Rapid Measurement of Adenosine Concentration in Human Blood Using Fixed Potential Amperometry: Comparison with Mass Spectrometry and High-Performance Liquid Chromatography

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Abstract

Background: Adenosine is a nucleoside that impacts the cardiovascular system during cardiovascular or inflammatory diseases. The rapid determination of adenosine in blood may be useful in emergency medicine especially in syncope diagnose or septic shock. We compare its measurement in blood using fixed potential amperometry (FPA), with usual methods: mass spectrometry (LC-MS/MS) or high performance liquid chromatography (HPLC).

Methods: Twenty healthy subjects (14 men and 6 women) and ten patients suffering from vasovagal syncope (VVS, 6 women and 4 men) were included. Blood samples were collected by vein puncture for plasma adenosine assay and in the same time using finger puncture for direct FAP measurement and on blotting paper for LC-MS/MS.

Results: Mean plasma adenosine concentration was 26% higher using HPLC compared with LC-MSMS; p<0.01. In whole blood, adenosine concentration was 35% higher using FPA compared with LC-MS/MS. We found a good correlation between adenosine values measured by FAP and LC-MS/MS in whole blood and between LC-MS/MS and HPLC in plasma. Mean adenosine concentration was higher in patients whatever the method used.

Conclusion: Adenosine measurement to the patient’s bed, using FPA may be useful in some cases where high adenosine is associated with pejorative outcome.

Keywords: Adenosine; Adenosine plasma concentration; Cardiovascular diseases; Fixed potential amperometry; Mass spectrometry; High performance liquid chromatography

Introduction

Adenosine is an ATP derivative that is implicated in a lot of cardiovascular and neurological diseases including neurocardiogenic syncope [1-3], coronary artery diseases [4,5], neuropathic pain [6], septic shock [7] and severe systemic inflammatory response [8]. Thus, measuring adenosine in blood may be helpful for some pathologies. As example measuring adenosine in blood help to distinguish patient's vasovagal syncope (VVS) that have high adenosine plasma concentration (APC) from patients with sudden syncope that have low APC and more pejorative prognostic [2].

Due to its short half-life in the blood, the sample collection and measurement of adenosine is not easy. We have developed a method using a stop solution to inhibit adenosine degradation in body fluid. After plasma deproteinization, adenosine concentration was evaluated using high performance liquid chromatography [9-12]. Some teams have developed a tandem mass spectrometry method that require no stop solution for adenosine measurement in whole blood collected on blotting paper in the perspective of adenosine deaminase deficiency screening [13]. Finally, some have developed a specific fixed potential amperometry (FPA) method using specific electrode to measure adenosine in extracellular cortical slice in real time in the perspective of seizure studies [14].

The aim of this study was to measure adenosine concentration in blood using fixed potential amperometry (FAP) and to compare the results with those obtained with high performance liquid chromatography (HPLC) or Mass spectrometry (LC-MS/MS).

Materials and Methods

Blood samples

Blood samples were collected from 20 healthy subjects (14 men and 6 women, mean age 49 ± 12, recruited among the medical staff) and from 10 patients suffering from VVS (6 women and 4 men, mean age 36 ± 7 years). Their VVS state was well documented by previous head-up tilt test (a test that reproduce the symptomatology), [15] and were recruited in the department of cardiology of the Timone hospital. Patients were included to have a broad range of APC range, because VVS patients are known to have basal high APC [16]. Healthy subjects and patients gave their written inform consent for blood collection. Whole blood was collected through a cubital vein using special tubes (5 mL) under vacuum containing 2 mL of cold stop solution as previously described [11,12]. The stop solution was composed of dipyridamole 0.2 mmol/l, ethylene diamine tetracetic acid disodium (4 mmol/l), erythrosine 9-2- hydroxy-3-nonyl adenine (5 mmol/l), α,β- methyleadenosine S’ diphosphate (79 mmol/l); cofomycine 10 µg/mL, and heparin sulfate 1
IU/mL (SIGMA Aldrich). In the same time, whole blood was collected using finger puncture followed by deposit a drop of blood (20 µL) using finnpipette®, on a blotting paper (Whatman 903 protein saver cards™) and dried over night at room temperature to obtain dried blood spot (DBS).

High performance liquid chromatography

The method has been previously described [9-12]. Briefly, after collection, blood samples were put in ice then centrifuged (4°C, 1500 g), and deproteinized (perchloric acid, 5%, 0.25 ml/ml of plasma), again centrifuged (1500 g for 10 min) and the supernatant was pipetted off and stored at -80°C until analysis. Defreezed samples were analyzed by chromatography. A modular system with a diode array detector (Chrom System®, Germany) was used. Samples were dissolved in 1 ml of phosphate buffer and eluted with a methanol gradient (0 to 100%) and deproteinized (perchloric acid, 5%, 0.25 ml/ml of plasma), again centrifuged (1500 g for 10 min) and the supernatant was pipetted off and stored at -80°C until analysis. Defreezed samples were analyzed by chromatography. A modular system with a diode array detector (Chrom System®, Germany) was used. Samples were dissolved in 1 ml of phosphate buffer and eluted with a methanol gradient (0 to 35% in 60 min) on a Merck LiChrospher C18 column (Nottingham, UK). Adenosine was identified by its elution time and by spectrum (pic absorption 254 nm) and quantified by comparison of peak areas with those of known quantities of adenosine. The sensitivity threshold was 5 pmol/ml of plasma matrix. The intra- and inter-assay coefficients of variation ranged from 3% to 6%.

LC-MS/MS

Extraction

Plasma: Internal standard solution with 2-Chloroadenosine was prepared at a concentration of 300 nM in water. One hundred microliters of plasma sample were transferred into a microfuge tube. Each sample was spiked with 50 µl internal standard solution and 300 µl methanol and vortexed for 1 min. Samples were then centrifuged for 10 min at 13300 × g at 4°C. Supernatant was then evaporated to dryness at 60°C under nitrogen, 150 µl of 0.1% formic acid in water were added and quickly vortexed before transferring into an HPLC auto sampler vial.

Whole blood spot: Six millimeters of dried blood spot (DBS) were cut out followed by extraction with mixture consisting of methanol (400 µl) and internal standard (50 µl) in 2 ml microfuge tubes then mixed for 90 min at 45°C. After extraction, an aliquot of 350 µl were transferred into a new 2-ml safe-lock tube and evaporated to dryness at 60°C under nitrogen, 150 µl of 0.1% formic acid in water were added and quickly vortexed before transferring into an HPLC auto sampler vial.

LC-MS/MS assay: Samples were analyzed using a Shimadzu UFLC XR system consisting of two LC-20ADXR binary pumps, a DGU-20A5R vacuum degasser, and a CT0-20AC thermostated column oven and a SIL-20ACXR cooled auto sampler (Shimadzu, Marne la Vallée, France). The LC system was interfaced with an ABSciex 4500 triple quadrupole mass spectrometer (Les Ulis, France) operating with an electrospray ionization source (ESI) using nitrogen (purity: 99.99%). Ten microliters of the extracted sample were injected onto a 2.1 × 100 mm, 3 µm Atlantis' T3 column, Waters (Guyancourt, France). The starting mobile phase consisted of 3% methanol and 97% acidified water (0.1% formic acid) with a flow of 0.7 ml/min for 3.5 min. Then, the gradient of methanol was increased to 30% for 3 min. The column was re-equilibrated for 2 min to starting conditions.

Reagents: LC-MS grade methanol and water were purchased from VWR (Fontenay-sous-Bois, France). Formic acid, Adenosine and 2-Chloroadenosine were from Sigma Chemical (Saint-Quentin Fallavier, France). Whatman 903 protein saver cards™ for sample collection and preparation were acquired from GE Healthcare (Cardiff, UK).

Fixed potential amperometry: We used fixed potential amperometry (FPA) described by Van Gompel et al. [14], with some modifications. FPA uses carbon-based microelectrodes to detect the current associated with electroactive compounds. Adenosine measurement was performed with commercial Pinnacle Biosensor (Pinnacle Biosensors 7001, Sarissa-Biomedical Ltd, Coventry UK). Amperometry biosensors were stored at 4°C until their use. Rehydration of biosensors was performed using special phosphate buffer (NaCl 100 mM, MgCl2 1 mM, glycerol 2 mM, pH 7.4) for 10 min as suggested by Sarissa-Biomedical. Biosensors were used only once. Calibrations were performed in 50 ml of Phosphate Buffered Saline (PBS, pH 7.4) relative to the internal reference. The biosensors were then polarized to +500 mV and again allowed to asymptote for 10 minutes. Averaged response to 1 µM of analyte was used for pre-in vivo calibration. In vivo data were not used if the sensor showed no post-in vivo calibration responsivity. After calibration procedure, using 7 increasing concentration points (Figure 1) whole blood collected on the finger was mixed with buffer (v/v). Height of the pic was chosen for adenosine quantification (Figure 1). Sensitivity threshold was 0.1 µM and inter assay CV was <10% in the range of 0.1 to 10 µM.

Statistical analysis: Data were expressed as means and standard deviation or median and interquartile range. A variance analysis (ANOVA two ways analysis) was used for comparison between adenosine level as a function of method used. We used the Pearson’s correlation coefficient for correlation studies.

Results

In plasma, mean adenosine concentration was significantly higher using HPLC compared with LC-MS/MS: 0.96 ± 0.79 µM vs 0.76 ± 0.58 µM, mean +26%, p<0.01 (Figure 2). However, a good correlation was found between the results obtained with these two methods (Figure 3A). In whole blood, mean adenosine concentration was significantly higher using FPA compared with LC-MS/MS: 1.9 ± 2.5 µM vs 1.4 ± 1.5 µM, +35%, p=0.01 (Figure 2). As previously, a good correlation was found between the values obtained with these two methods (Figure 3B).

Adenosine concentration evaluated with LC-MS/MS was significantly higher in whole blood compared with plasma; whole blood vs plasma: 1.4 ± 1.5 µM vs 0.76 ± 0.58 µM, p<0.01 (Figure 2). A correlation was also found even less strong between whole blood using LC-/MSMS or FPA and HPLC. LC-MS/MS vs HPLC: r=0.65, p<0.01; FPA vs HPLC: r=0.57, p<0.01 (Figures 3C and 3D).
Mean adenosine concentration was significantly higher in VVS patients whatever the method and the medium (plasma or whole blood) used. In plasma: LC-MS/MS; Patients vs Controls: 1.32 ± 0.71 μM vs 0.48 ± 0.16 μM, mean +275%, p<0.001. HPLC; 1.7 ± 1 vs 0.57 ± 0.16 μM, +298%, p<0.001. In whole blood: MS/MS; 3.1 ± 1.7 vs 0.6 ± 0.24; +516%, p<0.001. FPA: 4.3 ± 3.3 vs 0.74 ± 0.45 μM, +610%, p<0.001.

Finally, FPA method gives the higher concentration in adenosine whatever the medium. We choose FPA rather than fast scan cyclic.
voltametry (FSCV) because FPA is able to distinguish between adenosine and hypoxanthine [14].

**Discussion**

The main result of this work is that amperometry (FPA) may be a quick and useful method to measure adenosine concentration in whole blood collected after finger puncture. Indeed, an acceptable correlation was found between results obtained with FPA compared with mass spectrometry or HPLC. The use of spotting paper for whole blood analysis, without stop solution does not alter the results with the condition of placing quickly the drop of blood on the blotter. Adenosine concentration was higher in whole blood than in plasma whatever the method used. This may be explained by a release of adenosine by red blood cells.

Adenosine in plasma was higher using HPLC compared with LC-MS/MS suggesting that mass spectrometry is more specific than HPLC which cannot separate coelution in spite of the use of diode array detector. The most sensitive method remains LC-MS/MS, while FPA was the fastest method, since adenosine concentration may be obtained in less than 10 minutes after blood collection. In this context, we do not evaluate APC using FAP because the goal of this method is to evaluate adenosine in whole blood so to save time.

We found a high adenosine concentration in blood of VVS patients whatever the method and the medium (plasma or whole blood). High APC was previously reported in VVS [3,16]. This high APC is responsible for the drop in blood pressure and loss of consciousness. Conversely, low adenosine is associated with sudden syncope [2-3]. This last kind of syncope has a more pejorative prognosis because lacking prodromes and because the cause of loss of consciousness is often atrio-ventricular block [3]. Because syncope accounts for more than 1% of admissions in emergency department, (HAS data: http) the use of a quick measurement of adenosine using FPA in an emergency department, may help to distinguish VVS from others kind of syncope.

Due to the small size of the apparatus which is easily transportable, quick adenosine measurement to the patient's bed in critical care unit, using FPA may be also useful in the case of septic shock [7] where high adenosine predict outcome or during systemic inflammation response syndrome where high adenosine level is associated with vasoplegia [8]. We concluded that adenosine measurement using FPA may be useful in some cases where high adenosine is associated with pejorative outcome.

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