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Development and application of a LC–MS/MS method to quantify basal adenosine concentration in human plasma from patients undergoing on-pump CABG surgery

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ABSTRACT

A sensitive and robust LC–MS/MS method was developed to quantify basal adenosine concentrations in human plasma of patients undergoing on-pump coronary artery bypass grafting (CABG) surgery. A strong cation exchange (SCX) monolithic cartridge was used to enrich analyte, improve robustness, and reduce biological complexity. A simple modifier-free mobile phase was employed to improve sensitivity and reproducibility. This method exhibits consistent precision and accuracy, and the RSDs or REs of all the intraday and interday determinations were within 10%. The calibration curve was linear across the examined dynamic range from 1 nM to 500 nM (r^2 = 0.996). LOD and LOQ were determined to be 0.257 nM and 0.857 nM respectively, while LLOQ was below 10 nM. This method was used to monitor changes of adenosine levels in patient plasma drawn intraoperatively during on-pump CABG surgery. The analysis of 84 patients revealed that the mean concentration of adenosine in coronary sinus plasma after cardiopulmonary bypass (CPB) is higher than that in coronary sinus before CPB (p = 0.0024; two-tailed t-test) and that in radial artery plasma after CPB (p = 0.0409; two-tailed t-test). These findings suggest that the equilibrium between adenosine production and elimination has favored the elevation of adenosine basal level during on-pump CABG surgery and the change is specific to heart tissues. Evaluation of adenosine with a sensitive and robust analytical method has important implications on providing consistent results and meaningful insights into adenosine regulation, as well as its steady state and sustained action on the heart. Relating patient characteristics or clinical outcomes with basal adenosine concentration can be used to optimize the CABG–CPB maneuver by regulating adenosine level via pharmacological intervention, and differentiating adenosine's contribution to cardioprotection from other modulatory factors.

1. Introduction

Coronary artery bypass surgery has been shown to benefit patients with severe ischemic heart disease, as evidenced by improved long-term survival rates and reduction in perioperative ischemic syndromes [1]. A requirement for surgery is a motionless surgical field achieved by isolating the heart from the systemic circulation using cardiopulmonary bypass and then induction of cardioplegia during elective global ischemia. Revascularization of the heart at the end of the procedure may be inadequate. Reperfusion may be paradoxically associated with injury that prevents functional recovery in the short or longer term.

Cardioprotection against ischemia reperfusion injury have been actively pursued in the field of cardiac research. Cardioprotection strategies have employed pharmacologic and physical means in an effort to maintain the cellular metabolic milieu, avoid depletion of cellular cardioprotective substrates or activate endogenous triggers of intracellular mediators of protection. Examination of adenosine and adenosine receptor pharmacology during myocardial ischemia-reperfusion *in vivo* and *in vitro* has attracted significant interest. Unfortunately, a major methodologic challenge is the delineation of its role, given its quick turnover in patient samples.

Sustained elevation of adenosine basal level might be an attractive alternative to overcome the practical challenges faced by clinicians. Although adenosine is rapidly produced and metabolized, it has been suggested that its regulatory effect is only activated when the buffering effect of red blood cells is overcome, and basal adenosine level is significantly increased [2]. Proof of concept studies in animals utilizing adenosine inhibitors suggests the

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utility of adenosine modulation to salvage myocardium and reduce myocardial stunning. The translation of such findings clinically has yet to be established and quantification of basal levels of adenosine and its role in cardioprotection yet to be identified [3–5].

High performance liquid chromatography (HPLC) and radioimmunoassay techniques to quantify low basal adenosine levels (80–100 nM) have proven to be insufficient and associated with limitations to use. Analytical methods based on separation are highly preferable and several separation methods have been developed for adenosine quantitation. They include capillary electrophoresis [6,7], high performance liquid chromatography with UV absorbance detection (HPLC–UV) [2,8–11], HPLC-fluorescence detection [12,13], liquid chromatography–mass spectrometry (LC–MS) [7,14,15], and liquid chromatography–tandem mass spectrometry (LC–MS/MS)[16–21].

LC–MS/MS is generally considered to be the method of choice in bioanalytical field, offering superior selectivity and sensitivity to detect low concentration analytes in complex matrices, such as blood, plasma and tissue extracts. LC–MS/MS methods have been published for measuring adenosine in microdialysate [16], mouse kidney perfusate [21], urine [19], and cell culture medium [20]. An exploratory method for the analysis of adenosine in plasma has been described [18]. A fully validated method for detecting basal adenosine level in human plasma with adequate sensitivity and robustness is still needed.

The goals of this study were: (1) to develop a more sensitive, selective, and robust LC–MS/MS method for adenosine determination in CABG patient plasma; (2) use the fully validated method to monitor the adenosine changes throughout the on-pump CABG surgery; and (3) describe potential clinical relevance of basal adenosine levels in cardioprotection.

2. Experimental

2.1. Instrument and data processing software

All experiments were conducted on a Varian 1200L LC–MS/MS system (Varian Inc. now a part of Agilent Technologies, Palo Alto, CA, USA) with a triple quadrupole detector. The solvent delivery system consists of two single-piston rapid-refill pumps (ProStar 210 Dynamax System). Varian Mass Spectrometry Workstation Version 6 was employed to control and monitor the LC–MS/MS instrument, as well as acquire and process the spectra and chromatography information. Excel and Prism were used for statistical analysis.

2.2. Chemicals, reagents and consumables

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was purchased from Cayman Chemical (Ann Arbor, MI, USA). ACS grade acetic acid was purchased from Fisher Scientific (Nepean, ON, Canada). Adenosine standard was purchased from Sigma–Aldrich (St Louis, MO, USA). $^{13}C_5$-adenosine standard (>98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Redistilled trichloroacetic acid was purchased from Sigma–Aldrich (St Louis, MO, USA). 3 mL SPEC SCX column with monolithic solid phase extraction disk (Cat. No. A5310420) were purchased from Varian Inc. (Palo Alto, CA, USA). Pooled human plasma was purchased commercially from Innovative Research (Novi, MI, USA).

2.3. Preparation of calibration standards

Concentrations of calibration working solutions were 5000, 1000, 500, 100, 50, and 10 nM. Concentration of internal standard working solution was 2000 nM. To construct a calibration curve, 50 μL of the calibration working standards and 50 μL of the internal standard stock solution were spiked into 400 μL of commercial pooled human plasma before sample exaction and LC–MS/MS analysis. Zero concentration samples and blank samples were also prepared for the calibration.

2.4. Patients and sampling

This investigation conforms to the principles outlined in the Declaration of Helsinki [22]. The surgery was performed according to previously described procedure [23]. Two blood samples of 5 mL each were withdrawn from the coronary sinus 10 min before aorta clamping (denoted as C2 blood sample) and 5 min after declamping (denoted as C3 blood sample). Radial artery blood samples were obtained at the same time points (denoted as A2 and A3 blood samples respectively). The blood samples were treated with potassium EDTA and centrifuged at 4000 rpm for 15 min at 4 °C. Plasma was then quickly separated from blood cells and stored in 1.25 mL aliquots at −80 °C for subsequent adenosine analysis.

2.5. Sample pre-purification

Each of the 400 μL patient plasma samples was spiked with 50 μL of 2000 nM internal standard (IS) working solution. A solid phase extraction procedure was used to process the human plasma samples including calibration samples and patient samples spiked with IS before they were submitted to LC–MS/MS analysis. To avoid enzymatic degradation of adenosine, 20% trichloroacetic acid was added to the plasma samples at 2:10 (v/v). The mixtures were vortexed and then centrifuged at 13,000 rpm for 15 min. Supernatants were aspirated with a pipette and loaded to pretreated SCX cartridge.

The SPE procedure using SCX cartridge is as follows. The cartridge was (1) rinsed with 250 μL of methanol under gravity for 1 min; (2) rinsed with 250 μL of 0.1 M acetic acid under gravity for 1 min; (3) loaded supernatant under positive air pressure; (4) washed with 0.1 M acetic acid/methanol (50:50) under positive air pressure for 1 min; and (5) eluted with 250 μL of methanol/concentrated ammonium hydroxide (98:2) under positive air pressure.

Eluted methanol solution was concentrated by SpeedVac and the residue was resuspended in 70 μL 2% acetonitrile aqueous solution and submitted to LC–MS/MS analysis.

2.6. Liquid chromatography

A modifier-free mobile phase strategy was used in liquid chromatography. Mobile phase A contained 100% ultrapure water while mobile phase B contained 100% acetonitrile. The total run time was 12.5 min including column equilibration time with 3 linear gradient components: from 0 to 9.5 min, 2–15%B; from 9.5 to 10 min, 15–2%B; from 10 to 12.5 min, 2%B, where %B is a volume percentage of total mobile phase. A Gemini-NX 3 mm C18 100 mm × 2.0 mm column from Phenomenex (Torrance, CA, USA) was applied in the chromatographic separation. A guard cartridge system from Phenomenex, SecurityGuard™, was installed to protect the analytical column at all times. For each run a 25 μL of processed sample was injected. The flow rate was set to 0.2 mL/min.

2.7. Tandem mass spectrometry

The sensitivity was tuned according to manufacturer's recommendations. We have used positive electrospray ionization (ESI) as LC–MS interface. The specific conditions after tuning were as follows: needle voltage 3750 V; shield 375 V; capillary 30 V; drying

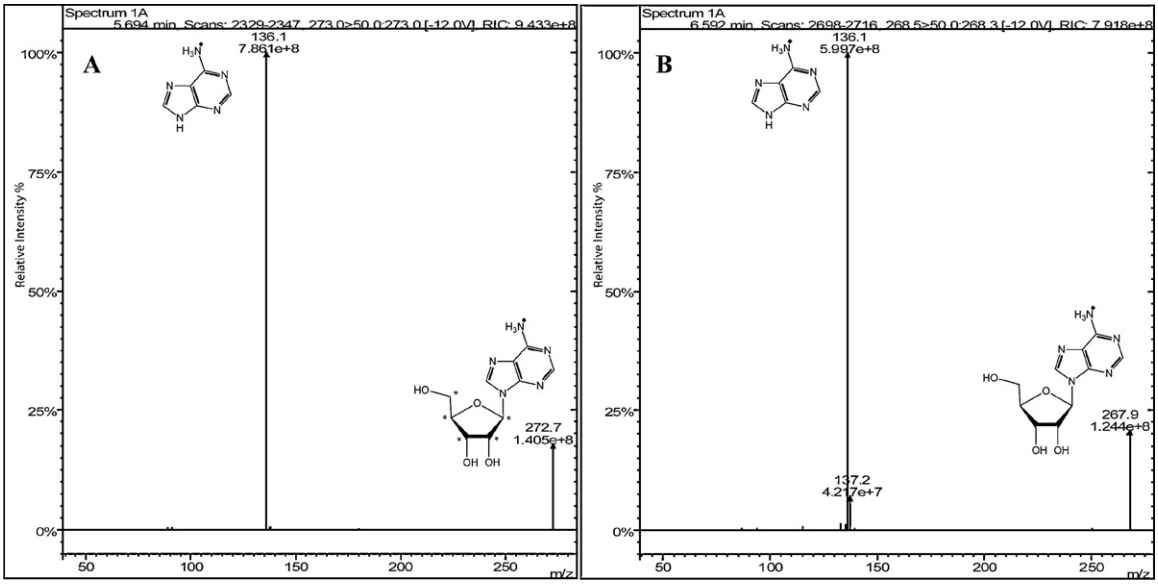

Fig. 1. Product ion spectra of $^{13}C_5$-adenosine (Panel A) and adenosine (Panel B). Base peaks were identified to be *m*/*z* 136.1 for both $^{13}C_5$-adenosine and adenosine. Asterisk (*) denotes the position of ^{13}C.

gas, nitrogen at 300 °C and 21 psi; neubulizer, nitrogen at 50 psi; electromultiplier 1700 V; collision energy −12.0 V; collision gas, ultrapure argon at 2.00 mTorr. Multiple reaction monitoring (MRM) was used to monitor the adenosine (*m*/*z* 268.0 to >136.1) and $^{13}C_5$-adenosine (*m*/*z* 273.0 to >136.1).

3. Results and discussion

A reliable and robust bioanalytical method requires full validation that addresses selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), lower limit of quantitation (LLOQ), and stability. The method development aspects for the quantitative analysis of adenosine in human plasma followed by its application to adenosine level determination for patient plasma are described below. Statistical analysis revealed significant changes of adenosine level during CABG surgery.

3.1. Optimization of mass spectrometer parameters

All MS parameters were adjusted to achieve optimal sensitivity according to manufacturer's recommendation. The optimal conditions are listed in Section 2.7. The product ion spectra of adenosine and $^{13}C_5$-adenosine were acquired through a product ion scan mode, where specific precursor ion (*m*/*z* at 268.0 or 273.0) was transmitted through the first quadrupole and a mass range was swept (*m*/*z* from 50.0 to 380.0). Major product ion peaks after gas collision by ultrapure argon at 2.00 mTorr for adenosine and internal standard were both at *m*/*z* 136.1, corresponding to a purine moiety of adenosine (Fig. 1).

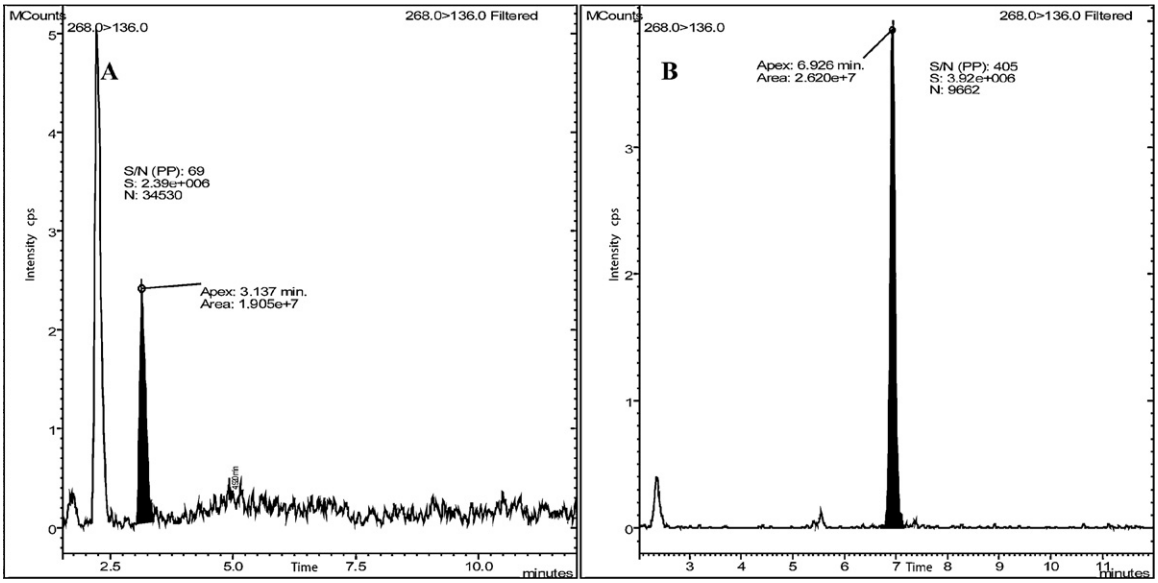

Fig. 2. Mobile phase with 0.1% formic acid versus modifier-free chromatograms of adenosine SPE extract. Chromatography condition: from 0 to 9.5 min, 2–15%B; from 9.5 to 10 min, 15–2%B; from 10 to 12.5 min, 2%B. A Gemini-NX 3 mm C18 100 mm × 2.0 mm column from Phenomenex (Torrance, CA, USA) was applied in the chromatographic separation. For Panel A, mobile phase A contains 0.1% formic acid in water; mobile phase B contains 0.1% formic acid in acetonitrile. For Panel B, mobile phase A contains ultra pure water; mobile phase B contains HPLC grade acetonitrile.

3.2. Modifier free mobile phase

Since the amino group on the purine ring of adenosine is easily protonated under acidic conditions, which is beneficial for electrospray ionization and subsequent MS/MS analysis, an acidic modifier was added to the mobile phase in the hope of achieving higher signal intensity and hence sensitivity. However, as shown in Fig. 2, the addition of 0.1% acid modifier (e.g. formic acid) did not provide a significant increase in sensitivity but led to shorter retention time of the analyte and higher background level compared to the modifier free mobile phase.

Adenosine is a relatively polar molecule, given that it bears three hydroxyl groups and one aromatic amine. Under acidic conditions, more amine groups of adenosine are protonated, making the analyte and nonpolar reverse stationary phase interact to a lesser degree. Although we have adapted a very low elution gradient, adenosine still comes out the column rather quickly (retention time around 3 min). At this retention time, three factors may come into play to cause reduced signal-to-noise ratio: ionization suppression, low organic content, and high noise level from other polar compounds. Plasma samples contain numerous polar organic metabolites and inorganic salts. During the first few minutes of reverse phase elution, these polar contaminants could foul the mass spectrometer's heated capillary, and cause severe ionization suppression. Matrix effects incurred by these endogenous substances are also a concern for the accuracy and reproducibility for LC–MS/MS methods, particularly rapid high-throughput analysis [24]. Organic content is known to significantly influence the ionization and higher organic content is usually preferred [25]. In this scenario, at an already low gradient, eluent with a lower retention time is accompanied with a low organic content at the point of analyte ionization, resulting in a low sensitivity. Lastly, the presence of an acid modifier increases the ionization efficiency of impurities, causing a higher background signal, as seen in Fig. 2 Panel A.

The modifier-free mobile phase provided superior performance compared to with modifiers, including acetic acid, ammonium acetate, and ammonium formate. On average, a 5–10-fold improvement in S/N ratio was observed for the modifier-free mobile phase system compared to those modified with an acid or acidic salt when analyzing SPE extracts spiked with equal amounts of adenosine. Despite lacking buffer capacity, modifier free mobile phase does not cause peak deterioration in essentially all the performed analyses. Retention times were consistent across the tested samples from various sources (RSD% < 1%, $n = 15$). An additional benefit for modifier-free mobile phase system is that it does not require any work associated with mobile phase preparation. Representative chromatograms of adenosine and $^{13}C_5$-adenosine are shown in Fig. 3.

3.3. Solid phase exaction

Although solid phase exaction is not always required prior to quantitative analysis of adenosine by LC–MS/MS [20], bypassing this step in our experience caused a significant increase in column pressure, leading to deviations of retention times. This could be a consequence of particle accumulation on the column head if no preventive extraction steps were used. In order to prevent irreversible damage to the expensive analytical column, and to keep retention times constant, we incorporated solid phase extraction into the protocol. Furthermore, unprocessed sample gave lower signal intensity than processed sample (Fig. 4). The difference in retention times between Panels A and B in Fig. 4 is possibly caused by different acid content in the suspension buffer. The sample without SPE contains high percentage of trichloroacetic acid while the sample with SPE contains no acid. Trichloroacetic acid drops the pH considerably so that the retention of adenosine in the beginning of chromatography by C18 column is predicted to be poor. Thus we concluded that it is important to carry out sample pre-treatment before the analysis for complicated matrices such as plasma.

Varian SPEC SCX monolithic disk cartridges were chosen for adenosine analysis because they have uniform flow properties, low bed mass, high mass transfer efficiency, and small processing volume. Their uniform flow properties allow viscous, particle-laden samples such as plasma to be processed quickly without clogging

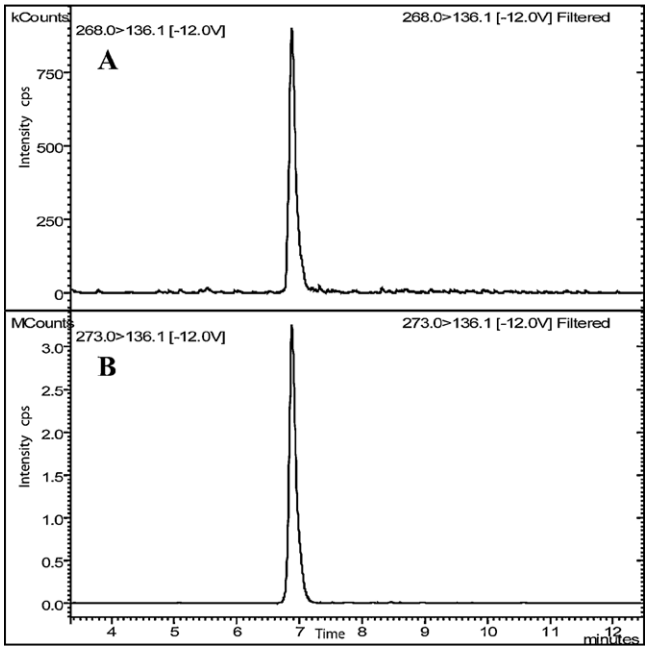

Fig. 3. A representative chromatogram of a patient sample spiked with 200 nM internal standard. Panel A chromatogram, the SRM m/z 268.0 to >136.1, represents endogenous adenosine from patient plasma sample; Panel B chromatogram, the SRM m/z 273.0 to >136.1, represents the spiked $^{13}C_5$-adenosine. The basal adenosine level in this patient is calculated to be 44.8 nM.

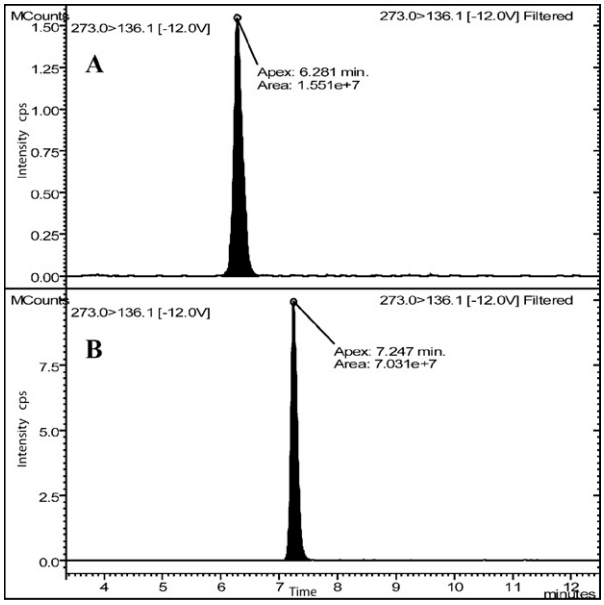

Fig. 4. The comparison of chromatograms of plasma samples spiked with same amount of IS before SPE (Panel A) and after SPE (Panel B). Chromatography condition: from 0 to 9.5 min, 2–15%B; from 9.5 to 10 min, 15–2%B; from 10 to 12.5 min, 2%B. Mobile phase A contains ultra pure water; mobile phase B contains HPLC grade acetonitrile.

the disk. Low bed mass resulted in less retention of interfering particles or contaminants, hence a cleaner sample. High mass transfer efficiency allows faster processing time with this type of SPE. Finally, only 250 μL of solvent in each individual step is needed to run the SPE due to its small processing volume, saving time, solvent and labor for sample pre-purification. After monolithic disk purification and further centrifuge sedimentation as described in the method in Section 2.5, the samples submitted to LC–MS/MS did not cause any build-up of backpressure or shift of retention time after repeated continuous runs ($n > 80$). The absolute recovery of adenosine off the SCX disc is 84.5 ± 1.56% (mean ± SD; $n = 12$).

3.4. Selectivity

We assigned the adenosine and $^{13}C_5$-adenosine peaks and matched them with their specific retention times by spiking either adenosine or $^{13}C_5$-adenosine reference substances into the human plasma matrix. Isotopic internal standard is always the analytical choice in terms of its ability to improve repeatability and accuracy, because the peak area ratio of adenosine and $^{13}C_5$-adenosine is independent upon differential ion suppression and variation of sample extraction. Furthermore, co-elution of adenosine along with its isotopic internal standard facilitates unambiguous peak identification and integration. Cross-talk issue was examined to avoid selectivity problem [24]. For standard aqueous samples, no appreciable peak in ion channel m/z 273.0 to >136.1 (for $^{13}C_5$-adenosine) was observed at the retention time of $^{13}C_5$-adenosine (~7.0 min) if only adenosine is spiked and vice versa.

3.5. Linearity

The basal adenosine concentration range was estimated to be between 20 nM and 200 nM in human plasma according to literature reports [2,18,26]. The linearity of the current method was assessed using the peak area of standard adenosine solutions between 1 nM and 500 nM relative to that of 200 nM internal standard. This ratio of peak areas of adenosine over that of its internal standard was plotted against the ratio of spiked adenosine concentrations over spiked internal standard concentration. This calibration curve has a goodness of fit of $r^2 = 0.996$ across the tested range, with the equation:

$$y = 1.1513x + 0.1399.$$

3.6. Precision

Precision was assessed by spiking three clinically relevant concentrations of adenosine (10, 50, and 500 nM) into the commercial human plasma, followed by trichloroacetic acid treatment, SCX cartridge extraction, and LC–MS/MS analysis. Intraday precision was determined by using six individually prepared plasma samples spiked with known amounts of adenosine and internal standard during the same day. Interday precision was determined by using eight individually prepared spiked plasma samples during 4 days in duplicates. Our determined values conform to the FDA requirements for bioanalytical method validation, with percentage of RSD below 6.33% in all cases (Table 1).

3.7. Accuracy

The accuracy of an analytical method is defined to be the closeness of mean test results determined by the analytical method to the true concentration of the analyte. The accuracy of this method was assessed by testing three concentrations in the range of expected adenosine basal concentration and using six to eight samples per concentration. Our calculated concentrations are reasonably close to the known concentrations, with relative error or inaccuracy no greater than 10% in all cases (Table 2). It is seemingly unexpected that at 500 nM level the calculated value deviates from the true value more than those at 50 nM or 10 nM level. However, this may be explained by the fact that the mass spectrometry response is non-linear at high concentrations. This is also known as high concentration related analyte "saturation" phenomena [27,28].

Table 1
Intraday and interday precision parameters of the described method.

	Conc. (nM)	Area ratio average	Std deviation	RSD (%)	No. of samples
Intraday	500	2.76	0.17	6.33	6
	50	0.44	0.02	3.80	6
	10	0.20	0.01	3.33	6
Interday	500	2.76	0.14	4.92	8
	50	0.45	0.03	6.00	8
	10	0.20	0.01	3.78	8

All the samples were prepared by individually spiking standard solution and internal standard into commercial human plasma followed by acidic protein precipitation, solid phase extraction and LC–MS/MS analysis. Basal adenosine concentration in the commercial plasma is calculated to be 30.39 ± 2.72 nM.

3.8. LOD, LOQ and LLOQ

The LOD and LOQ are defined as the concentration at which the signal is 3 times and 10 times the standard deviation of the background, respectively. We determined the LOD and LOQ were 0.257 nM and 0.857 nM, respectively, for this method. Our method is approximately 100–400-fold more sensitive than LC–MS methods previously developed in human synovial fluid [15] or in the venoms from viperinae snakes [7], and 6 fold more sensitive than LC–MS/MS method [29] developed in Cordyceps sinensis, based on this estimate of the LOD.

LLOQ is defined to be a concentration at which the analyte peak is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. Experimentally, we determined that the LLOQ was below 10 nM since 10 nM was tested to be acceptable with precision and accuracy (<15%). This is at least 6 times lower than what is a recently reported LC–MS/MS method [20] developed in serum-free cell culture medium.

3.9. Stability

The stability of adenosine under conventional storage conditions was assessed by subjecting the spiked samples to three freeze–thaw cycles as well as 24 h room temperature storage. Six high concentration samples and six low concentration samples

Table 2
Intraday and interday accuracy parameters of the described method.

	True conc. (nM)	Calculated conc. (nM)	Relative error (%)	No. of samples
Intraday	500	454.70	−9.06	6
	50	53.05	6.10	6
	10	10.34	3.43	6
Interday	500	455.06	−8.99	8
	50	51.57	3.15	8
	10	10.93	9.29	8

Calculated concentration is derived by using: [cal. conc.] (nM) = (average area ratio − a)/b × [spiked internal standard], where a is the y-intercept and b is the slope of the calibration curve. Relative error is derived by using: relative error (%) = ([cal. conc.] − [true conc.])/[true conc.] × 100. Basal adenosine concentration in the commercial plasma is calculated to be 30.39 ± 2.72 nM.

were prepared in parallel. To check the freeze–thaw stability, one of the three samples of each group was analyzed immediately, freeze-thawed once, or freeze-thawed three times. To check the room temperature storage stability, one of the remaining three samples of each group was analyzed immediately, 8 h later, or 24 h later. The RSD percentages of the freeze–thaw series were 4.94 and 9.31 for high and low concentrations respectively, while the RSD percentages of the prolonged room temperature storage series were 2.55 and 6.57 for high and low concentrations, respectively. Overall, no obvious changes were observed for area ratio of adenosine/IS under freeze–thaw test and prolonged time test.

3.10. Adenosine basal level in A2, C2, C3 and A3 samples

We used this LC–MS/MS method to analyze the concentration of adenosine in the plasma of 84 patients receiving either propofol or isoflurane during cardiopulmonary bypass. The mean plasma concentration of adenosine in coronary sinus blood prior to and following CPB was 36.02 ± 2.02 nM vs. 45.92 ± 2.49 nM (mean value ± SEM) respectively (two-tailed Student's *t*-test; $p = 0.0024$), as shown in Fig. 5.

Due to adenosine's ultrafast turnover in human blood [2], stopping solution was often immediately added into blood sample at collection [3,30–32]. However, even with stopping solution to trap adenosine instantly, Becker et al. still could not detect any change of adenosine in coronary sinus or arterial blood ($n = 11$) after CABG surgery [32]. In contrast, Kerbaul et al. noticed a 50% increase in the concentration of adenosine following CABG–CPB compared to at the beginning of this surgery ($n = 30$) [33]. This disagreement reflected considerable variations in patient profiles, analytical methods, sample number and sampling techniques. Our results from testing relatively large number of samples are in agreement with Kerbaul's findings.

At the point of aorta declamping, the average coronary adenosine level is also significantly higher than the average radial artery adenosine level (two-tailed Student's *t*-test; $p = 0.0409$), suggesting adenosine changes are cardiogenic. Our results are consistent with Kerbaul's findings [33] that upon CABG–CPB, plasma adenosine level is higher in coronary sinus than in radial artery.

Adenosine production and release have been associated with ischemia following coronary occlusion or stenosis [3,34]. Increased plasma adenosine levels in mixed venous blood have also been observed during CABG–CPB surgery [30]. The inverse proportional relationship between oxygen supply and adenosine production [3] might be one of the reasons accounting for the increased coronary sinus adenosine level. Other factors such as elimination rate can also contribute to an altered adenosine balance equation. Although large amounts of adenosine output may not be detected using our method because of adenosine's ultrafast turnover, this sensitive LC–MS/MS method has allowed us to see small but significant changes of adenosine basal level.

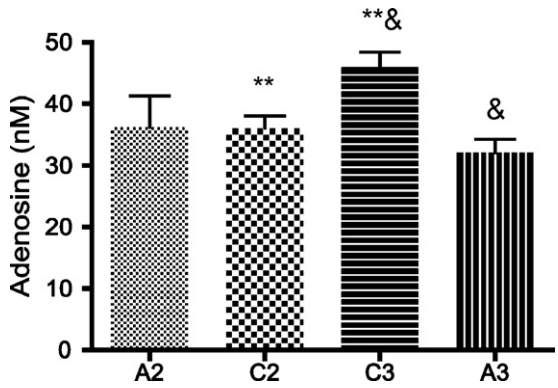

Fig. 5. The comparison of adenosine levels in A2, C2, C3 and A3 plasma samples. C2 and C3 plasma samples were obtained from the coronary sinus 10 min before aorta clamping and 5 min after aorta declamping respectively. A2 and A3 samples drawn from radial artery were obtained at the same time points. Asterisk (**) denotes statistical significance between C2 and C3 ($p = 0.0024$; two-tailed *t*-test). Dollar sign $ denotes statistical significance between C3 and A3 ($p = 0.0409$; two-tailed *t*-test).

3.11. Sensitive measurement of adenosine basal level without stopping solution

The detection limit of most HPLC methods is at best approximately 20 nM if using 2 mL of plasma sample. These methods also require affinity gel pre-purification involving tedious washing steps, identification of the adenosine peak by adding deaminase, and sometimes two columns in tandem for cleaner separation [10,31,35]. Adenosine basal levels ranging from 130 to 360 nM in human plasma have been reported in literature [36]. Here we observed a lower basal adenosine level than what has been seen with HPLC–UV methods. However, more sensitive methods such as LC–MS/MS [18] or radioimmunoassay [26] give similar ranges to this method. The discrepancy can be explained by the fact that HPLC–UV methods are usually not very selective and co-eluting interfering substance might have contributed to a higher baseline [31].

It has been shown that the change of basal adenosine level at steady state was minimal even when incubating plasma sample at 37 °C for 1 h and plasma adenosine in freshly drawn human blood (~80–100 nM) was at a similar level as adenosine sample collected in dipyridamole stopping solution (72 nM). The *in vitro* and *in vivo* measurements agreed with each other because the formation of adenosine is mostly from extracellular AMP degradation and adenosine elimination is mostly through red blood cell uptake, which might reach the equilibrium extremely fast [2]. In the same paper, the half-life of radiolabeled adenosine was about 1.5 s and this fast kinetics has made stopping solution a popular choice when adenosine release needs to be instantly captured to prevent elimination. However, there are three limitations to studying kinetic changes of adenosine: (1) researchers have used variety of cocktails [3,30–32] to inhibit either red blood cell uptake or deaminase degradation of adenosine so that the results are difficult to compare; (2) the ultrafast kinetic change of adenosine makes sampling and timing extremely challenging [30–32]; (3) the presence of a short adenosine spike after ischemia condition might not be pharmacologically meaningful. In contrast, determination of the sustained elevation or depression of adenosine basal level by sensitive LC–MS/MS methods may provide consistent results and meaningful insights regarding adenosine steady state, regulation and sustained action on heart.

Furthermore, the application of LC–MS/MS to monitor adenosine basal level may find wide spread applications in relating patient characteristics or clinical outcome with basal adenosine concentration, optimizing the CABG–CPB maneuver by regulating adenosine level via pharmacological intervention, and differentiating adenosine's contribution to cardioprotection from those of other modulatory factors. For example, quantitation of basal adenosine level can give accurate feedback to surgeons in cardioprotective dosing finding studies of either adenosine [36–42] or adenosine modulators [4,5,17,43,44], as well as help explain clinical outcome with other cardioprotective measures.

4. Conclusion

In conclusion, we have developed a simple, sensitive, selective, and robust LC–MS/MS method for the determination of basal adenosine level in patients undergoing on-pump CABG surgery.

Due to its high sensitivity, the method may be also applied to detect trace adenosine in other biological fluids.

Without using stopping solution, this method might be useful to resolve the conflicting findings around adenosine changes during on-pump CABG surgery. The increase of basal adenosine level in coronary sinus and different basal adenosine levels detected between the coronary sinus and radial artery plasma at reperfusion imply that the equilibrium of adenosine production and elimination has been altered in the heart upon CPB. These global changes can further help to understand the sustained action of adenosine on the heart and delineate cardioprotective strategies via adenosine modulation against ischemia-reperfusion injury.

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References

[1] C. Rihal, D. Raco, B. Gersh, S. Yusuf, Circulation 108 (2003) 2439.
[2] G. Möser, J. Schrader, A. Deussen, Am. J. Physiol. 256 (1989) C799.
[3] H. Bardenheuer, A. Fabry, B. Höfling, K. Peter, Cardiovasc. Res. 28 (1994) 656.
[4] K. Teoh, R. Weisel, J. Ivanov, S. Teasdale, M. Glynn, Thromb. Res. 57 (1990) 91.
[5] L. Cortigiani, M. Baroni, E. Picano, C. Palmieri, A. Boni, M. Ravani, A. Biagini, E. Nannini, Am. Heart J. 136 (1998) 37.
[6] Y. Jiang, Y. Ma, Anal. Chem. 81 (2009) 6474.
[7] R. Graham, S. McClean, E. O'Kane, D. Theakston, C. Shaw, Biochem. Biophys. Res. Commun. 333 (2005) 88.
[8] R. Gayden, B. Watts, R. Beach, C. Bendict, J. Chromatogr. 536 (1991) 265.
[9] D. Mei, G. Gross, K. Nithipatikom, Anal. Biochem. 238 (1996) 34.
[10] T. Yamamoto, Y. Moriwaki, S. Takahashi, T. Fujita, Z. Tsutsumi, J. Yamakita, K. Shimizu, M. Shioda, S. Ohta, K. Higashino, J. Chromatogr. B 719 (1998) 55.
[11] E. Sottofattori, M. Anzaldi, L. Ottonello, J. Pharmaceut. Biomed. 24 (2001) 1143.
[12] W. Wojcik, N. Neff, J. Neurochem. 39 (1982) 280.
[13] M. Pazzagli, C. Corsi, S. Latini, F. Pedata, G. Pepeu, Eur. J. Pharmacol. 254 (1994) 277.
[14] E. Dudley, F. Lemiere, W. Van Dongen, R. Tuytten, S. El-Sharkawi, A. Brenton, E. Esmans, R. Newton, Rapid Commun. Mass Spectrom. 18 (2004) 2730.
[15] L. Huang, F. Guo, Y. Liang, B. Li, B. Cheng, J. Pharmaceut. Biomed. 36 (2004) 877.
[16] Y. Zhu, P. Wong, Q. Zhou, H. Sotoyama, P. Kissinger, J. Pharmaceut. Biomed. 26 (2001) 967.
[17] T. Dorheim, A. Hoffman, D. Vanwylen, R. Mentzer, Surgery 110 (1991) 136.
[18] P. Dolezalova, J. Krijt, J. Chladek, D. Nemcova, J. Hoza, Rheumatology 44 (2005) 74.
[19] W. Hsu, W. Chen, W. Lin, F. Tsai, Y. Tsai, C. Lin, W. Lo, L. Jeng, C. Lai, Clin. Chim. Acta 402 (2009) 31.
[20] A. Van Dycke, A. Verstraete, K. Pil, R. Raedt, K. Vonck, D. Boison, P. Boon, J. Chromatogr. B 878 (2010) 1493.
[21] J. Ren, Z. Mi, E. Jackson, J. Pharmacol. Exp. Ther. 325 (2008) 920.
[22] Br. Med. J. 2 (1964) 177.
[23] D. Ansley, K. Raedschelders, D. Chen, P. Choi, Contemp. Clin. Trials 30 (2009) 380.
[24] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[25] N. Weng, J. Chromatogr. B 796 (2003) 209.
[26] C. Hamm, W. Kupper, R. Bredehorst, H. Hilz, W. Bleifeld, Cardiovasc. Res. 22 (1988) 236.
[27] K. Tang, J. Page, R. Smith, J. Am. Soc. Mass Spectrom. 15 (2004) 1416.
[28] C. Enke, Anal. Chem. 69 (1997) 4885.
[29] Z. Yang, S. Chi, C. Zhang, A. Wu, Zhongguo Zhong Yao Za Zhi 32 (2007) 2018.
[30] R. Mueller, L. Saltzman, M. Isley, R. Hunt, E. Norfleet, J. Cardiothor. Vasc. Anesth. 5 (1991) 444.
[31] M. Feldman, C. Ayers, M. Lehman, H. Taylor, V. Gordon, P. Sabia, D. Ras, T. Skalak, J. Linden, Clin. Chem. 38 (1992) 256.
[32] B. Becker, S. Zahler, T. Freyholdt, P. Massoudy, Drug Dev. Res. 45 (1998) 159.
[33] F. Kerbaul, F. Collard, F. Benard, Y. By, F. Paganelli, A. Saadjian, J. Ruf, P. Lejeune, B. Mallet, L. Carrega, I. Zouher, C. Monserrat, J. Bonnet, R. Guieu, J. Invest. Med. 55 (2007) 195.
[34] F. Paganelli, A. Saadjian, J. Sampol, J. Maixent, S. Levy, R. Guieu, Eur. J. Clin. Invest. 30 (2000) 105.
[35] H. Liebich, C. DiStefano, A. Wixforth, H. Schmid, J. Chromatogr. A 763 (1997) 193.
[36] A. Sollevi, Prog. Neurobiol. 27 (1986) 319.
[37] S. Fremes, S. Levy, G. Christakis, S. Walker, J. Iazetta, H. Mallidi, R. Feder-Elituv, K. Deemar, E. Cohen, B. Wong, B. Goldman, Circulation 94 (1996) 370.
[38] S. Fremes, S. Levy, G. Christakis, S. Walker, J. Iazetta, H. Mallidi, K. Deemar, E. Cohen, W. Wong, B. Goldman, Circulation 92 (1995) 3664.
[39] G. Cohen, R. Feder-Elituv, J. Iazetta, P. Bunting, H. Mallidi, J. Bozinovski, C. Deemar, G. Christakis, E. Cohen, B. Wong, R. McLean, M. Myers, C. Morgan, C. Mazer, T. Smith, B. Goldman, C. Naylor, S. Fremes, Circulation 98 (1998) II225.
[40] R. Mentzer, V. Birjiniuk, S. Khuri, J. Lowe, P. Rahko, R. Weisel, H. Wellons, M. Barker, R. Lasley, Ann. Surg. 229 (1999) 643.
[41] T. Rinne, J. Laurikka, I. Penttila, S. Kaukinen, J. Cardiothor. Vasc. Anesth. 14 (2000) 18.
[42] A. Shalaby, T. Rinne, O. Jarvinen, A. Saraste, J. Laurikka, H. Porkkala, P. Saukko, M. Tarkka, Eur. J. Cardio-Thorac. 33 (2008) 639.
[43] M. Zughaib, A. Abdelfattah, M. Jeroudi, J. Sun, S. Sekili, X. Tang, R. Bolli, Circulation 88 (1993) 2359.
[44] D. Blumenthal, G. Hutchins, B. Jugdutt, L. Becker, Circulation 64 (1981) 915.