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HILIC-MS/MS Analysis of Adenosine in Patient Blood

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Abstract: Adenosine is a purine ribonucleoside with important roles in various physiological processes. A number of studies have indicated the importance of adenosine in cardiovascular diseases including syncope; however, the accurate determination of adenosine in human blood is challenging due to the molecule's instability. In the present study, we report a simple method for the pre-treatment of blood samples and the development of a fast and efficient hydrophilic interaction chromatographic tandem mass spectrometry method for the analysis of adenosine in patient blood. During collection, samples were mixed directly with a solvent mixture containing 95% acetonitrile and 10 mM ammonium formate in a Vacutainer tube, resulting in successful prevention of adenosine metabolic processes and direct blood sample deproteinization. The method was validated according to bioanalytical industry guidelines and found to be accurate, repeatable, specific and sensitive with LLOQ 0.005 μg/mL, thus allowing its application in the analysis of real clinical samples.

Keywords: adenosine; HILIC-MS/MS; blood; bioanalysis; syncope

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1. Introduction

Adenosine is a purine ribonucleoside that plays important roles in many physiological processes, acting as an extracellular regulatory molecule [1]. Cellular signalling by adenosine occurs through four known adenosine receptor subtypes (i.e., A1, A2A, A2B and A3). Cells of the immune system express these receptors and are responsive to the modulatory effects of adenosine in an inflammatory environment [2].

Numerous pre-clinical studies have suggested the implication of adenosine in a number of cardiovascular and neurological diseases [3–6] including syncope [7]. However, little is known about the concentration ranges of adenosine in human blood or factors that influence adenosine levels, due mainly to the fact of technical limitations during blood collection. The short half-life of circulating adenosine, which is counted in seconds, makes sample collection and detection of adenosine a challenging task [8]. In general, the determination of the concentration of extracellular adenosine in blood is complicated due to the rapid formation and rapid clearance of adenosine in the blood [9]. Since the half-life of adenosine in blood is short, various collection protocols have been developed for the prevention of adenosine metabolism. Vacutainer tubes enable the direct mixing of venous blood with a "STOP" solution that prevents adenosine uptake by red cells. The most frequently used STOP solution contains dipyridamole, erythron-9-(2-hydroxy-3-nonyl) adenine (EHNA) and heparin in 0.9% saline [9,10]. Instead of using STOP solution, simple

protein precipitation with perchloric acid has also been proposed in the literature for the effective quantitation of adenosine in blood [11]. However, validation experiments revealed that adenosine concentrations in deproteinised blood samples were unstable.

Until now numerous methods have been reported for the quantitation of adenosine using HPLC with either ultraviolet (UV) [12] or fluorescence detection [13], mass spectrometry (MS) [14] and MS/MS detection [15–17]. Among them, only few publications report the quantitation of adenosine in plasma [9,18–22], while most of the publications report the quantitation of adenosine in either brain tissue cell media or other matrix [15,23,24]. LC-MS/MS can be considered the method of choice for the detection of adenosine since it offers increased sensitivity and specificity compared to UV detection; in comparison to HPLC with fluorescence detection, LC-MS/MS does not require sample derivatisation thus providing simpler and straightforward analysis.

The reported publications utilise the RPLC (reversed-phase liquid chromatography) mode on columns that are designed to retain polar analytes. Only two publications report the use of HILIC (hydrophilic interaction chromatography) for the determination of adenosine [21,22]. As a rule, HILIC is expected to provide stronger retention for polar analytes, such as adenosine, and increase chromatographic selectivity; detection sensitivity also increases as a result of mobile phases rich in organic solvent typically utilised in the HILIC mode. In a recent investigation, Olecsti et al. reported the determination of six small polar compounds (polar neurotransmitters and related compounds including adenosine) in rat brain and plasma using HILIC-MS/MS technology [22]. They did not report special treatment of blood samples with STOP solution.

The aim of the present study was to develop a method capable of providing trustworthy results for the analysis of adenosine in blood samples of clinical patients. Herein, we present a simple pre-treatment method and subsequent analysis using HILIC–ESI–MS/MS for the determination of adenosine in human blood. Separation was performed in the HILIC mode resulting in a sensitive MS/MS response. The developed method was finally applied for the quantitation of adenosine in the blood of syncopal patients with the aim of find a correlation between adenosine plasma levels and risk stratification of patients with syncope.

2. Results and Discussion

2.1. LC-MS/MS Method

Adenosine is a polar molecule (Supplementary Materials Figure S1) with low molecular weight, 267.2413 g/mol. Such molecules typically show better retention on HILIC columns in comparison to reversed-phase materials. This increased retention is expected to minimise the impact of interferences from the analysis of real-life samples: the analyte does not elute in the solvent front together with various other sample components. HILIC uses high organic solvent ratios in the mobile phase, thus enhancing analyte ionisation which also increases detection sensitivity. For the purposes of this study, a BEH Amide column was selected for chromatographic separation. A simple and fast gradient elution program was developed via the modification of a method previously described [25]. The gradient program started at 100% A (3 min isocratic step with organic-rich solvent), then rose to 85% B (aqueous-rich solvent) linearly over 2 min. Next, the column was equilibrated for 4 min in the initial conditions. A satisfactory resolution and peak shape were achieved within a total gradient run of 9 min (including column equilibration). The chromatographic system provided good retention with a satisfactory peak shape, which is needed for good detection sensitivity. Adenosine was eluted in a symmetric, well-shaped peak at 4.6 min. MS-MS data were obtained for adenosine after direct infusion of the standard solution (1 μg/mL), and the predominant single charged precursor ion $[M+H]^+$ of the analyte at *m/z* 268 was selected. The product ion with *m/z* 136 was selected for analyte quantification. The optimum MS-MS transition parameters for the pair *m/z* 268 → 136 were 20 and 15 V for cone and collision voltage, respectively. The fragmentation pattern of adenosine using the

MS1 scan mode and daughter scan of *m/z* 268 are presented in Supplementary Materials Figure S2a,b, respectively.

2.2. Method Validation

Analysis of the adenosine-free sample revealed no peaks in the retention time and MS/MS transition of the analyte, ensuring a specific analysis with no interferences. Figure 1A illustrates the chromatogram of a blank sample analysed after the HQC sample.

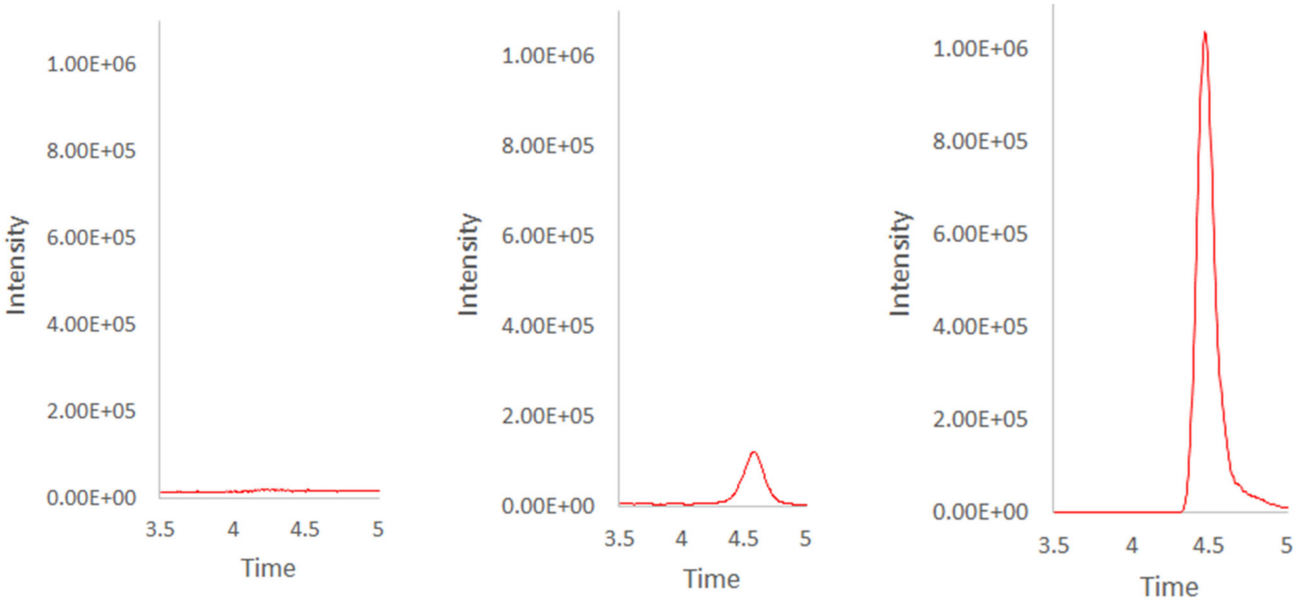

Figure 1. HILIC-MS/MS of (**A**) a "blank" blood sample, a sample that was left for 60 min at room temperature; (**B**) blank sample spiked at LLOQ; (**C**) a real clinical blood sample (concentration 0.05 µM).

The linearity of the method was assessed by the correlation of determination (R^2) of the constructed nine-point calibration curve and was found to be linear with $R^2 > 0.9927$. Working ranges for adenosine in mM are provided in Supplementary Materials Table S1. The chromatographic peaks of the analyte of interest at the LLOQs and in a real blood sample can be seen in Figure 1B,C.

For validation of the method, blank blood samples were spiked with adenosine at four concentration levels (i.e., LLOQ, LQC, MQC and HQC) and were analysed with the LC-MS/MS method as described above. The percentage recovery of adenosine, calculated as the mean peak area of quality control samples (QCs) spiked before extraction divided by the mean peak area of QCs spiked after extraction, ranged from 96% to 114% (Table 1).

Table 1. Recovery and matrix effect (ME%) of adenosine in blood samples at four concentration levels. Extraction was performed three times and analysis in triplicates.

Level	Concentration		Matrix Effect	Recovery
	µg/mL	µM	(%)	(%)
LLOQ	0.005	0.019	104	104
LQC	0.02	0.075	94	96
MQC	0.5	1.873	95	98
HQC	1.8	6.742	85	114

Matrix factors (MFs) should be calculated for each of the investigated matrices, and the CV of the MFs was found to be less than 15%. The matrix effect ranged between 85% and 104% (Table 1). In addition to the above, the slopes of the external and matrix-matched calibration curves were compared, and the slope ratio was found to be 0.98, indicating good

parallelism between the two calibration curves; thus, indicating the absence of matrix effect. Since no interferences from the matrix were observed, an external calibration was used for the quantitation of the analyte in the real samples. Intra- and inter-assay accuracy in blood expressed as recovery % was found to be 120% and 119% for LLOQ and 85 < %R < 115 for low, medium and high QCs. Regarding the intraday precision of adenosine in blood, %RSD was found to be 2% for LLOQ, 4% for LQC and 5% and 1% for medium and high QCs, respectively, while for inter-day the precision values were found to be <11% with the exception of LQC 20%, although it was within acceptable limits (Table 2).

Table 2. Intra- and inter-day precision and accuracy of adenosine in spiked blood samples at four concentration levels.

QC Level	Concentration		Intraday (n = 5)			Inter-Day (n = 15)		
	μg/mL	μM	Mean (μg/mL) ± SD	Accuracy %R	Precision %RSD	Mean (μg/mL) ± SD	Accuracy %R	Precision %RSD
LLOQ	0.005	0.019	0.006 ± 0.0001	120	2	0.006 ± 0.0002	119	3
LQC	0.02	0.075	0.020 ± 0.001	100	4	0.023 ± 0.004	115	20
MQC	0.5	1.873	0.445 ± 0.05	89	5	0.485 ± 0.05	97	11
HQC	1.8	6.742	1.566 ± 0.08	87	1	1.233 ± 0.10	85	7

The short-term stability of the spiked blood samples, expressed as percentage recovery, was found to be within acceptable limits (within ±15% of the nominal concentration) after storage at 4 °C for 24 h and at −22 °C for three days with an %RSD < 15%. The stability of the deproteinated extracts indicated that the adenosine concentrations remained stable, ensuring the effectiveness of the sample collection and preparation. Data on the stability assessment can be found in Table 3.

Table 3. Stability data of adenosine at four concentration levels. Analysis was performed in triplicates, and mean recovery (%) was assessed as the percentage concentration of that determined at t0, when samples were freshly analysed.

Concentration		Stability %R			
μg/mL	μM	24 h (4 °C)	%RSD	3 Days (−22 °C)	%RSD
0.005	0.019	104	0.4	101	4
0.02	0.075	106	4.2	109	4.3
0.5	1.873	100	1.4	115	13
1.8	6.742	113	0.9	92	5.6

2.3. Application to the Analysis of Real Samples

Head-up tilt table tests (HUTT) and occasionally adenosine tests (ADTs) are used as part of the diagnostic algorithm in patients with syncope. The purinergic signalling system, including adenosine plasma (ADP) levels and its receptors, has been proposed to be involved in neurally mediated syncope or syncope of unknown origin. We investigated if the clinical characteristic of syncope and the response to HUTT and ADT were correlated to the baseline adenosine plasma level (APL). During HUTT, an assay of APL in the supine position, immediately after tilting the table and during syncope in the case of a positive test, was performed.

Blood was collected from 124 patients (71 women, aged 46.78 ± 21.01 years) [26,27]. Adenosine levels ranged at various time points of examination from 0.33 ± 1.03 to 0.70 ± 1.89 μM. The results are in accordance with those from the literature [28,29], indicating the potential of the method to be used together with other clinical and other laboratory tests in support of clinical decision making.

3. Materials and Methods

All solvents used were of LC/MS grade. Acetonitrile and methanol were obtained from Carlo Erba (CARLO ERBA Reagents S.A.S, Val de Remil, France). Water (18.2 MΩ) was purified in a Milli-Q device, Millipore Purification System (Merck, Darmstadt, Germany). Ammonium formate and formic acid were purchased from Sigma–Aldrich (Gillingham, Dorset, UK). All other standards used in the study were of analytical or higher grade and were obtained from various vendors. Standards of adenosine were prepared from the stock solutions by appropriate dilution with acetonitrile–water (95:5% v/v). Concentration of working standard solutions ranged from 0.005 to 2 μg/mL (0.02–7.48 μM).

3.1. Standards and Sample Preparation

An adenosine stock solution with a concentration of 1000 μg/mL was prepared in MeOH:H_2O, 50:50% v/v and was stored at −22 °C. Working standard solutions were prepared by diluting stock solution in acetonitrile–water (95:5% v/v). An adenosine-free matrix was prepared by keeping the freshly collected blood for approximately 60 min at room temperature in order to extinguish endogenous levels of adenosine.

Two calibration curves were prepared: an external calibration curve using neat standard solvents and a matrix-matched calibration curve by spiking an adenosine-free matrix sample with working standard solutions. The final concentrations of the calibration curve standards were 0.005, 0.01, 0.018, 0.03, 0.09, 0.3, 0.6, 1.2 and 2 μg/mL. In addition to the above fortified "blank" blood sample, QCs were prepared at four concentration levels: a lower limit of quantitation (LLOQ) at 0.005 μg/mL, a low QC (LQC) at 0.02 μg/mL, a medium (MQC) at 0.5 μg/mL and a high (HQC) at 1.8 μg/mL in order to assess the validation of the method including accuracy precision, matrix effect and recovery. To spike standards and QC samples, 150 μL of blood was directly mixed and diluted with 250 μL of acetonitrile–water (95:5% v/v) and 50 μL standard solution. Samples were vortexed and centrifuged at 11.700× g and 4 °C. Supernatant (200 μL) was transferred to an LC/MS vial and evaporated to dryness under a stream of nitrogen. Dry extracts were reconstituted in 50 μL acetonitrile–water (95:5% v/v, 10 mM ammonium formate).

3.2. Sample Collection and Preparation

A volume of 6 mL ice-cold acetonitrile–water (95:5% v/v, 10 mM ammonium formate) was added to a Vacutainer tube, under vacuum with the use of a syringe. For blood sampling, 3 mL of blood was directly infused in the tube in order to precipitate proteins and simultaneously quench adenosine metabolism. Samples were vigorously vortex mixed for 1 min at room temperature. All samples were stored at −20 °C immediately after collection. Before analysis, the samples were allowed to thaw at room temperature. After thawing at room temperature, samples were centrifuged for 10 min (4.375× g) at 4 °C. An aliquot of the supernatants (280 μL) was transferred to an LC/MS vial and evaporated to dryness under a stream of nitrogen. Dry extracts were reconstituted in 70 μL of acetonitrile–water (95:5% v/v, 10 mM ammonium formate).

3.3. HILIC- MS/MS Targeted Analysis

Chromatographic separation was performed on an Acquity BEH Amide Column (2.1 × 150 mm, 1.7 μm) (Waters Ltd., Elstree, UK) with an Acquity UPLC System (Waters Corporation, Milford, Massachusetts, USA) under gradient elution. The mobile phase system consisted of solvent A: acetonitrile–water (95:5%, v/v) and solvent B: water–acetonitrile (70:30%, v/v), both containing 10 mM ammonium formate. The flow rate was 0.5 mL/min, and the column temperature was set at 40 °C. The injection system was subjected to two washing cycles with a weak solvent (95:5%, v/v acetonitrile–water, 0.1% HCOOH) and a strong solvent (70:30%, v/v water–methanol, 0.1% HCOOH) prior to injection and one cycle for 6 s with the strong solvent for post wash.

Mass spectrometry was performed on a Xevo TQD system (Waters Corporation, Milford, Massachusetts, USA). Electrospray ionisation was operating in the positive mode

during analysis. Capillary voltage was set at +3.5 kV. The block and desolvation temperatures were 150 and 350 °C, respectively, the desolvation gas flow rate was 650 L/h and the cone gas was set at 50 L/h. The cone voltage and collision energy were optimised for the detection of adenosine in the direct infusion mode.

3.4. Method Validation

Method validation was based on existing guidelines for endogenous compound analysis [30,31]. The method's precision, accuracy, recovery, matrix effect, linearity, sensitivity, selectivity and stability were assessed. The calibration curve for adenosine was prepared and studied at nine concentration levels in a range from 0.005 to 2 μg/mL. To assess extraction analyte recovery, the mean peak area of the blank blood spiked before extraction was divided by the mean peak area of blood spiked with adenosine after the extraction. This experiment was performed in triplicate at four concentration levels: LLOQ, LQC, MQC and HQC. Any potential interferences from the blood matrix on the adenosine signal was measured by calculating the matrix effect as the ratio of the response of extracted blank blood spiked after the extraction to the response of standard solvent solutions. Calculations were made at the concentration levels LLOQ, LQC, MQC and HQC. A total of six lots of unique sources of blank matrix were used for the preparation of QCs for method validation experiments. Quality control samples were analysed in five replicates within one day for intra- (n = 5) and fifteen replicates from three different days (n = 15) for inter-day precision and accuracy. Accuracy was estimated as recovery (%) using Equation (1). Precision is expressed as %RSD.

$$Recovery\ \% = \frac{Mean\ of\ experimental\ concetrations}{Nomimal\ concetration} \times 100 \quad (1)$$

Short-term stability was assessed at the four studied concentrations (i.e., LLOQ, LQC, MQC and HQC) by analysing spiked blood samples in triplicates. Fortified samples were analysed directly after sample preparation and were kept at 4 °C for 24 h and at −22 °C for 3 days. Analyses of the extracts were performed using freshly prepared calibration curves. Short-term stability was expressed as recovery %.

4. Conclusion

The developed LC-MS/MS method provided reliable and accurate determination of adenosine in blood samples in a concentration range of 0.005–2 μg/mL. Considering the short lifetime of circulating adenosine, a sampling method was studied and successfully tested and, subsequently, it was applied in the analysis of clinical samples. The developed sample collection and preparation scheme is simple and cost effective compared to previously reported methods and "stop solutions" in the literature. Chromatographic and MS conditions were optimised to achieve appropriate levels of accuracy, sensitivity and robustness. The method was validated and successfully applied to the analysis of blood samples from patients at the Aristotle University Cardiology clinic. Adenosine plasma and adenosine receptor levels warrant further investigation, as they may predict the response to cardiac tests thereby contributing to the diagnostic evaluation and risk stratification of patients with syncope.

Supplementary Materials: The following are available at https://www.mdpi.com/article/10.3390/separations8110222/s1. Table S1, Calibration curve concentration range in μM. Figure S1, Molecular structure of Adenosine. Figure S2, a. MS1 Scan of adenosine (10 μg/mL, cone energy 20V) and b. Daughter scan of adenosine (m/z 268, collision energy 15V).

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The great majority of data are presented within this article, tables and figures. More data supporting reported results may be available on request from the corresponding author.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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