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Short communication

An improved LC–S/MS method for the quantitation of adenosine concentration in mice brain microdialysates

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ABSTRACT

A sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of adenosine concentrations in mouse brain microdialysis samples was developed. High method sensitivity (LLOQ of 1.25 fmol) was achieved by on-line switching column. A C18 was employed as enrichment column and a cyano based (CN-SB) as analytical column. The method was fully validated for its sensitivity, selectivity, matrix effect and stability. It was successfully applied to measure quantitatively adenosine in brain of freely moving mice after different stimuli.

1. Introduction

Adenosine is an important neuromodulator that plays a crucial role in the central nervous system (CNS) pathophysiological processes like sleep, arousal, locomotion, neuroprotection and seizure susceptibility [1]. It is known that adenosine depresses excitability of CNS neurons and inhibits presinaptical release of various neurotransmitters [1]. Moreover, several studies show an increase of adenosine levels in different brain areas during ischemia and other metabolic insults [2–6]. Therefore, methods to detect and monitor adenosine brain levels are important tools in the ongoing understanding of adenosine functions. Since the extracellular concentration of adenosine measured by microdialysis technique is typically in the low nanomolar range, highly sensitive analytical methods are required to detect and quantify the adenosine extracellular brain levels. Notwithstanding different LC–MS/MS methods to evaluate adenosine in different biological matrix have been reported [7,8], at the best of our knowledge, only the LC–MS/MS method developed by Zhu et al. has been applied to measure endogenous adenosine in rat brain dialysates with a method sensitivity of 10 ng/mL (150 fmol) that was insufficient to evaluate low basal adenosine levels in discrete brain areas like cortex [9]. The aim of the present work is to develop a method to quantify low adenosine levels in brain microdialysates of freely moving mice under basal conditions and after different stimuli.

2. Materials and methods

2.1. Reagents and chemicals

Adenosine, 2-chloroadenosine, LC-grade water, acetonitrile, methanol, ammonium acetate, acetic acid and kainate were purchased from Sigma–Aldrich (Milan, Italy).

2.2. Sample preparation

Stock solution of standard adenosine was prepared in a concentration of 10 mM in water. A stock solution of an internal standard (IS) was prepared in a concentration of 1 mM in water. The stock solutions were stored at −80 °C. The samples were prepared by spiking 5 μl IS (50 nM) into 20 μl of dialysate.

2.3. Method validation

The analytical method was validated following the criteria suggested by the US Food and Drug Administration (FDA) bioanalytical method validation guidance [10] (see Supplementary Material).

The specificity of the method was established by comparing the chromatograms of artificial cerebrospinal fluid (aCSF) samples with

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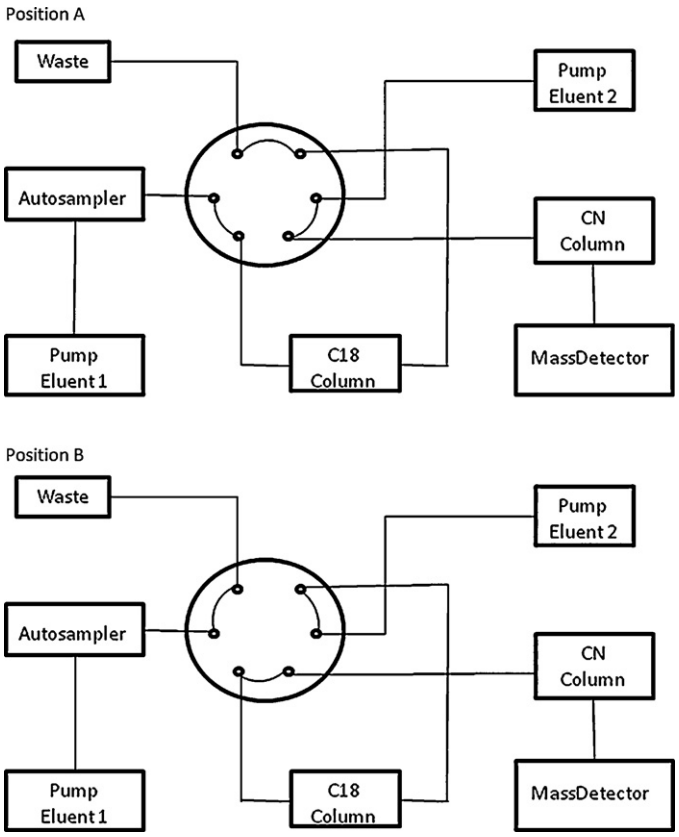

Fig. 1. Schematic representation of the column switching system.

those spiked with analytes to find out interference from endogenous components.

2.4. Liquid chromatography

The experiments were performed using an Agilent HP 1200 liquid chromatograph (Hewlett-Packard) consisting of a binary pump as pump 1, an autosampler and a thermostated column compartment. A Merck Hitachi L-6200A pump (Merck KGaA, Darmstadt, Germany), was employed as pump 2. A Zorbax SB-C18 column (30 mm × 2.1 mm, 3.5 μm) (Hewlett-Packard) was employed as enrichment column (eluent: 0.1% acetic acid and acetonitrile 98:2 (v/v), 0.4 mL/min). Chromatographic separations were carried out using a Zorbax SB-CN column (150 mm × 2.1 mm, 5 μm). The HPLC analyses were carried out isocratically using a mobile phase composed of 0.1% acetic acid and acetonitrile 90:10 (v/v). The flow rate was 0.2 mL/min. The injection volume was 5 μl. A Rheodyne 7000 valve was used to switch the mobile phase flow (Jasco Europe, Italy, Milan).

2.5. Mass spectrometry

An Agilent 6410 triple quadrupole-mass spectrometer with an electrospray ion source operated in positive mode was used for detection. Flow injection analysis was used to optimize the fragmentor and source parameters. The optimized source parameters for MS analysis were: drying gas temperature 350 °C and gas flow 10 L/min, nebulizer gas flow pressure 35 psi and capillary voltage 4500 V. The optimized fragmentor voltage values were 88 and 90 V for adenosine and 2-chloroadenosine, respectively. Subsequently, positive ion mass spectra for adenosine and 2-chloroadenosine were generated by flow injection analysis in full scan mode at the mass range *m/z* 50–350. In order to determine the characteristic mass fragments for selected reaction monitoring (SRM) analysis, the product spectra of adenosine and 2-chloroadenosine were recorded in full scan mode by varying the offset voltage between 5 and 20 eV. The optimal collision energy values were 12 and 16 eV for adenosine and 2-chloroadenosine, respectively. The quantitative analyses were carried out using selected reaction monitoring (SRM) following the reactions: *m/z* 268 → 136 for adenosine and *m/z* 302 → 170 for 2-chloroadenosine.

3. Results and discussion

3.1. Optimization of mass spectrometer conditions

Adenosine and 2-chloroadenosine (IS) were detected by LC–MS/MS with ESI source in positive ion mode. The protonated molecules [M+H$^+$], *m/z* 268 for adenosine and *m/z* 302 for 2-chloroadenosine, were selected as precursor ions. The abundances of precursor ions increase with the increasing of ESI capillary voltage reaching the highest value at 4500 V. Subsequently, MS/MS spectra in product ion mode of operation were acquired to obtain information on fragment ions. Collision-induced dissociation of molecular ions of adenosine and 2-chloroadenosine generates only two fragment ions for each analyte. Fragment ions for adenosine are *m/z* 136 [M-ribosyl+H$^+$] and 119 [M-ribosyl-NH$_3$+H$^+$], that correspond to adenine and to the deaminated base, respectively. Fragment ions for 2-chloroadenosine are *m/z* 170 [M-ribosyl+H$^+$] and 134 [M-ribosyl-HCl+H$^+$], that correspond to 2-chloroadenine and to dechlorinated base, respectively. SRM transitions *m/z* 268 → 136 for adenosine and *m/z* 302 → 170 for IS were used as quantifiers; and *m/z* 268 → 119 for adenosine and *m/z* 302 → 134 for IS were used as qualifiers.

3.2. Optimization of chromatographic conditions

Zhu et al. have reported a chromatographic method for detection of adenosine in rat brain microdialysate samples employing a SB-CN column and acetate buffer/methanol as mobile phase [9]. The sensitivity of the method (10 ng/mL) was insufficient to quantify the lower adenosine concentrations in mice brain microdialysate samples under basal conditions [9]. In a first attempt the Zhu et al. method sensitivity was improved employing a mobile phase 0.1% acetic acid and acetonitrile 90:10 (v/v) that led to an increase of adenosine ionization. Despite the positive effect obtained on ionization, the reached sensitivity was still not sufficient to quantify low adenosine levels in mice cerebral microdialysis samples.

Notwithstanding larger compounds (>6–20 kDa) such as proteins and enzymes in the microdialysate samples are physically removed by the membrane dialysis probe, quantitative analysis of endogenous compounds is often made difficult by matrix-induced interference due to co-elution of aCSF inorganic salts that can cause ionization suppression and foul the ESI-MS source. Hence matrix effect was investigated by post-column infusion of adenosine (100 nM) and IS (50 nM) together with the mobile phase or aCSF. The results obtained indicate the presence of matrix effects for adenosine and IS measurements in aCSF compared to mobile phase leading to a dramatic decrease of ionization. Therefore, to the aim to remove matrix effect and increase method sensitivity an on-line column switching HPLC method was developed (Fig. 1). While inorganic salts and polar endogenous compounds in microdialyis samples are discharged, adenosine was retained on the C18 enrichment column employing water:acetonitrile 98:2 (v/v) as mobile phase (Fig. 1a). The retained adenosine was transferred on SB-CN analytical column by backing flush of the C18 column using a mobile phase with a major percentage of acetonitrile (water:acetonitrile 90:10 (v/v)). Subsequently post-column