Determination of Excitatory Amino Acids in Biological Samples by Capillary Electrophoresis

PhD thesis

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1. Introduction

Glutamate and aspartate are the two major excitatory neurotransmitters in the central nervous system. While the role of glutamate is widely investigated, the function of aspartate is less well understood. Neither the mechanism of its release nor the exact functions in higher cognitive processes are clarified.

Amino acids except glycin are chiral molecules, thus two physically and chemically identical forms can be distinguished. These forms are non-superimposable mirror images of each other and denoted with L- or D-prefix. It was believed for a long time, that only L-amino acids can be found in living organisms. However, along with the increasing sensitivity of the analytical methods the presence of numerous D-amino acids including D-aspartate was found in higher organisms, such as avian or mammals. The accurate quantification of D-aspartate in biological samples may be compromised by the excessive presence of the corresponding L-enantiomer. Based on experimental data, neuromodulator role of D-aspartate in the early neurogenesis and memory forming is hypothesized.

Microdialysis has gained a wide recognition as a valuable sampling tool for *in vivo* monitoring of extracellular neurotransmitter levels in certain brain regions. In order to achieve the required high temporal resolution to follow the rapid changes in neurotransmitter concentrations, short sampling times are needed. Because of the short sampling time combined with low flow rate used during microdialysis, only a couple of microliter sample can be collected. Analytical techniques fitting to small sample volumes thus should be used.

Capillary electrophoresis is an analytical separation technique that separates charged molecules based on their electrophoretic mobility. The electrically charged analytes move in a capillary filled with conductive liquid medium under the influence of an electric field. The separated sample components can be selectively detected. Because of its high sensitivity, small required sample

volume and the relatively fast analysis time, capillary electrophoresis is a suitable analytical technique for analyzing biological samples.

Moreover, because of the achievable high separation efficiency of capillary electrophoresis it is widely used to separate enantiomers. To achieve chiral resolution, inclusion of a chiral additive into the separation buffer is needed. This chiral selector interacts with the enantiomers in different extent, thus differentially modifying their electrophoretic mobility. Due to their versatile complex forming ability, cyclodextrins and their derivatives are the most commonly used chiral selectors. One of the difficulties of amino acid analysis is the lack of easily detectable moiety, thus prior to analysis sample derivatization providing either a chromophore or a fluorophore group is needed. Laser induced fluorescence (LIF) detection offers much better sensitivity compared to the conventional UV absorbance.

2. Aims

In this work my aim was to develop and validate capillary electrophoresis methods capable of quantification of excitatory neurotransmitters in various biological samples. The particular aims were the follows:

1. High performance capillary electrophoresis method development and validation for simultaneous achiral determination of aspartate and glutamate in microdialysates.
2. Investigation of the differential changes of extracellular aspartate and glutamate in the striatum of domestic chicken with *in vivo* microdialysis sampling in cooperation with the Institute of Anatomy, Histology and Embryology of Semmelweis University.
3. High performance chiral capillary electrophoresis method development and validation capable of simultaneous separation and quantification of D-aspartate and D-glutamate and high excess of their L-enantiomers in tissue samples.
4. Investigation of D-aspartate levels in various brain tissue samples of domestic chickens in further cooperation.

3. Methods

3.1 All experiments were performed on a P/ACE MDQ capillary electrophoresis system. The instrument was coupled with a LIF detector, equipped with an argon-ion laser. Excitation and emission wavelengths were 488 and 520 nm, respectively. Separations were carried out in fused silica capillaries 75 μm i.d., 365 μm o.d., coated with polyacrylamide by in situ polymerization. The precapillary derivatization was carried out in sealed microvials. Briefly, 5 μL of amino acid solution or biological sample was mixed with 5 μL derivatization buffer containing the internal standard and 5 μL labeling. The labeling reaction was carried out at given temperature for given time in case of each reagent. The derivatized samples were stored frozen at -20 °C and diluted prior to analysis. All separations were carried out in 100 mM borate buffer. The separation of the fluorescein-5-isothiocyanate (FITC) and the 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) derivatives were carried out at pH 8.5, while the separation of the carboxyfluorescein succinimidyl ester derivatives were carried out at pH 8.0. The background electrolyte (BGE) contained 8 mM β-cyclodextrin (β-CD) and 20 mM sodium dodecyl sulphate in case of NBD-F and FITC derivatives, respectively. Typically 300-500 V/cm electric field was applied and the total and effective capillary lengths were varied between 30-50 cm and 10-40 cm, respectively. Samples were introduced into the capillary by pressure (5 s, 3447 Pa). All the validation data have been determined according to the FDA Guidance for Industry. Intra- and inter-day accuracy have been determined at three different concentrations, which covered the expected concentration range of analytes in the biological samples. The detection limit (LOD) has been determined as the concentration when the analyte peaks reached 3:1 signal-to-noise ratio. Both intra- and inter-day repeatability have been investigated, and the quantitation limit (LOQ) has been determined as the concentration of amino acids that can be derivatized and measured with acceptable accuracy and precision.

3.2 The animal experiments were conducted in the Institute of Anatomy, while the analysis of the microdialysis samples was performed in our laboratory. Forty one-day-old unsexed Hunnia broiler chicks purchased from a local company were used. Microdialysis probes were implanted in the left medial striatum. The microdialysis experiments were started after a 12 h recovery. Artificial cerebrospinal fluid (ACSF) was perfused through the probe by using 1 μL/min flow rate. After a stabilization period (usually about 2-3 h), samples were collected in polypropylene tubes at 5 min intervals, resulting in 5 μL final sample volume. As a behavioral stimulation mild chasing was applied, while the chemical treatments (KCl, tetrodotoxin (TTX), Ca^{2+}-deprivation) were carried out by the rapid change of the perfusion media. The microdialysis samples were derivatized with FITC prior to capillary electrophoresis.

3.3 Chiral separations were carried out under the same basic conditions (instrument, detector, coated capillary) likewise the former achiral separations. The parameters of the derivatization with NBD-F were the same as described previously. Hundred mM borate buffer pH 8.0 was used. The background electrolyte contained 8 mM heptakis (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and 5 mM 6-monodeoxy-6-mono(3-hydroxy)propylamino-β-cyclodextrin (HPA-β-CD). The effective capillary length was 50 cm and 400 V/cm electric field was applied. Samples were introduced into the capillary by pressure (20 s, 6.89 kPa). All the validation data have been determined according to the FDA Guidance for Industry.

3.4 One-day old Hunnia broiler chicks were used for D-amino acid analysis. The dissected tissue samples were frozen immediately in pre-weighted vials on dry ice and stored on -80 °C until further process. Samples were mixed with 10 μL/mg wet tissue ice-cold acetonitrile/water (2:1) mixture. The samples were homogenized by sonication, followed by centrifugation. Supernatants were collected and further diluted by ten times with ACSF prior to derivatization.

4. Results

4.1 Our analytes, the excitatory amino acid transmitters carry two carboxyl groups. As they have considerably more negative charge at alkaline pH then the other amino acids and amines, they can be selectively separated. In uncoated capillaries these analytes possess the least apparent electrophoretic mobilities and migrate behind the biogenic amines as well as the other amino acids found in biological samples. This makes the analysis time relatively long, although they can selectively be analyzed. However, using coated capillary and reversed polarity of separation voltage, the migration order can be reversed. This way while keeping selectivity, the separation time can be reduced, which is especially useful when large number of microdialysis samples is to be analyzed. In contrast to other amino acid profiling studies performed in commercially available CE instruments the analysis time of a single run is reduced to 3-5 min. In order to gain proper sensitivity, LIF detection was applied. Three different fluorescent tags (NBD-F, FITC, and CFSE) have been compared for the quantitative analysis of aspartate and glutamate in brain microdialysate samples.

4.1.a The reaction condition of the derivatization with NBD-F were optimized. Maxima in the amino acid peak areas were observed at 15 min reaction time by using pH 8.5 derivatization buffer. Hundred-millimolar borate buffer of pH 8.5 was chosen as BGE. Some minor peaks deriving from the reagent migrated in the range of analytes of interest. Adding 8mM-β-CD to the BGE, the interfering side products could be resolved from the analyte peaks. Using 10 cm effective capillary length, satisfactory resolution has been obtained within 3.5 min, which is a considerably faster separation than those previously reported in similar offline methods. In the lower concentration part of the calibration curves significant deviation of the fitted line from the measured points has been observed. LOD values of 9.8 and 7.8 nM have been found for aspartate and glutamate, respectively. However, accuracy and precision measurements have

revealed 0.1 μM LOQ for both amino acids, which is more than one order of magnitude higher than LOD.

4.1.b FITC is the most commonly used fluorescein-based amine-reactive probe. Being a fluorophore label, much more interference can be expected from the high excess of the reagent itself, in addition to its hydrolysis products. Hundred-millimolar borate buffer of pH 8.5 has been found appropriate for the separation, although the peaks were rather broad. Significant improvement of the peak shape has been achieved by inclusion of 20mM SDS into the BGE. Using 20 cm effective capillary length, these FITC derivatives could be separated within 5 min. The calibration curves of the FITC labeled analytes were linear between 0.01 and 1 mM with R^2 value of 0.9988 for aspartate and 0.9986 for glutamate. The LOD values have been found 3.5 nM for both analytes. However, the validation data have revealed, tahat under 0.1 μM analyte concentration the measurements could not be performed with the required accuracy. Both intra- and inter-day accuracy and precision data were within the acceptable range between 0.1 and 1 μM analyte concentration

4.1.c In order to explore the possibility of gaining further improvement in detection sensitivity, another fluorescein based labeling compound, CFSE was also investigated. According to some previous reports, more efficient labeling at low sample concentration and less side product formation could be expected using this labeling tag. However, co-migration of the analytes with the reagent excess has been experienced at pH 8.5. We have found that at pH 8.0 and below the analytes migrated ahead the excess of reagent, while their apparent mobility was similar at higher pH. Hundred-millimolar pH 8.0 borate buffer was thus chosen as BGE for the separation of aspartate and glutamate derivatized with CFSE. Forty centimeter effective capillary length was needed to achieve separation, although some interference with minor hydrolysis products has been still remained. Several buffer additives, such as cyclodextrins, detergents and

organic solvents, have been tried, but none of them could provide better resolution. The dependence of interfering to analyte peak ratio on derivatization time has also been studied. The most favorable results could be achieved when 4 h derivatization time was used. From the calibration lines (R^2 values 0.9985 and 0.9986, respectively) LOD values of 1.3 and 1.5 nM were determined for aspartate and glutamate, respectively. Based on accuracy and precision measurements, similar to FITC labeling LOQ values of 0.1 μM have been established.

4.1.d Based on its superior stability FITC derivatization was chosen for the analysis of the biological samples. Pooled striatal microdialysate has been used to assay the recovery of amino acids from biological sample. The measurement of spiked samples has revealed that the concentration of the added amino acids around the LOQ could be determined with accuracy between 101 and 109% (Fig. 1).

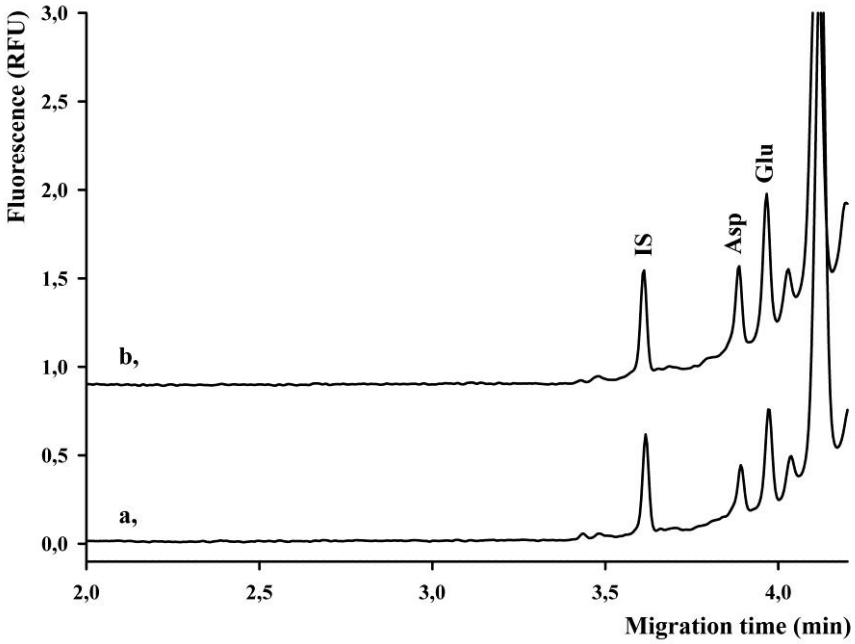

Fig. 1 Typical electropherogram of a pooled brain microdialysate sample derivatized with FITC (the found concentrations are 0.12 and 0.2 μM for aspartate and glutamate, respectively), and the same sample spiked with 0.06 μM aspartate and 0.1 μM glutamate before derivatization. Separation conditions: BGE: 100mM borate pH 8.5 containing 20mM SDS; 20/30 cm x 75 mm id coated fused-silica capillary, -9 kV. Sample introduced by 3,447 kPa pressure for 5 s.

4.2 To standardize the variation of the excitatory amino acids between the individual animals, all stimulation induced changes in amino-acid levels were calculated as percentages of the basal concentration (mean ± SEM). The basal release (set as 100 %) was determined as the average concentration of the three samples that were measured immediately before chemical or behavioral stimulation. Using 1 μL/min flow rate, fractions were collected in polypropylene tubes at 5 min intervals, resulting in 5 μL of final sample volume.

4.2.a The behavioral stimulation was carried out by the experimenter applying mild chasing for 15 min. This stress induced significant increase in the concentration of both amino acids. During the sodium channel blocker TTX administration the distress had no effect on the levels of aspartate and glutamate. The apparent elevation of glutamate level during Ca^{2+}-free ACSF perfusion was not significant.

4.2.b Similarly to the behavioral stimulation, the perfusion of 50 mM KCl induced marked elevation in the levels of both neurotransmitters. Excessive release was observed after KCl stimulation if applied 1 h after distress. The sudden surge of excitatory amino acids on high potassium was rapidly reversed despite of a persistent stimulus. This effect was likely due to a rapid depletion of the amino acid pools. When KCl stimulation was combined with TTX infusion, there was no significant elevation in aspartate or in glutamate. Glutamate level was seemingly increased when KCl stimulation was combined with EGTA and Ca^{2+}-free ACSF medium, although this elevation was not significant.

The ratio of aspartate strongly correlated with the amount of glutamate released before and during stimulation. During the stimulation, the ratio of the released aspartate was between 50-60% to the total released excitatory amino acids. While in the absence of stimuli the aspartate ratio varied between 10 and 60% in the animals. These findings may suggest that some parts of the mechanisms underlying the aspartate release are independent from the glutamate release.

4.3 In order to achieve chiral resolution of aspartate and glutamate enantiomers, several native cyclodextrins and their derivatives have been screened. Majority of them provided only partial chiral resolution. Only an amino modified cyclodextrin derivative, HPA-β-CD has been proved to be appropriate for the enantioseparation of both aspartate and glutamate, probably the ionic interaction between the carboxyl and the amino group provided the differential complex formation of the enantiomers. Using 100 mM pH 8.0 borate buffer and 3 mM HPA-β-CD baseline separation of the amino acid enantiomers could be achieved. Since in the biological sample large excess of L-enantiomers are present, further improvement of the separation selectivity was needed. During the cyclodextrin screening we have found that DM-β-CD has shown chiral recognition only for glutamate with a strong interaction, indicated by the considerable change of its migration time. As the concentration of the DM-β-CD was elevated, the difference in migration times of aspartate and glutamate increased. DM-β-CD thus seems to be appropriate to adjust the chemical selectivity. Five different combinations of the two cyclodextrins have been tested. The higher concentrations of both cyclodextrins gave better resolution but provided longer separation time, while at lower concentrations less resolution of the analytes could be achieved. As a compromise a mixture containing an intermediate concentration of both selectors were chosen. After setting the optimized separation conditions, the method was validated. Previous literature data indicate about one to two orders of magnitude lower concentration of D-aspartate compared with its L-isomer in various biological samples. As in our preliminary studies, we have found about 50-fold concentration difference of D- and L-aspartate; this ratio of enantiomers was kept throughout the entire validation process. LOQ for both D-amino acids was 0.05 μM, which is the lowest quantification limit reported so far for their NBD-tagged derivatives obtained by validated CE-LIF method. The LOD was found to be 17 and 9 nM for D-aspartate and D-glutamate, respectively.

4.4 The applicability of the method was tested by analyzing samples from various brain regions of 1-day-old domestic chickens. Three different subregions, the subventricular zone (SVZ), the nidopallium and the cerebellum were investigated. The concentration of D-aspartate found in extracts from different areas of chicken brain ranged from 0.17 to 0.45 μM. corresponding to 17 to 45 nmol/g wet tissue concentrations, while D-glutamate was not detected in these samples. The D-enantiomer represented 1-2 % of the total aspartate content in all the studied brain areas.

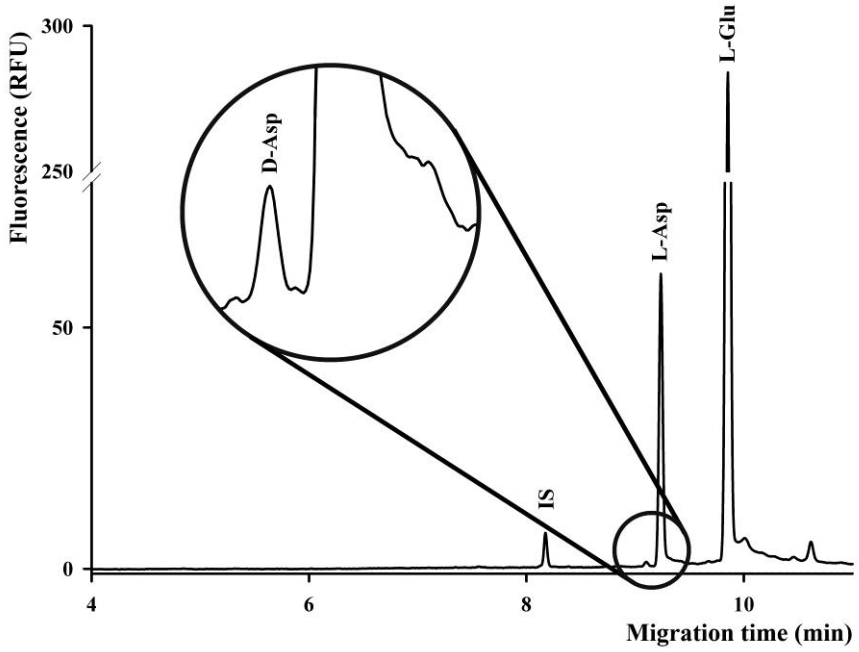

Fig. 2 Typical electropherogram of chicken brain tissue sample derivatized with NBD-F. Background electrolyte: 100 mM borate pH 8.0 containing 5 mM HPA-β-CD and 8 mM DM-β-CD; voltage: -24 kV; temperature: 25 °C; capillary: 50/60 cm×75 μm i.d. polyacrylamide-coated fused silica.
Sample introduced by 6,89 kPa pressure for 20 s

5. Conclusions

5.1 In our work, we investigated the applicability of the three commonly used fluorescent labeling reagents in quantification of aspartate and glutamate in brain microdialysate samples. All the separation was carried out in a coated capillary at alkaline pH. These conditions provided rapid and highly selective separation of the analytes. Method validation has revealed similar quantification limit of 0.1 μM of analytes using either of the labels, although LOD values were different. Our results suggest that accurate measurements below 0.1 μM concentration of these analytes cannot be achieved using either of these tags. The almost two orders of magnitude difference between LOD and LOQ values is likely due to the unreliable derivatization reaction at low sample concentration. This quantification sensitivity has been found appropriate for the measurement of excitatory amino acids in brain microdialysate samples.

5.2 In collaboration with the Institute of Anatomy, differential changes in the concentration of excitatory amino acids in domestic chickens were investigated by microdialysis. Event-related elevations of extracellular glutamate and aspartate concentrations in response to handling stress and to high KCl were observed. When KCl stimulation was combined with TTX infusion, there was no significant elevation in asparate or in glutamate suggesting that most of the extracellular excitatory amino acids were released by synaptic mechanisms. In most cases, the amino acids showed correlated changes, asparate concentrations being consistently smaller at resting but exceeding glutamate during stimulation. The results support the suggestion that aspartate is co-released with glutamate and may play a signaling role (as distinct from that of glutamate) in the striatum of birds. The ratio of aspartate as compared to glutamate is considerably higher in the avian striatum than in mammals, therefore birds appear to be a more appropriate model for studying the function and dynamics of aspartatergic mechanisms.

5.3 A rapid and highly selective method has been developed for the analysis of D-aspartate and D-glutamate in brain tissue samples. Similarly to the achiral methods laser induced fluorescence detection was applied. Dual cyclodextrin system containing HPA-β-CD and DM-β-CD was applied for simultaneous chiral separation of NBD-derivatives of the excitatory amino acids. During the method development and validation 50-fold L-to-D ratio of the analytes was applied. To best of our knowledge, this is the first validated CE-LIF method capable of simultaneous quantification of aspartate and glutamate enantiomers in brain tissue samples using NBD-F as labeling reagent. Compared to the previous achiral separations the method validation revealed lower LOQ for D-amino acids. The large excess of the L-enantiomers may increase the derivatization efficiency.

5.4 D-aspartate concentration found in chicken brain tissue are lower compared to those found in chicken embryos and newborn rats, but higher than the concentrations found in adult rats. Remarkable scatter was found among the D-aspartate content of the individual animals, however the D-aspartate concentration of the SVZ was always higher compared to the control regions (nidopallium, cerebellum). In 1-4 days old chickens significant cell proliferation can be observed in the SVZ region whereas by this time in the cerebellum and in the nidopallium neuronal stem cells are not present anymore. The results may suggest the role of D-aspartate in neurogenesis. In order to confirm the significant difference more samples are to be analyzed.

6. Summary

Glutamate and aspartate are the primary excitatory neurotransmitters in the central nervous system, playing an important role, e.g. in learning including memory formation and memory retrieval. While glutamate is widely investigated, the exact role of aspartate is less well understood. In the last few decades, the D-enantiomers of some amino acids were also detected in higher living organisms. The transient high concentration of D-aspartate found in embryonic brain suggests its crucial role in the early development of the nervous system. However, the quantification of the excitatory amino acids and their enantiomers requires proper analytical methods. Because of its high sensitivity, small required sample volume and the relatively fast analysis time, capillary electrophoresis is a suitable analytical technique for analyzing various biological samples. In our work we have developed new capillary electrophoresis methods capable of quantifying excitatory amino acids in biosamples. In order to increase the sensitivity, laser induced fluorescence was applied. Because of the lack of proper fluorophore, sample derivatization prior to analysis was required. Three capillary electrophoretic separation method based on different fluorescent labeling reagents were developed. Method validation has revealed similar quantification limit of 0,1 μM of analytes using either of the labels, although detection limits were different. The almost two orders of magnitude difference between the detection and the quantification limits is likely due to the unreliable derivatization reaction at low sample concentration. Based on its superior stability, fluorescein isothiocyanate (FITC) was chosen in order to analyze striatal brain microdialysates. A chiral capillary electrophoresis method using 7-fluoro-4-nitro-2,1,3-benzoxadiazole (NBD-F) as a labeling reagent has also been developed. Using dual cyclodextrin system, the baseline separation of aspartate and glutamate enantiomers has been achieved within 10 minutes. This method was also validated for biological application. In cooperation with the Institute of Anatomy, Histology and Embryology at the Semmelweis University, the release mechanism of aspartate and glutamate were investigated using one-day-old domestic chickens and brain microdialysis. The applicability of the chiral method was demonstrated by analyzing various brain regions of one-day-old chickens.

7. Publication list

7.1 Publications related to the thesis

Zs. Wagner, T. Tábi, G. Zachar, A. Csillag, É. Szökő (2011) Comparison of quantitative performance of three fluorescence labels in CE-LIF analysis of aspartate and glutamate in brain microdialysate. Electrophoresis, 32: 2816–2822. **IF: 3,303**

G. Zachar, Zs. Wagner, T. Tábi, E. Bálint, É. Szökő, A. Csillag (2012) Differential Changes of Extracellular Aspartate and Glutamate in the Striatum of Domestic Chicken Evoked by High Potassium or Distress: An In Vivo Microdialysis Study. Neurochem Res 37: 1730-1737. **IF: 2,24**

Zs. Wagner, T. Tábi, T. Jakó, G. Zachar, A. Csillag, É. Szökő (2012) Chiral separation and determination of excitatory amino acids in brain samples by CE-LIF using dual cyclodextrin system. Anal Bioanal Chem, 404: 2363-2368. **IF: 3,778**

7.2 Publications not directly related to the thesis

B. Balogh, B. Jójárt, Zs. Wagner, P. Kovács, G. Máté, K. Gyires, Z. Zádori, G. Falkay, Á. Márki, B. Viskolcz, P. Mátyus (2007) 3D QSAR models for α2A-adrenoceptor agonists. Neurochem Int, 51: 268-276. **IF: 2,975**

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