous system involved in various physiological functions. A simple high performance thin layer chromatography (HPTLC) method has been developed for the simultaneous estimation of L-glutamic acid and L-aspartic acid in the rat brain tissue samples. The method was validated in terms of precision, reproducibility, variability, and accuracy. Instrumental precision was found to be 0.5321 and 1.1961 (% CV) and reproducibility of the method was found to be 2.612 and 2.883 (% CV) for L-glutamic acid and L-aspartic acid, respectively. The accuracy of the method was checked by the recovery study. Average recovery of L-glutamic acid was found to be 98.89% and that of the L-aspartic acid was 97.25%. The proposed HPTLC method for the simultaneous estimation of L-glutamic acid and L-aspartic acid was found to be simple, precise, reproducible, sensitive, and accurate. No doubt, this proposed method will be a useful tool for the estimation of these excitatory

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amino acids in the brain tissue samples and for the researchers in the field of neuroscience.

Keywords: L-Glutamic acid, L-Aspartic acid, Cerebral stroke, Rat brain tissue, HPTLC

INTRODUCTION

Excitatory neurotransmitters/amino acids (EAA) (glutamic acid, Aspartic acid, glycine etc.,) mediates most of the synaptic excitatory activity in the central nervous system, they are released by an estimated (~) 40% of all synapses[1] in the mammalian brain. Most of these EAA are implicated in cognitive function, synaptic plasticity (both LTP & LTD), and neurotrophic activity.[2] Among these glutamic acids is the major excitatory amino acid neurotransmitter in the brain, which plays an important role in the pathogenesis of neuronal injury and death induced in cerebral ischemia condition.[3] Brain concentration of glutamic acid, and related EAA–aspartic acid increases rapidly during ischemic episodes, contributing to neuronal depolarization.[4–6] In animal models of stroke, elevated concentration of glutamic acid, aspartic acid, and glycine are detectable in the biological samples soon after focal cerebral ischemia.[7,8] So, precise quantification of these amino acids, glutamic acid, and aspartic acid, in the different regions of the brain in the experimental animals, is expected to reflect the pathophysiological/metabolic status of the neurons and, also ischemic/therapeutic intervention evoked release/inhibition of neurotransmitter activity. Therefore, it is clear that measurement of excitatory amino acids in different pathological conditions is necessary to correlate their role in neurodegenerative conditions and to understand the mechanism of neurodegeneration.

In this regard, various analytical methods such as UV spectrophotometer,[9] HPLC,[10] and "on-line" or "off-line" microdialysis techniques,[11] were employed to estimate EAA in the biological samples. The experimental procedures, volume and complexity of biological samples for the above mentioned techniques were tedious. So far, further protocol for simultaneous estimation of these EAA has not been carried out. In view of the biological significance of these amino acids in cerebrovascular disorders, there has been a spurt of interest in the development of a simple, rapid, precise, reproducible, and accurate high performance thin layer chromatography (HPTLC) assay. A rat cerebral ischemic model was selected to determine the brain concentration of glutamic acid, and related EAA–aspartic acid, because in this condition a rapid increase in both gluatamic and aspartic acid level was observed in different parts of the brain soon after focal cerebral ischemia induction.[7] Hence, the objective of the present study is to develop a simple, feasible, cost effective HPTLC method for simultaneous estimation of glutamic and aspartic acid in rat brain tissue based on the previously reported method of Raju and co-workers.[12]

EXPERIMENTAL

Chemicals

All the chemicals and solvents used in the experiment were of analytical grade, purchased from Qualigens Fine chemicals, India. L-glutamic acid and L-aspartic acid were purchased from Sigma, USA.

Animals

Male Sprague-Dawley rats, weighing 250 to 280 g were obtained from Central Animal House Facility, Sri Ramachandra University, Chennai, India and used in this study. The animals were kept under laboratory standard conditions on a 12 h light/dark cycle with light from 6:00 am to 6:00 pm, at 23 $\pm$ 2°C room temperature, and 55% relative humidity. The animals were acclimatized for 7 days before the experiments were performed. They were housed in colony cages, with free access to standard rat chow pellet and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC).

Preparation of Standard Solutions

0.1 N HCl was prepared in 80% ethanol (mentioned hereafter as 0.1 N HCl), which was used to dissolve L-glutamic acid and L-aspartic acid.

Preparation of Stock Solution of L-Glutamic Acid/L-Aspartic Acid

Stock solutions of L-glutamic acid/L-aspartic acid were prepared by dissolving 10 mg of the respective amino acid in 10 mL of 0.1 N HCl (1 mg/mL). From this stock solution, working standard solutions of concentration 1 ng to 500 ng in 10 μL was prepared by transferring aliquots made up to desired concentration in 10 mL volumetric flasks and adjusting the volume with 0.1 N HCl.

Preparation of 0.2% Ninhydrin Solution

In a 100 mL standard flask, 200 mg of ninhydrin was taken and dissolved in a small amount of acetone. To this, 1 mL of pyridine was added and the volume was made up to 100 mL with acetone.

Preparation of Brain Tissue Samples

The overnight fasted rats were sacrificed under deep choral hydrate anesthesia and their brains was excised out quickly in ice cold conditions, and the different brain regions (cortex, striatum, and hippocampus) were identified and dissected as described by Glowinsky.[13] Each region was homogenized in 0.1 N HCl (for every 10 mg tissue/200 μL) in a manual homogenizer. The homogenates were transferred to polypropylene tubes and centrifuged at 4500 rpm for 20 min at 25°C. The supernatant was then transferred into micro centrifuge tubes and used at the earliest for spot application.

Chromatographic Conditions

Stationary phase: Silica gel GF254; Mobile phase: n-butanol: glacial acetic acid: water (65:15:25 v/v); Chamber saturation time: 3 hr; Instrument: HPTLC (Camag–version 1.3.4); Applicator: Linomat V; Scanner: Camag TLC scanner III; Developing chamber: Twin trough glass chamber (20 × 10); Developing mode: Ascending mode (multiple development); Detection reagent: 0.2% ninhydrin in acetone; Scanning wavelength: 486 nm; Experimental condition: 25 ± 2°C; Temp/RH: 55–65%.

Method Validation

The present method was validated in terms of recovery, precision, reproducibility, and variability. Limit of detection (LOD) and limit of quantification (LOQ) was also evaluated.

Calibration Curve

A 10 μL volume of the standard solutions (1 ng to 500 ng) of L-glutamic acid/L-aspartic acid was applied in triplicate on a precoated HPTLC plate. Spots were dried in a hot air oven at 60–65°C for 1–2 min and the plate was developed in the mobile phase n-butanol: glacial acetic acid: water (65:15:25). When the solvent front reached the top of the plate, the plates were removed and dried at 60–65°C for 3–4 min in a hot air oven. A second run was performed in a similar way; once the solvent front reached about 8.0 cm (marked previously), the plates were dried as mentioned above. The plates were then dipped (1 sec) in 0.2% ninhydrin reagent and dried in a hot air oven at 60–65°C for 3–4 min. The spots were scanned at 486 nm and the peak areas were recorded. Calibration curves of L-glutamic acid and L-aspartic acid were prepared by plotting areas vs. concentration.

Limit of detection (LOD) and limit of quantification (LOQ) was evaluated from the calibration curve.

Recovery

The accuracy of the method was tested by performing the recovery studies at two levels. To 50 mg of the rat brain tissue a known amount of L-glutamic acid (100 ng) and L-aspartic acid (100 ng) were added, and the efficiency of the present extraction method was evaluated for the optimized method in terms of % recovery (Table 1).

Precision

The instrumental precision for the present estimation was checked by repeated scanning of the same spot of L-glutamic acid (100 ng) and L-aspartic acid (100 ng) five times, and the result was expressed in coefficient of variance (% CV). Results were tabulated (Table 1):

$$\% \text{ CV} = (\text{SD}/\text{M}) \times 100$$

M = mean value of the scanned area; SD = standard deviation for the mean.

Reproducibility

The reproducibility of the present method was validated by analyzing standard solutions of L-glutamic acid and L-aspartic acid in the concentration of 100 ng in triplicate three times, in freshly prepared and saturated mobile phase. The results were expressed in % CV (Table 1).

Table 1. Method validation parameters for the quantification of L-glutamic acid and L-aspartic acid by HPTLC method

Parameters	L-glutamic acid	L-aspartic acid
Instrumental precision (% CV) (n = 7)	0.5321	1.1961
Reproducibility	2.612	2.883
Variability	1.83	1.62
Limit of detection	10 ng	5 ng
Limit of quantification	40 ng	20
Specificity	Specific	Specific
Linearity (correlation coefficient)	0.9992	0.9997
Range (ng/spot)	20–200 ng	10–80 ng

Variability

The variability of the present method was studied by analyzing the standard solution of L-glutamic acid (100 ng) and L-aspartic acid (100 ng) on the same day (intra day assay), and on different days (inter days assay), and the results were expressed as % CV (Table 1).

RESULTS AND DISCUSSION

Estimation of excitatory amino acids in the biological fluids, brain tissue samples is of great important in the field of neuroscience. Accurate estimation of these amino acids depends on several aspects, such as efficiency of extraction, the specificity, and sensitivity of the method employed.

The extraction step of amino acids from the biological fluids or brain tissue samples, apart from precipitation of proteins, should also be effective in extracting the bound forms of amino acids. Further, the method should be able to resolve the amino acids and, also, sensitive enough to quantify them. In the present study, the use of 0.1 N HCl in 80% ethanol was found to be more efficacious than conventional solvents (data not shown) in the separation of amino acids (L-glutamic and L-aspartic acid) from the brain tissue samples, and to solubilize the standards, which has been validated and recommended.

Of the various proportions of the solvent systems tried, n-butanol: glacial acetic and water (65:15:25 v/v) with double elution, gave the best resolution of L-glutamic acid (Rf = 0.4) and L-aspartic acid (Rf = 0.3). The bands of L-glutamic acid and L-aspartic acid were detected by derivatizing the plate in 0.2% ninhydrin in acetone. The purity of the L-glutamic acid and L-aspartic acid bands in the brain tissue samples was confirmed by comparing the UV absorption spectra at the start, middle, and end position of the bands. The identity of the bands of L-glutamic acid and L-aspartic acid in the brain tissue samples was confirmed by overlaying their UV absorption spectra with those of the standard L-glutamic acid and L-aspartic acid (Figures 1 and 2, respectively) using Camag TLC Scanner 3. The present method for the estimation of L-glutamic acid and L-aspartic acid in the brain tissue sample was validated in terms of recovery, precision, reproducibility, and variability (Table 1). In the present study, the limit of detection (LOD) for L-glutamic acid and L-aspartic acid was found to be 10 ng and 5 ng, respectively.

Glutamic acid content was reported to be 8–16 u moles/g in the rat brain[14] and our results was found to be consistent with the report. L-glutamic acid and L-aspartic acid peaks in the brain tissue sample were shown in Figures 3a–e.

The relationship between the concentration of standard solutions and the peak response was found to be linear within the concentration range of

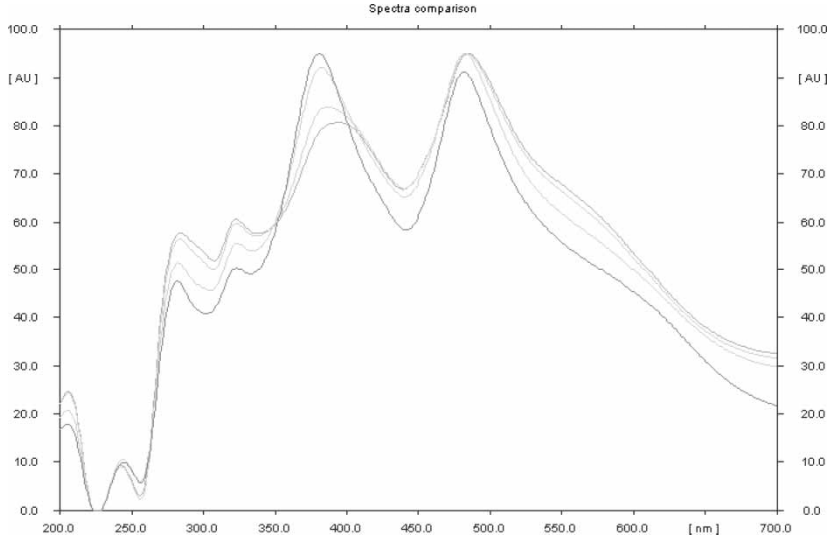

Figure 1. Overlay UV absorption spectra of standard L-glutamic acid along with L-glutamic acid in the brain tissue samples.

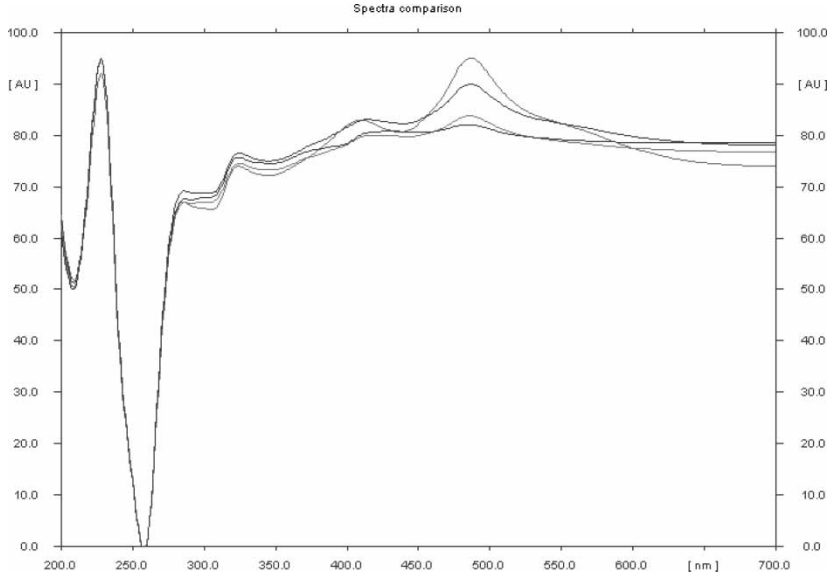

Figure 2. Overlay UV absorption spectra of standard L-aspartic acid along with L-aspartic acid in the brain tissue samples.

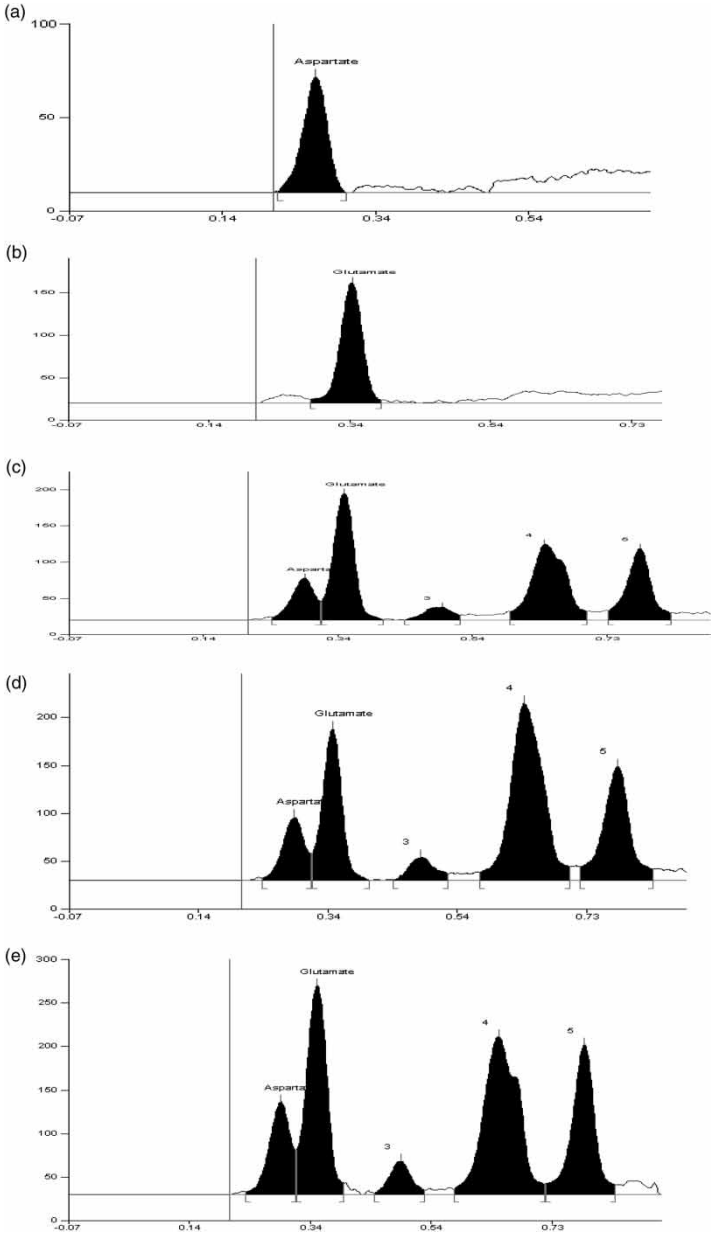

Figure 3. HPTLC chromatogram of L-glutamic acid and L-aspartic acid in the different brain regions of rats. a) Standard L-aspartic acid's HPTLC chromatogram. b) Standard L-glutamic acid's HPTLC chromatogram. c) L-glutamic acid and L-aspartic acid peaks in cortex region of the rat brain. d) L-glutamic acid and L-aspartic acid peak in the striatal region of the rat brain. e) L-glutamic acid and L-aspartic acid peak in the hippocampal region of the rat brain.

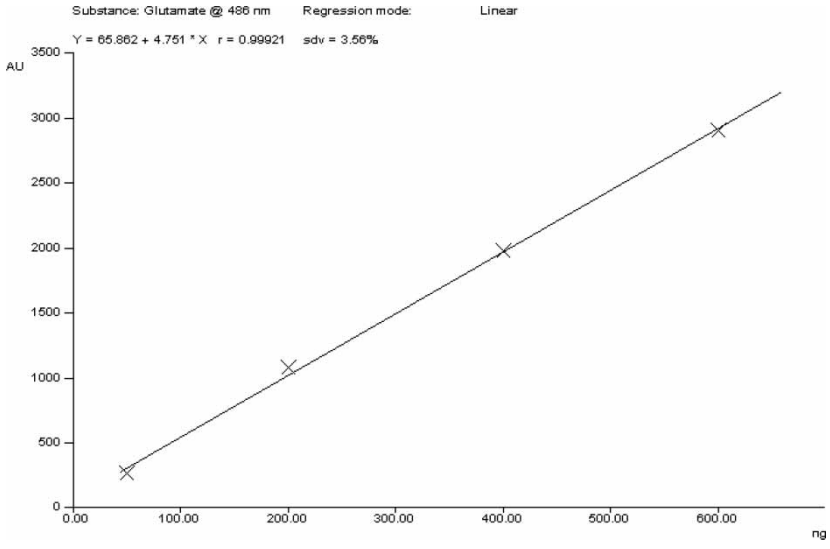

Figure 4. Calibration curve of L-glutamic acid.

20–200 ng/spot with a correlation coefficient of 0.99921 for L-glutamic acid (Figure 4) and 10–80 ng/spot with a correlation coefficient of 0.99997 for L-aspartic acid (Table 1/Figure 5). The percentage recovery of L-glutamic acid was found to be 99.01% and 98.76%, with an average of 98.89% and that of L-aspartic acid was found to be 96.05% and 98.45%, with an

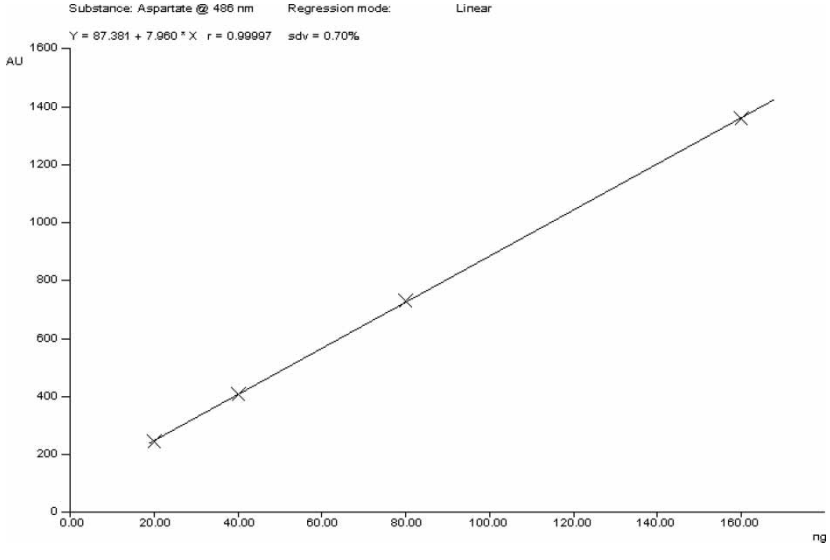

Figure 5. Calibration curve of L-aspartic acid.

Table 2. Recovery study of L-glutamic acid and L-aspartic acid by the proposed HPTLC method

Amino acid	Amount present in 50 mg tissue (ng)	Amount added (ng)	Amount found (ng)	Recovery (%)	Average recovery (%)
L-Glutamic acid	592	100	685.2	99.01	98.89
	587.5		679	98.76	
L-Aspartic acid	172.07	100	262.31	96.05	97.25
	184.03		280.18	98.45	

Table 3. L-Glutamic acid and L-aspartic acid content found in different brain regions by HPTLC method

Brain region	Content of L-glutamic acid (mg/g wet tissue)	Content of L-aspartic acid (mg/g wet tissue)
Cortex	1.29 ± 0.06	0.94 ± 0.05
Striatum	1.10 ± 0.1	0.75 ± 0.08
Hippocampus	1.05 ± 0.03	0.84 ± 0.02

Values were expressed in mean $\pm$ SEM ($n = 6$).

average of 97.25% (Table 2). L-glutamic acid and L-aspartic acid levels in the cortex, striatal, and hippocampal region of the rat brain were quantified by the proposed method and the results were shown in Table 3.

The best resolution and result was obtained in the estimation of L-glutamic and L-aspartic acid in rat brain, by applying the test sample immediately after the sample preparation, saturating the developing chamber for at least 3 h, and pre/post derivatisation of TLC plates at the recommended temperature (60–65°C) and period (2–3 min). The advantages of the present method over existing techniques for the estimation of L-glutamic acid and L-aspartic acid are: the present method is simple, robust, cost effective, and less time consuming for large numbers of samples.

CONCLUSION

To our knowledge, the present method is the first of its kind to estimate, simultaneously, the L-glutamic acid and L-aspartic acid level in the rat brain tissue by HPTLC. The present method for simultaneous estimation of L-glutamic acid and L-aspartic acid is simple, rapid, precise, accurate, and economic.

This proposed HPTLC method will be a useful tool, for the estimation of EAA in brain tissue samples.

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