Journal of Chromatography B, 880 (2012) 58–65

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

In vivo monitoring of cerebral agmatine by microdialysis and capillary electrophoresis

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ARTICLE INFO

Article history:
Received 5 July 2011
Accepted 9 November 2011
Available online 18 November 2011

Keywords:
Capillary electrophoresis
Neurotransmission
Chemical messengers
Bioamines
Probe recovery

ABSTRACT

Agmatine is a putative neurotransmitter in the brain. Current analytical techniques do not allow the detection of agmatine in extracellular fluid, making it difficult to study its physiological role. However, a new method for *in vivo* monitoring agmatine in the brain was developed. Capillary zone electrophoresis and laser induced fluorescence detection (CZE–LIFD) was used to measure nanomolar concentrations of agmatine in submicroliter sample volumes. This analytical technique proved to detect 0.49 attomole of agmatine improving the sensitivity of previous analytical techniques. On the other hand, the hippocampus is a brain region well known for having a population of agmatine containing neurons. Therefore, intracerebral microdialysis was performed in the hippocampus and agmatine was extracted from the extracellular environment. Detectable amounts of agmatine were found in dialysates from probes located in the hippocampus but not from the probes located in the lateral ventricle. Furthermore, extracellular agmatine was calcium and impulse dependent and depolarization of hippocampal neurons increased extracellular agmatine concentration. The methods reported here are sensitive enough to study the physiological role of brain agmatine in freely moving animals.

1. Introduction

Agmatine (N-(4-amminobutyl) guanidine) is an endogenous diamine synthesized from L-arginine by the action of arginine-decarboxylase in living organisms [1,2]. Agmatine has been comprehensively studied in bacteria, plants and in some invertebrates. In these organisms agmatine is an intermediary in the synthesis of polyamines [3,4] and intervenes in growth regulation [5,6] cell proliferation [7,8] and differentiation [9]. Agmatine has been found in mammalian tissues [10] including the nervous tissue [11] where it is believed to function as a neurotransmitter or neuromodulator [12,13].

Agmatine containing neurons are distributed throughout the brain especially in the hippocampus, hypothalamus, the telencephalon and the cerebral cortex [14,15]. These brain regions are involved in memory, emotions, pain perception, neuroendocrine control and cognition.

The neurobiological relevance of agmatine is supported by experiments in which exogenous administration of agmatine inhibited hyperalgesia [16] as well as tolerance to opioids and opioid abstinence syndrome [17,18]. These actions have been attributed to the capacity of agmatine to block glutamatergic NMDA channels [19,20], to inhibit nitric oxide synthase [21] and to bind to imidazoline receptors (I1 and I2) [22–24] and to alpha-2-adrenoceptors [25–27]. In addition, agmatine has been shown to exert an antidepressive and anticonvulsant action [28–33] and also acts as a neuroprotector [34–36]. These effects suggest that agmatine has potential for the treatment of chronic pain, addiction, depression, epilepsy and brain damage.

Despite all these findings it is still unknown whether the actions of agmatine are physiological or not. Obviously a method to monitor agmatine in the extracellular compartment of the brain in freely moving animals might help answer this question. Brain microdialysis is an *in vivo* technique appropriate for monitoring changes of agmatine in the extracellular fluid. However, to the best of our knowledge, this technique has not been used to study physiological changes of agmatine in the brain. One of the reasons is that brain microdialysis requires analytical techniques for small volume samples with small masses of agmatine. To date, agmatine has been quantified using different techniques (see Table 1) but no one is sensitive enough to measure extremely low masses of agmatine in brain microdialysates.

The combination of microdialysis and CZE and laser induced fluorescence detection (LIFD) for agmatine has not been attempted

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doi:10.1016/j.jchromb.2011.11.016

Table 1
Analytical methods for agmatine comparing separation and detection methods and their sensitivity.

Method	Sensitivity	Reference
Fluorimetry	LoD 219×10^{-9} M LomD 0.547×10^{-9} mole	[48,49]
Thin layer chromatography	LoD 2.19×10^{-6} M LomD 21.9×10^{-12} mole	[50]
GC/mass spectrometry alkali flame ionization detection	LoD 13×10^{-6} M LomD 65×10^{-12} mole	[51]
GC/mass spectrometry negative ion chemical ionization	LoD 0.5×10^{-9} M LoQ 0.3×10^{-6} M LomD 25×10^{-15} mole LomQ 15×10^{-12} mole	[52]
High performance liquid chromatography Fluorescence detection	LoD 5×10^{-9} M LomD 0.1×10^{-12} mole	[45]
High performance liquid chromatography Fluorescence detection	LoD 50×10^{-9} M LomD 0.5×10^{-12} mole	[53]
High performance liquid chromatography transmittance at 510 nm	LoD 3.73×10^{-6} M LomD 3.73×10^{-9} mole	[54]
High performance liquid chromatography Fluorescence detection	LoD 40×10^{-6} M LomD 10×10^{-12} mole	[55]
High performance liquid chromatography Fluorescence detection	LoD 10×10^{-6} M LomD 0.1×10^{-9} mole	[56]
High performance liquid chromatography UV detection	LoD 0.25×10^{-6} M LomD 5×10^{-12} mole	[57]
High performance liquid chromatography Fluorescence detection	LoD 250×10^{-6} M LomD 20×10^{-12} mole	[58]
High performance liquid chromatography Fluorescence detection	LoD 5×10^{-9} M LomD 0.1×10^{-12} mole	[59]
High performance liquid chromatography UV absorption	LoD 248×10^{-9} M LomD 4.96×10^{-12} mole	[60]
Ultrahigh pressure chromatography Fluorescence detection	LoD 219×10^{-9} M LomD 219×10^{-15} mole	[63]
Agmatine sensor	LoD 5×10^{-6} M LomD 0.25×10^{-9} mole	[61]
CZE-conductimetric detection	LoD 2×10^{-6} M LoQ 5×10^{-6} M LomD 0.4×10^{-12} mole	[44]
CZE-chemiluminescence	LoD 4.3×10^{-6} M LomD 8.6×10^{-15} mole	[38]
CZE-LEDIF	LoD 4.1×10^{-9} M LomD 8.2×10^{-18} mole	[47]
CLC/mass spectrometry	LoD 2.6×10^{-9} M LomD 13×10^{-15} mole	[46]

yet. The main advantage of CZE–LIFD is its limit of detection for amino acids in the subnanomolar range and the low limit of mass detection in the attomolar range [37]. In the present report we measured agmatine by CZE–LIFD and obtained limits of concentration detection in the nanomolar range and limits of mass detection in the zeptomolar range. Furthermore, we successfully applied the technique to measure agmatine in brain dialysates from the hippocampus where agmatine acts as a putative neurotransmitter.

2. Materials and methods

2.1. Chemicals and reagents

Agmatine sulphate salt, fluorescein isothiocyanate isomer I (FITC), sodium bicarbonate, sodium carbonate, sodium hydroxide, sodium chloride, calcium chloride, potassium chloride, magnesium chloride, dibasic sodium phosphate, monobasic potassium phosphate and Tetrodotoxin (TTX), were purchased from Sigma Chemical Co. Acetone and acetonitrile were purchased from J.T. Baker. Formaldehyde was purchased at a drug store. Milli-Q water was used throughout.

2.2. Apparatus, electrophoretic conditions and data acquisition

Measurements were done on a R2D2-1 CZE–LIFD apparatus (Meridialysis Co., Merida, Venezuela) equipped with an argon ion laser of 20 mW and a 64 cm long, 25 μm I.D. and 350 μm O.D. fused silica capillary. The 488 nm line was used.

The samples were hydrodynamically injected at the anodic end of the capillary applying a negative pressure of −10 psi at the cathodic end of the capillary for 0.5 s. Then 23 kV were applied between both ends of the capillary. The data were obtained at 41 points by second and digitally filtered with the moving average method. Both the height and the area of the peaks were measured but only the peak height is reported because it proved to be satisfactory and reliable enough while peak area was harder to

estimate due to fronting of the peaks when agmatine was dissolved in ACSF. The results were analyzed by curve fitting with the SPSS.9.0 for Windows package. The level of significance was set at $p < 0.05$. After each run the capillary was flushed with 1 M NaOH (8 min) followed by 18 mΩ water (8 min) and 20 mM carbonate buffer (8 min) prepared by mixing 5 ml of 200 mM sodium carbonate solution with 5 ml of 200 mM sodium bicarbonate solution and water up to 100 ml. All solutions were filtered through 0.22 μm pore size membranes before they were injected into the capillary.

2.3. In vitro measurement of agmatine: sensitivity and accuracy

One milligram of agmatine sulphate was dissolved in 1 ml of 20 mM carbonate buffer. This solution was diluted ten times in carbonate buffer. A derivatizing solution was prepared dissolving 1 mg of FITC in 1 ml of acetone and mixed with 1 ml of 20 mM carbonate buffer. Five microliters of the FITC derivatizing solution were added to the agmatine dilution. This dilution had higher pH than the 1 mg/ml solution and consequently allowed to deprotonate the amine group at the alpha carbon (which pK_a value is 9.07) facilitating the formation of fluorescein thiocarbamate-agmatine derivative [39] but preserving a strong positive charge due to the guanidinium group whose pK_a value is >13 i.e. more than 3 logarithmic units above the pH of the 20 mM carbonate buffer which was 10.0 and, as a consequence remains protonated at alkaline pH. A blank solution was prepared by adding 5 μl of the FITC derivatizing solution to 1 ml of 20 mM carbonate buffer.

To determine the dynamic range of the method we derivatized a 3.9 μM agmatine solution in 20 mM carbonate buffer as described above and diluted it to obtain 3.16 μM, 2.42 μM, 1.72 μM, 970 nM, 270 nM, 215 nM, 107 nM, 53 nM, 26 nM, 13 nM and 6 nM concentrations. The peak heights were measured and fit to a concentration vs. arbitrary units of fluorescence (mV) by means of regression analysis.

The limits of detection and quantitation (LoD and LoQ) were determined according to the Protocols for Determination of Limits of Detection and Limits of Quantitation of the Clinical and Laboratory Standards Institute [40]. First we determined the limit of blank (LoB) according to LoB = mean blank + 1.645 (SD blank). For this purpose we dissolved 5 μl of derivatizing solution in 1 ml of 20 mM carbonate buffer at pH 10. After 21 h the solution was diluted and injected into the CZE–LIFD instrument. The procedure was repeated 10 times. The noise fluctuations at the place where agmatine should migrate if the sample contained agmatine was measured and the mean height of the 10 peaks as well as the standard deviation was calculated. Then we derivatized a 3.9 μM solution and diluted with 20 mM carbonate buffer to obtain 60 nM, 12 nM, 6 nM, 3 nM and 2 nM solutions. Then, these solutions were run. This whole procedure of derivatization at 3.9 μM, dilution and CZE–LIFD run was repeated 10 times. The peak heights for each concentration were averaged and the standard deviations were calculated. The limit of detection was estimated according to LoD = LoB + 1.645 (SD low concentration sample). The means were analyzed by regression analysis and the best fitting linear equation as well as the value of R obtained. Finally, we prepared 43 nM, 28 nM, 12 nM, 6 nM agmatine solutions in artificial cerebrospinal fluid (ACSF, the matrix of the dialysates, for composition see below) and mixed 100 μl of each with 100 μl of derivatizing solution. A blank solution was prepared by mixing 100 μl of ACSF with 100 μl of derivatizing solution. After 21 h, the mixture was run in the CZE–LIFD instrument and the peak heights were measured. This procedure was repeated 10 times. The average of the peak heights and the standard deviations was calculated. The limit of quantitation (LoQ) was estimated as the minimal measurable concentration above the LoD. Again, means were analyzed by regression analysis and the best fitting linear equation as well as the value of R obtained. The limit of mass detection (LomD) and of mass quantitation (LomQ) were estimated multiplying the LoD and the LoQ times the injected volume. The injected volume was calculated as follows. The distance of the anodic end of capillary to the detection window was 468 mm. This end of the capillary was immersed into a fluorescent solution and −10 psi applied at the cathodic end of the capillary. Then we measured the time it took for the fluorescence to reach the detection window. This procedure was repeated 10 times. Then we calculated the average time. We then calculated the travel speed and the distance (d) that the fluorescent solution ran in 0.5 s. With this distance and the cross section of the 12.5 μm radius (r) capillary we calculated the injected volume as:

$$\text{injected volume} = d\pi r^2$$

2.4. Optimal agmatine/FITC relationship

The optimal molar relation of [FITC] vs. [Agmatine] was calculated by mixing 1 ml of 4.0 μM agmatine with increasing volumes of a mixture of 2.5 mM FITC in acetone and 20 mM carbonate buffer. The final solutions contained 1, 2, 4, 8, 16, 32, 62, 125, 500 and 1000 molecules of FITC in excess for each agmatine molecule.

2.5. In vitro recovery of the probes

In the *in vitro* recovery experiment we prepared a 438 nM solution of agmatine sulphate in ACSF and filled six different vials with this solution. Then we perfused six microdialysis probes with ACSF at a flow rate of 1 μl/min and placed one probe in each vial. The probes were made of concentric stainless steel tubes and a fused silica capillary tubing (150 μm outside diameter and 75 μm inside diameter) inside with a piece of 4 mm long cellulose hollow fiber of 13,000 Da Molecular Weight Cutoff attached to the tip [41]. Then we collected the dialysates and derivatized both the solution contained in each vial and the corresponding dialysate following our derivatization protocol. After measuring the agmatine signal we compared each dialysate with its respective vial solution and calculated a percent of *in vitro* recovery.

2.6. In vivo recovery of the probes

To determine the extracellular agmatine concentration and *in vivo* recovery in a manner independent of probe kinetics, the method of zero net flow (ZNF) was used [64]. Standard stereotaxic procedures were used to implant a 10 mm long guide shaft, made of 21 gauge stainless steel tubing aimed to the dorsal hippocampus (coordinates: 3.5 mm lateral to the midsagittal suture, 4.0 mm posterior to Bregma and 1.5 mm ventral to the surface of the brain, at 30° of inclination) in five rats. After a minimum of six days we inserted a microdialysis probe that protruded 4.0 mm from the tip of guide shaft. A basal sample was obtained 4 h after the microdialysis probe was inserted. Then we intercalated an 11.5 cm long PE-50 piece of tubing containing a 22 nM solution of agmatine sulphate in ACSF. After 10 min we collected a microdialysis sample. Then we repeated the procedure with three more pieces of tubing containing 44 nM, 87 nM and 146 nM agmatine sulphate in ACSF solutions and collected the dialysate the same way as we did for the 22 nM intercalated solution. We then derivatized both the perfused agmatine sulphate solutions and the dialysates. After reading the peak heights we plotted the concentration of the perfused solutions vs. the peak height of the perfused solution minus the peak height of its corresponding dialysate. We calculated the best fitting straight line and solved the equation for $y = 0$ finding the extracellular concentration of agmatine. We then solved the equation for $x = 0$ and found the peak height of the dialysate (PHD). In addition

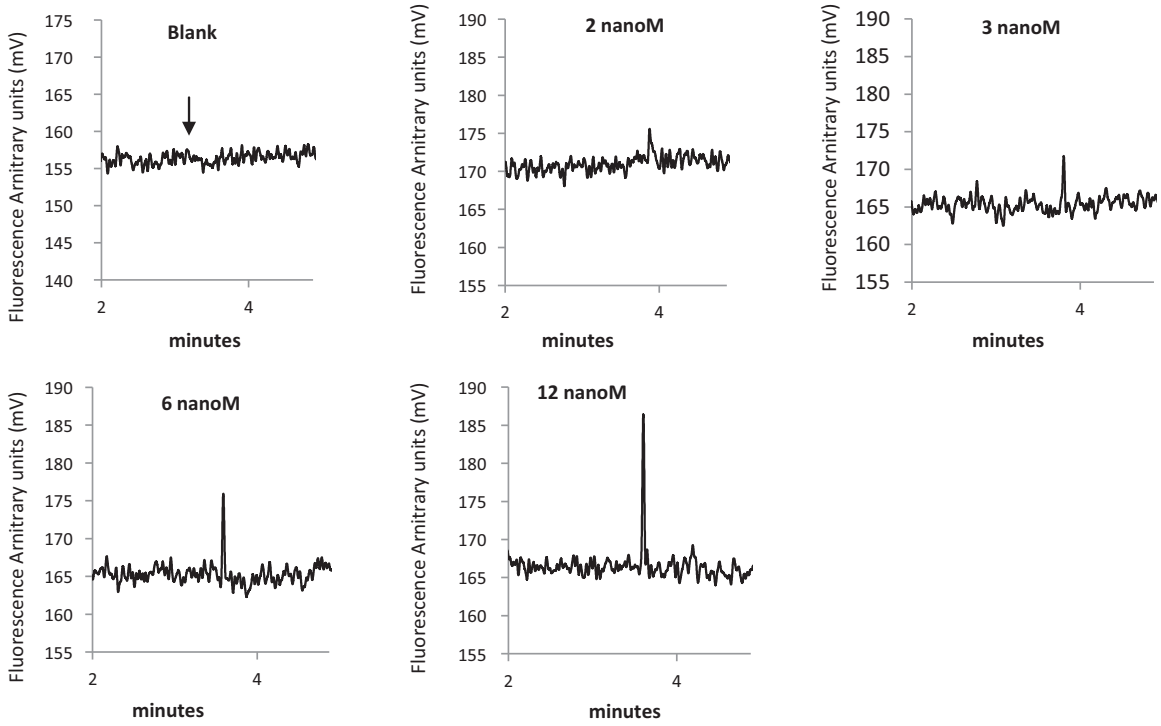

Fig. 1. Electropherograms of blank, 2 nM, 3 nM, 6 nM and 12 nM agmatine solutions.

we plotted the concentration of the perfused (standard) solution vs. the peak heights of agmatine (in dialysates) and with the linear equation we calculated the peak height of the estimated concentration in the extracellular fluid (PHEC). Solving (PHD/PHEC) × 100, we calculated the *in vivo* percent recovery.

2.7. In vivo agmatine measurement

Fourteen Wistar male rats weighing between 250 and 300 g were anesthetized with Xylazine (10 mg/kg) and Ketamine (25 mg/kg) and placed in a stereotaxic instrument. A 10 mm long guide shaft, made of 21 gauge stainless steel tubing was inserted into the brain and aimed to the dorsoventral hippocampus (coordinates: 4.5 mm lateral to the midsagittal suture, 5.0 mm posterior to Bregma and 4.0 mm ventral to the surface of the skull). The guide shaft was attached to the skull by jeweler screws and cemented with dental acrylic.

After 10 days of recovery the rats were microdialyzed by inserting into the guide shaft a microdialysis probe. The inlet of the probe was connected to a syringe pump filled with a modified aqueous Ringer's solution (artificial cerebrospinal fluid: ACSF): made of 136 mM NaCl, 3.7 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$ at pH 7.4. The dialysis probe was inserted 16 h before the experiment and the perfusion flow rate was set at 0.2 μl/min overnight. The flow rate was later raised to 1 μl/min for 3 h before three samples were collected every 30 s (sample volume = 0.5 μl) in collection tubes made of hematocrit tubing [42,43].

The samples were mixed 1:1 with the derivatizing solution. A standard of agmatine was prepared by dissolving 0.1 mg of agmatine in 1 ml 20 mM carbonate buffer and mixing with 5 μl of derivatizing solution to obtain a 6 μM solution of fluorescein thiocarbamate-agmatine. Both, samples and agmatine standard were left 21 h in darkness and then diluted in 18 mΩ water in a 1:10 ratio. The electrophoretic run was made as previously described. Spiking of the samples was used to verify the identity of the putative agmatine peak. For this purpose, an injection of each sample followed by an injection of standard solution was loaded in the anodic end of the capillary and run.

2.8. The tracks of the probes were localized by histology

In the *in vivo* recovery experiment brains were mounted on a Leica vibratome. Sections (40 μm) were taken through the brain areas of cannula placement, mounted onto slides and visualized in a microscope without stain. Animals showing signs of tissue damage other than cannula track, or those in which the probe was placed outside the hippocampal formation, were discarded. Histology was performed after data analysis to prevent any bias in data selection.

At the end of the *in vivo* measurement experiment all rats received an overdose of chloroform and were perfused transcardially with 0.1 M PBS [NaCl 80 g (137 mM), KCl 2 g (2.7 mM), $Na_2HPO_4{\cdot}7H_2O$ 11.5 g (4.3 mM), KH_2PO_4 2 g (1.4 mM)], at pH 7.4 followed by 4% of formaldehyde in 0.1 M PBS at pH 7.4. The skulls were removed and maintained in 4% of formaldehyde in PBS solution for 48 h. Then the brains were dissected out, fixed for 4 more days, embedded in paraffin and sectioned in 5 μm slices in a rotary microtome. Then they were stained with a cresyl violet staining method, and the tracks of the dialysis probes were localized.

2.9. Neuronal origin of extracellular agmatine

ACSF was perfused through the probes at a flow rate of 1 μl/min. Perfusions of microdialysis probe with calcium-free ACSF (without $CaCl_2$), infusion of 10 μM Tetrodotoxin (TTX) in ACSF and ACSF with 100 mM KCl by reverse microdialysis were carried out to test whether or not agmatine in hippocampus microdialysates was calcium dependent, nerve impulse dependent and released by depolarization of neurons. Three 1-min samples were collected before treatment to establish baseline levels. Then either calcium-free ACSF, 10 μM TTX in ACSF or ACSF plus KCl was infused for 60 min and, after that, 3 more samples were collected.

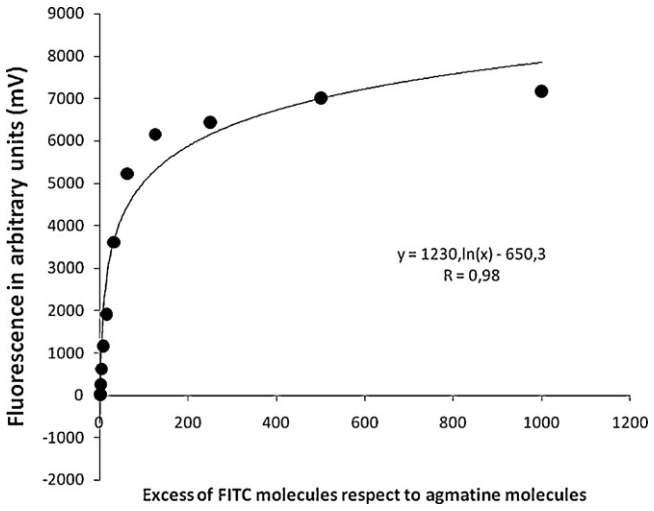

Fig. 2. Optimal FITC/agmatine molar relationship was about 400 FITC molecules in excess respect 1 molecule of agmatine.

The samples were derivatized and measured according to the protocol described in the *in vivo* agmatine measurement experiment.

3. Results

3.1. In vitro measurement of agmatine: sensitivity and accuracy

Fig. 1 shows the electropherograms obtained by the blank, 2 nM, 3 nM, 6 nM and 12 nM agmatine solutions of the LoD experiments. In the electropherogram agmatine appears as a peak at 230 s absent in the electropherogram of the blank (place where the peak should be is indicated by the black arrow).

The regression analysis of the curve obtained from 12 standard agmatine concentrations ranging from 6 nM to 3.9 μM and the height of the signal emitted by each one of them, yielded the linear equation: $y = 0.876x - 0.037$, with a regression coefficient $R = 0.998$, and significant fitting to the straight line ($F(1, 10) = 932.95$; $p < 0.00001$). The LoB was 3.733 arbitrary units of fluorescence (mV). The LoD was 2 nM corresponding to the signal of 4.518 mV. A *t*-test showed that the difference between the mean of the blank and the mean of the 2 nM solution was statistically significant at the level of $p < 0.0001$. The 60 nM, 12 nM, 6 nM, 3 nM, 2 nM and blank solutions yielded a linear relationship with the magnitude of the signals ($y = 2.1639x + 2.241$; $R = 0.997$) and a statistically significant goodness of fit ($F(1, 4) = 11.300$, $p < 0.0001$). The LoQ was 6 nM corresponding to the signal of 7.082 mV. The 43 nM, 28 nM, 12 nM, 6 nM and blank solutions yielded a linear relationship with the magnitude of the signals ($y = 0.8668x + 3.1742$; $R = 0.998$) and a statistically significant goodness of fit ($F(1, 3) = 39.3$, $p < 0.01$). The noise of the ACSF solution was very similar to the LoB, but the shape of the agmatine peak derivatized at 6 nM concentration in the ACSF solution exhibited band broadening due to fronting. As a consequence the peak height for the 6 nM solution in ACSF was significantly smaller than the peak height for the 6 nM solution in 20 mM carbonate buffer (7.08 ± 1.43 vs. 15.11 ± 2.78 (mean ± SD). A *t*-test showed a statistical difference at the level of $p < 0.0002$). The average travel time from the anodic end of the capillary to the window was 465 s and the average speed was 1 mm/s. Therefore the injection volume was 2.45×10^{-10} l. For the LoD this volume contained a mass of 0.5 attomole and for the LoQ a mass of 1.47 attomole.

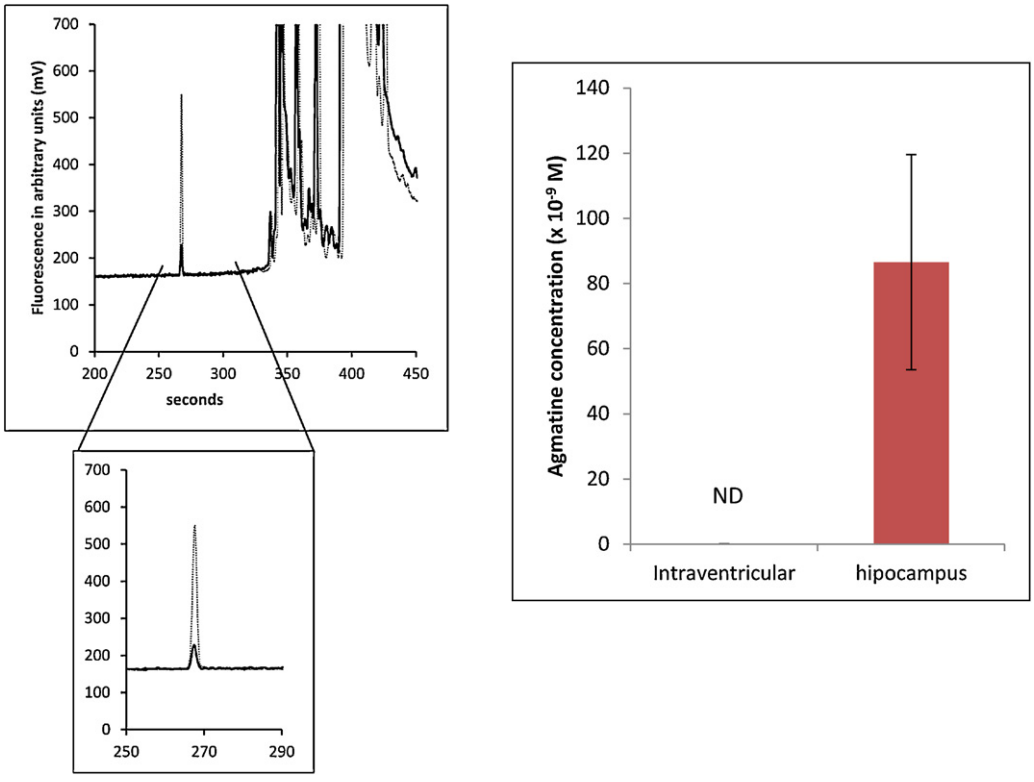

Fig. 3. Results of one of the spiking experiment can be seen in the electropherograms (left). The agmatine peak migrated before the other peaks of the sample (solid line electropherogram). After spiking (dashed line electropherogram) only the agmatine peak height increased. The bar graphic at the right shows the agmatine concentration in both the hippocampus and the lateral ventricle (mean ± SEM).

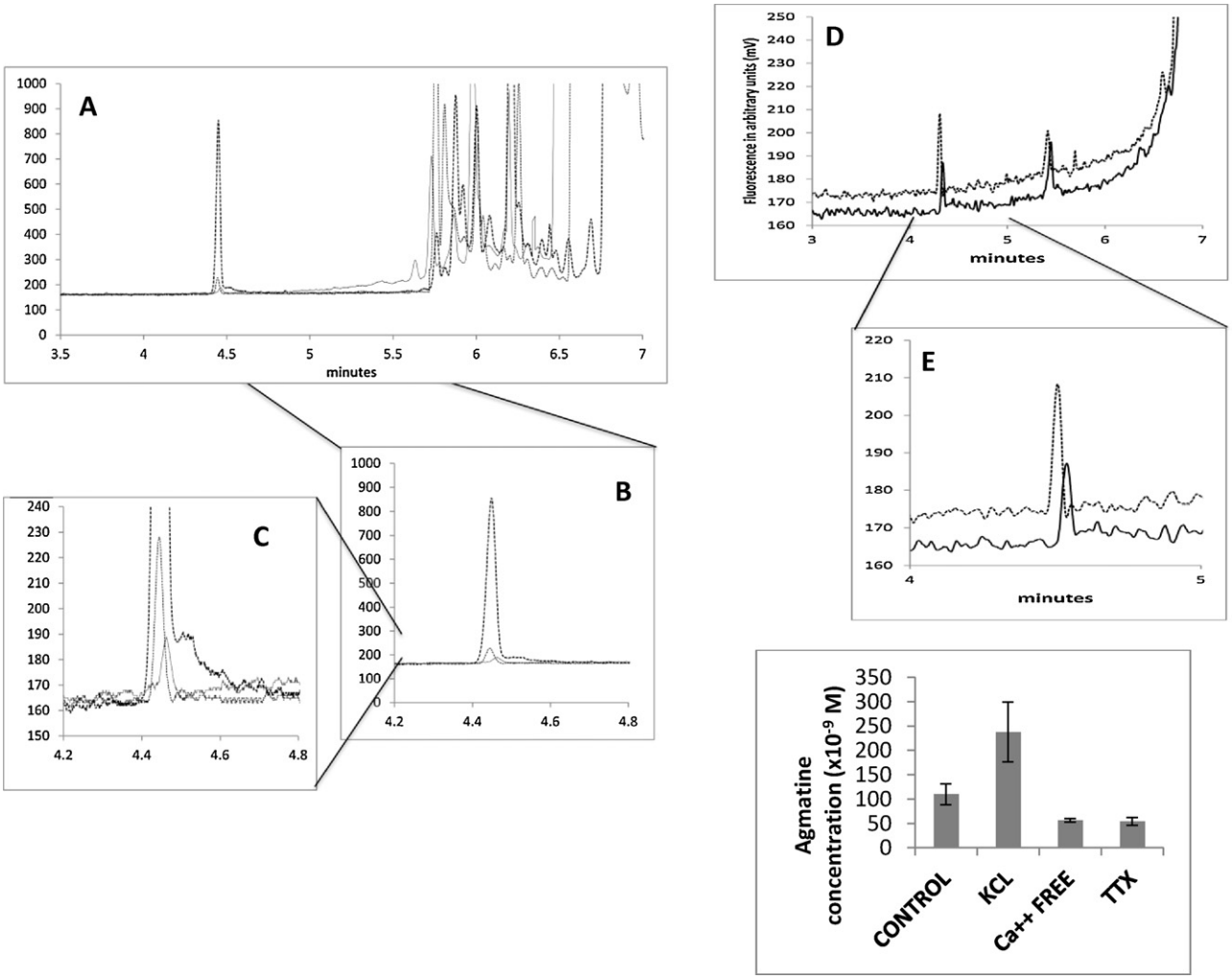

Fig. 4. Electropherograms showing that 100 mM KCl infusion by reverse microdialysis, increased extracellular agmatine (dashed line electropherogram showing the largest agmatine peak) respect to the baseline level (dashed line electropherogram with the short agmatine peak, A and B) and that perfusion with Ca^{2+}-free ACSF diminished extracellular agmatine (solid line electropherogram, C). 10 mM TTX in ACSF decreased agmatine in hippocampus dialysates (baseline, dashed line electropherogram; TTX, solid line electropherogram at the right; D and E). Bar graph showing the results of the three experiments to compare control, K^+ infusion by reverse microdialysis, perfusion with Ca^{2+}-free ACSF and TTX infusion by reverse microdialysis (bottom, right).

3.2. Optimal agmatine/FITC molar relationship

The result of the experiment is shown in Fig. 2. The amplitude of the agmatine signals fit logarithmic function. The curve reached an asymptotic level when there was an excess of 400 molecules of FITC respect to a molecule of agmatine.

3.3. In vitro recovery of the probes

The mean concentration of agmatine in the dialysates was 112 ± 13 nM and the concentration in the standard solution was 438 nM. The recovery calculated for the 6 probes was $23 \pm 3\%$.

3.4. In vivo recovery of the probes

The mean recovery for the 5 probes was $53 \pm 7\%$.

3.5. In vivo agmatine measurement

Eleven probes were localized in the hippocampus and three were localized in the lateral ventricles. As can be seen in Fig. 3, the concentration of agmatine in the dialysate obtained from the hippocampus probes ranged from 11 nM to 250 nM with a mean of 83 ± 26 nM (mean $\pm$ standard error of the mean). The figures of concentration range corrected for recovery of the probes yielded from 21 nM to 472 nM. Agmatine in the dialysates obtained from probes located in the lateral ventricles was undetectable. The spiking experiment showed that the mixture of sample with standard solutions had a larger agmatine peak.

3.6. Neuronal origin of agmatine

Fig. 4 (bottom right) shows the average concentration of agmatine in the nine control samples in the KCl depolarization, calcium-free ACSF perfusion and TTX infusion by reverse microdialysis experiments. The basal agmatine concentration of the nine rats was pooled and it was 109 ± 11 nM. Depolarization of neurons by reverse microdialysis infusion of KCl enhanced agmatine concentration to 238 ± 34 nM. Calcium-free ACSF decreased agmatine concentration to 56 ± 2 nM and TTX infusion decreased it to 49 ± 9 nM. The electropherograms at A, B, C, D and E show typical results.

4. Discussion

The present report shows that it is possible to measure extracellular agmatine in the brain by means of microdialysis and

CZE–LIFD. In the first experiment agmatine was detected in the nanomolar to the micromolar range (see Fig. 1). A linear correlation between agmatine concentration and magnitude of fluorescence signal was found. Since the lowest detectable concentration was 2 nM it was clear that submicromolar concentrations of agmatine in brain microdialysates were detectable by this method. The LoD of the method was 2 nM and the LoQ was 6 nM. These results suggest that the technique here reported is acceptable when compared with currently available analytical techniques to measure agmatine.

Microdialysis experiments showed that the basal concentration of agmatine in the dorsoventral hippocampus fits the range of concentration detection of CZE–LIFD. The *in vitro* recovery was about one half of the *in vivo* recovery. The *in vivo* recovery was about one half of the estimated extracellular concentration of agmatine. The reason for such discrepancy are that the *in vivo* recovery experiment was run at 37.5 °C and the *in vitro* recovery experiment was run at 23 °C (room temperature in Merida). The greatest temperature enhances the diffusion coefficient which makes more likely for the agmatine molecules to diffuse from the extracellular compartment through the dialysis membrane into the dialysate.

The agmatine contained in the dialysates probably was of neuronal origin. Depolarization of neurons with 100 mM KCl enhanced 218% agmatine in the extracellular fluid suggesting that stimulation of the hippocampus released agmatine. Perfusing the hippocampus with calcium-free ACSF decreased 51% agmatine concentration in the extracellular fluid suggesting that agmatine was released by a synaptic calcium dependent exocitotic process. Perfusing the hippocampus with 10 mM TTX in ACSF decreased 55% the concentration of agmatine suggesting that it is nerve impulse dependent and also pointing towards a neuronal origin.

Various techniques using CZE to monitor agmatine have been developed (see Table 1). One of them uses conductimetric detection with a 2 μM sensitivity limit [44]. Another technique is based on chemiluminescence detection and permitted agmatine detection in the 4.3 μM range of concentration detection and 42 picomole limit of mass detection [38]. Both techniques have the advantage of being fast and simple, however, their sensitivity is not enough to detect agmatine in brain microdialysates.

A method based on HPLC and laser induced fluorescence detection (HPLC-LIFD) was developed [45]. One disadvantage of HPLC techniques for brain micro dialysis analysis is that they require sample volumes of 20 μl or more. These large volumes are needed in order to increase the mass of analyte available to the detector and in that way to improve the limit of detection. However in the HPLC techniques, the mass of analyte becomes unacceptably small when the volume of sample to be analyzed is smaller than 1 μl. Nevertheless, with this technique, Zhao et al. obtained a 100 femtomole limit of mass detection and a 5 nM limit of detection [45]. This sensitivity might be useful for low time resolution brain microdialysis. Typical perfusion flow rate in brain microdialysis is 1 μl/min so, 20 min collection time is required to obtain enough sample volume containing detectable amounts of agmatine. The technique here reported (CZE–LIFD) has a 2 nM limit of detection and 6 nM limit of quantitation. The limit of mass detection was 0.5 attomole and the limit of mass quantitation was 1.47 attomole. This limit is three orders of magnitude smaller than the limit of mass quantitation of the CZE-chemiluminescence and five orders of magnitude smaller than the limit of mass quantitation of the HPLC-LIFD methods [38,45]. Perhaps the method that best fulfilled the requirements of sensitivity for *in vivo* measurement of agmatine in the brain is the capillary liquid chromatography with tandem mass spectrometry (CLC/MS) and CZE with Light Emitting Diode Induced Fluorescence (CZE-LEDIF) developed by Song et al. [46] and by Zhao et al. [47], respectively. In the first method (CLC/MS) the biogenic amines are separated by means of a (C8) 5 μm particle column and then injected into the mass spectrometer. The calibration curve was linear in the range of 15 ng/ml to 1 μg/ml and the limit of detection was estimated at 4.6 nM. Since the volume injected was 5 μl, the limit of mass detection might have been about 23 femtomole which is more than three orders of magnitude less sensitive than the CZE–LIFD technique that we report here. If this is correct, the CLC/MS technique does not have enough sensitivity to detect agmatine in brain dialysates. The authors of this paper reported that they were able to measure agmatine in brain homogenates. This is possible because agmatine in brain homogenates comes from both the intracellular pool and the extracellular pool. It is well known that intracellular agmatine can be anything between three and five orders of magnitude larger than the agmatine present in the extracellular fluid. In the second method (CZE-LEDIF) the authors report a LomD of 8.2 attomole. By contrast, the technique of CZE–LIFD that we report here has a LomD of 0.5 attomole which is 16 times higher making it better fit to monitor extracellular agmatine *in vivo* [47]. The differences might be due to the emission source (Laser vs. LED) the high magnification objective (60× vs. 40×) and the larger Numerical Aperture of the objective (85 NA vs. 60 NA) [41].

Due to its high positive charge the agmatine molecule migrates as the first peak in the electropherogram. This has a prominent technical advantage when FITC is used as derivatizing agent. This dye possesses a high quantum emission efficiency and good absorption at 490 nm that makes it ideal for the 488 nm line of the Argon ion laser. But the FITC molecule fragments itself in multiple fluorescent byproducts. Since agmatine migrates before the ghost peaks of the FITC products, it is easier to measure.

The estimated volume of injection is 0.245 nl since we used a 25 μm internal diameter capillary. Therefore, very small volumes of sample are required to monitor agmatine *in vivo* with the present combination of microdialysis and CZE–LIFD. Previously, we developed a technique to measure chemical compounds in a 30 nl dialysate samples [62] and the current technology that we utilized in this report shows that agmatine can be detected and measured in equivalent small volumes.

In conclusion, the present report shows the successful development of a technique to measure agmatine in brain dialysates collected from freely moving animals which will allow explore the physiological role of agmatine in the central nervous system.

Acknowledgement

This research was supported by Grants M-1000-10-03-B and M-967-080-7-F from the CDCHT.

References

[1] R.D. Slocum, H.E. Flores (Eds.), Biochemistry and Physiology of Polyamines in Plants, CRC Press, Boca Raton/Ann Arbor/London, 1991, p. 25.
[2] U.L. Bachrach, Y.M. Heimer, I. Pashev, J.S. Zlatanova, B. Bachrach (Eds.), The Physiology of Polyamines, CRC Press, Boca Raton/Ann Arbor/London, 1989, p. 93.
[3] C.A. Panagiotidis, S. Blackburn, K.B. Low, E.S. Canellakis, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 423.
[4] I. Hummel, I. Couee, A. El Amrani, J. Martin-Tanguy, F. Hennion, J. Exp. Bot. 53 (2002) 1463.
[5] K. Higashi, K. Yoshida, K. Nishimura, E. Momiyama, K. Kashiwagi, S. Matsufuji, A. Shirahata, K. Igarashi, J. Biochem. 136 (2004) 533.
[6] J. Satriano, S. Matsufuji, Y. Murakami, M.J. Lortie, D. Schwartz, C.J. Kelly, S. Hayashi, R.C. Blantz, J. Biol. Chem. 273 (1998) 15313.
[7] G.J. Molderings, B. Kribben, A. Heinen, D. Schroder, M. Bruss, M. Gothert, Cancer 101 (2004) 858.
[8] B. Kribben, J. Heller, J. Trebicka, T. Sauerbruch, M. Bruss, M. Gothert, G.J. Molderings, Naunyn. Schmiedebergs Arch. Pharmacol. 369 (2004) 160.
[9] G. Sturgill, P.N. Rather, Mol. Microbiol. 51 (2004) 437.
[10] W. Raash, S. Regunathan, G. Li, D. Reis, Life Sci. 56 (1995) 2319.
[11] G. Li, S. Regunathan, C.J. Barrow, J. Eshraghi, R. Cooper, D.J. Reis, Science 263 (1994) 966.
[12] D.J. Reis, S. Regunathan, Ann. N. Y. Acad. Sci. 881 (1999) 65.
[13] D.J. Reis, S. Regunathan, Trends Pharmacol. Sci. 21 (2000) 187.
[14] D.J. Reis, X.C. Yang, T.A. Milner, Neurosci. Lett. 250 (1998) 185.

[15] K. Otake, D.A. Ruggiero, S. Regunathan, H. Wang, T.A. Milner, D.J. Reis, Brain Res. 787 (1998) 1.
[16] C.A. Fairbanks, K.L. Schreiber, B.K.L. Brewer, Y.C. Gu, L.S. Stone, K.F. Kitto, H.O. Nguyen, B.M. Grocholski, D.W. Shoeman, L.J. Kehl, S. Regunathan, D.J. Reis, R.P. Yezierski, G.L. Wilcox, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10584.
[17] R.B. Su, J. Li, B.Y. Qin, Acta Pharmacol. Sin. 24 (2003) 631.
[18] R.B. Su, J. Li, X. Li, B.Y. Qin, Acta Pharmacol. Sin. 22 (2001) 639.
[19] X.C. Yang, D.J. Reis, J. Pharmacol. Exp. Ther. 288 (1999) 544.
[20] M. Kalloniatis, D. Sun, L. Foster, S. Haverkamp, H. Wassle, Vis. Neurosci. 21 (2004) 587.
[21] E. Galea, S. Regunathan, V. Eliopoulos, D.L. Feinstein, D.J. Reis, Biochem. J. 316 (1996) 247.
[22] N.##S.R. Wu, B. u, B. Xu, X.Q. Lu, Y. Liu, J.Q. Zheng, J.E. Piletz, J. Li, B.Y. Qin, Biochem. Pharmacol. 70 (2005) 1079.
[23] A. Halaris, J.E. Piletz, Ann. N. Y. Acad. Sci. 1009 (2003) 1.
[24] J.E. Piletz, D.N. Chikkala, P. Ernsberger, J. Pharmacol. Exp. Ther. 272 (1995) 581.
25 U. Schafer, C. Burgdorf, A. Engelhardt, W. Raasch, T. Kurz, G. Richardt, Ann. N.Y. Acad. Sci. 1009 (2003) 265.
[26] D. Pinthong, I.K. Wright, C. Hanmer, P. Millns, R. Mason, D.A. Kendall, V.G. Wilson, Naunyn. Schmiedebergs Arch. Pharmacol. 351 (1995) 10.
[27] B. Szabo, R. Urban, N. Limberger, K. Starke, Naunyn. Schmiedebergs Arch. Pharmacol. 351 (1995) 268.
[28] A.D. Zomkowski, A.R. Santos, A.L. Rodrigues, Neurosci. Lett. 381 (2005) 279.
[29] F. Aricioglu, H. Altunbas, Ann. N.Y. Acad. Sci. 1009 (2003) 136.
[30] J.E. Piletz, A. Halaris, P.R. Ernsberger, Crit. Rev. Neurobiol. 9 (1994) 29.
[31] Y. Feng, M.H. LeBlanc, S. Regunathan, Neurosci. Lett. 390 (2005) 129.
[32] K. Riazi, H. Honar, H. Homayoun, N. Rashidi, S. Kiani, M.R. Ebrahimkhani, A.R. Noorian, K. Ghaffari, A. Jannati, A.R. Dehpour, Epilepsy Res. 65 (2005) 33.
[33] R.B. Su, X.L. Wei, J.Q. Zheng, Y. Liu, X.Q. Lu, J. Li, Pharmacol. Biochem. Behav. 77 (2004) 345.
[34] W.P. Wang, A.H. Iyo, J. Miguel-Hidalgo, S. Regunathan, M.Y. Zhu, Brain Res. 1084 (2006) 210.
[35] J.H. Kim, M.A. Yenari, R.G. Giffard, S.W. Cho, K.A. Park, J.E. Lee, Exp. Neurol. 189 (2004) 122.
[36] G.M. Gilad, K. Salame, J.M. Rabey, V.H. Gilad, Life Sci. 58 (1996) 41.
[37] L. Hernandez, N. Joshi, E. Murzi, P. Verdeguer, J.C. Mifsud, N.A. Guzman, J. Liq. Chromatogr. A 652 (1993) 399.
[38] S. Zhao, C. Xie, X. Lu, Y.M. Liu, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 832 (2006) 52.
[39] D. Gründeman, C. Hahne, R. Berkels, E. Schömig, J. Pharmacol. Exp. Ther. 304 (2003) 810.
[40] Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline, CLSI, Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2004, EP17.
[41] L. Hernandez, B.G. Stanley, B.G. Hoebel, Life Sci. 39 (1986) 2629.
[42] P. Rada, S. Tucci, E. Murzi, L. Hernandez, Brain Res. 768 (1997) 338.
[43] L. Hernandez, S. Rossell, S. Tucci, D. Paredes, P. Rada, in: B.H.C. Westerink, T.I.F.H. Cremers (Eds.), Hand Book of Microdialysis, Academic Press, 2007, p. 267.
[44] F. Kvasnicka, M. Voldrich, J. Chromatogr. A 1103 (2006) 145.
[45] S. Zhao, Y. Feng, M.H. LeBlanc, J.E. Piletz, Y.-M. Liu, Anal. Chim. Acta 470 (2002) 155.
[46] Y. Song, Z. Quan, J.L. Evans, E.A. Byrd, Y.M. Liu, Rapid Commun. Mass Spectrom. 18 (2004) 989.
[47] S. Zhao, B. Wang, H. Yuan, D. Xiao, J. Chromatogr. A 1123 (2006) 138.
[48] M.C. Goldschmidt, B.M. Lockhart, Anal. Chem. 43 (1971) 1475.
[49] V.H. Cohn Jr., P.A. Shore, Anal. Biochem. 2 (1961) 237.
[50] J. Lapa-Guimaraes, J. Pickova, J. Chromatogr. A 1045 (2004) 223.
[51] T. Kawabata, H. Ohshima, T. Ishibashi, M. Matsui, T. Kitsuwa, J. Chromatogr. 140 (1977) 47.
[52] D. Stickle, A. Bohrer, R. Berger, J. Morrissey, S. Klahr, J. Turk, Anal. Biochem. 238 (1996) 129.
[53] C. Buteau, C.L. Duitschaever, G.C. Ashton, J. Chromatogr. 284 (1984) 201.
[54] N. Sayem-el-Daher, R.E. Simard, L. L'Heureux, A.G. Roberge, J. Chromatogr. 256 (1983) 313.
[55] M.L. Patchett, C.R. Monk, R.M. Daniel, H.W. Morgan, J. Chromatogr. 425 (1988) 269.
[56] K.J. Hunter, A.H. Fairlamb, Anal. Biochem. 190 (1990) 281.
[57] K. Kotzabasis, M.D. Christakis-Hampsas, K.A. Roubelakis-Angelakis, Anal. Biochem. 214 (1993) 484.
[58] M.T. Veciana-Nogues, T. Hernandez-Jover, A. Marine-Font, M.C. Vidal-Carou, J. AOAC Int. 78 (1995) 1045.
[59] Y. Feng, A.E. Halaris, J.E. Piletz, J. Chromatogr. B: Biomed. Sci. Appl. 691 (1997) 277.
[60] J. Kirschbaum, K. Rebscher, H. Bruckner, J. Chromatogr. A 881 (2000) 517.
[61] Y. Inaba, S. Tokishita, N. Hamada-Sato, T. Kobayashi, C. Imada, H. Yamagata, E. Watanabe, Biosens. Bioelectron. 20 (2004) 833.
[62] S. Rossell, L.E. Gonzalez, L. Hernandez, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 784 (2003) 385, 2149.
[63] M.L. Latorre-Moratalla, J. Bosch-Fusté, T. Lavizzari, S. Bover-Cid, M.T. Veciana-Nogués, M.C. Vidal-Carou, J. Chromatogr. A 1216 (2009) 7715.
[64] P. Lönnroth, P.A. Jansson, U. Smith, Am. J. Physiol. 2 (1987) 253.