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Highly sensitive, selective and rapid LC–MS method for simultaneous quantification of diadenosine polyphosphates in human plasma

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ABSTRACT

Background: Diadenosine polyphosphates (Ap_nAs) are endogenous mediators involved in large number of physiologic and pathophysiologic processes. The quantification of diadenosine polyphosphates in plasma and biological matrices is still challenging. Therefore, there is an urgent need for a simple and reliable quantification method suitable for clinical studies. The classical quantification of diadenosine polyphosphates is based on chromatographic separation and UV adsorption of the resulting fractions. These procedures are associated with low selectivity due to co-eluting plasma components. Therefore, we developed and validated a highly sensitive, selective and rapid LC–ESI–MS method for simultaneous quantification of Ap_nAs (with $n = 3–6$) in human plasma within this study. The identities of the endogenous Ap_nAs (with $n = 3–6$) were revealed by comparison of ESI-MS/MS fragment spectra of isolated endogenous compounds with those of authentic Ap_nAs.
Methods: Diadenosine polyphosphates were extracted from 100 μl human plasma using weak anion-exchange extraction cartridges. The separation of Ap_nAs was achieved using capillary C18 columns. ESI-HCT mass spectrometer (Bruker Daltonik, Germany) operated in negative ion mode was used for detection and quantification of Ap_nAs.
Results: A calibration curve was established for diadenosine polyphosphate free plasma in the concentration range 1.9–125 nM ($r^2 > 0.998$) for all analytes. The intra- and inter-day accuracies were in the range of 91.4% and 110.9%. The intra- and inter-day precisions were determines as 0.1% and 11.4%, respectively. The mean plasma concentrations of Ap_nAs were quantified as 31.9 ± 5.9 nM for Ap_3A, 40.4 ± 6.6 nM for Ap_4A, 10.7 ± 1.5 nM for Ap_5A and 10.0 ± 18.9 nM for Ap_6A.
Discussion: The developed and validated ESI MS-based method for quantification of diadenosine polyphosphates in human plasma was successfully evaluated within the study. Conclusion Since the quantification is based on a volume of 100 μl plasma, this method is highly applicable for clinical applications aiming at the validation of the impact of highly physiological and pathophysiological active diadenosine polyphosphates.

1. Introduction

Diadenosine polyphosphates (Ap_nAs) are endogenous mediators, isolated from e.g. blood platelets [1,2], adrenal medullary chromaffin granules [3,4], the central nervous system [4,5] and human plasma [6]. They are formed by two adenosine nucleosides interconnected by a phosphate chain, which varies from 3 up to 6 phosphates [7,8]. The schematic chemical structure of Ap_nAs is shown in Fig. 1A. Ap_nAs have been demonstrated to control numerous physiological and pathophysiological functions like neurotransmission, homeostasis as well as vascular regulation [9–11]. Furthermore, they are involved in cardio-renal processes and may have an impact on the progression of chronic kidney disease (CKD) [12,13]. Therefore, the quantification of the endogenous Ap_nAs concentrations is of utmost importance since Ap_nAs might be potential biomarker or target for specific therapy in the progression of chronic kidney disease.

Until now, just a few methods have been described for the determination of Ap_nAs in human plasma. These analytical methods are mostly based on liquid chromatography coupled with detection of the UV absorbance at 254 nm. The sensitivity is not exactly specified in those methods. Furthermore, for the quantification of Ap_nAs

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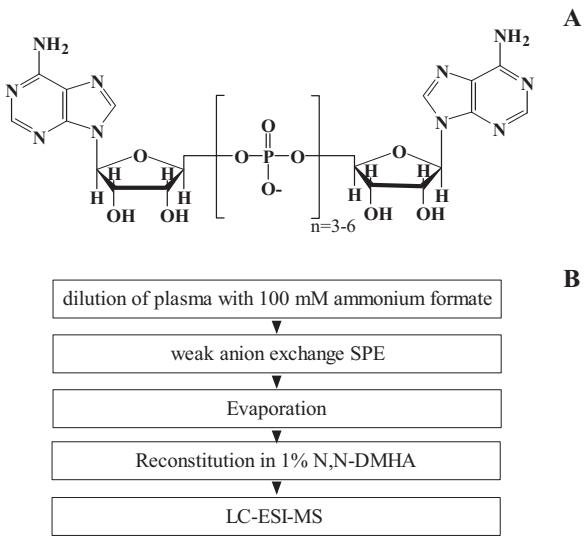

Fig. 1. (A) Molecular structure of diadenosine polyphosphates ($Ap_{3-6}A$) and (B) scheme of the preparation steps for the quantification of diadenosine polyphosphates.

by using those methods a high plasma volume is required. Since the CKD patients are suffering from anaemia the plasma volume should be minimized.

In general, the quantification of endogenous Ap_nAs levels and the validation of the analytical method are still challenging. The limitations are: (a) the concentration of diadenosine polyphosphates is in the nanomolar range, (b) short half-life times of diadenosine polyphosphates, and (c) the minimal differences in the chemical characteristics of diadenosine polyphosphates. In addition, since diadenosine polyphosphates are endogenous components of plasma, a diadenosine polyphosphates-free matrix is not directly available, which has an impact on the determination of the lower limit of quantification. Since chromatographic fractionation diadenosine polyphosphates are highly polar, the use of buffer systems and/or ion pair reagent to ensure a sufficient retention by chromatographic media are mandatory. To avoid the co-detection of eluting substances from the chromatographic column, a highly sensitive and selective method for detection of the eluting substances is essential. UV adsorption as used in former studies does not fulfil this criterion.

In recent years, liquid chromatography tandem mass spectrometry has been characterized as a selective and highly robust method for quantification of metabolites and endogenous mediators [14]. Therefore, we developed, established and validated a liquid chromatography electrospray-ionization mass-spectrometric-based method for simultaneous quantification of Ap_nAs in human plasma in this study.

2. Materials and methods

2.1. Chemicals and reagents

Diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A, Ap_6A), analytical grade ammonium formate, formic acid, ammonium hydroxide and *N,N*-dimethylhexylamine (DMHA) were purchased from Sigma Aldrich (Seelze, Germany). Liquid chromatography-mass spectrometry (LC–MS) grade water and acetonitrile were purchased from Fisher Scientific (Fair Lavn, United States). Oasis WAX cartridges were obtained from Waters (Eschborn, Germany).

2.2. High performance liquid chromatography conditions

An Agilent 1200 (Agilent, Böblingen, Germany) capillary LC system equipped with micro vacuum degasser (G1379B) and capillary pump (G1376A) along with an autosampler (G1377A) was used to inject 2 μl aliquots of prepared samples. The separation was carried out on a Zorbax C18 XDB 0.5 mm × 35 mm column with 3.5 μm particle size (Agilent, Böblingen, Germany). The temperature of the column was maintained at constant 50 ± 0.5 °C. The eluent A was consisted of 0.1% N,N-DMHA in 1 mM ammonium formate prepared in LC–MS grade water. The pH of eluent A was adjusted using formic acid to 9.0. Eluent B was LC–MS grade acetonitrile. The injection volume was 2 μl. The following gradient was used to separate Ap_nAs: 0 min, 0% B, 0–1 min 0–12% B, 1–1.75 min 1–16% B, 1.75–2.5 min 16–22% B, 2.5–5.5 min 22–98% B, 5.5–6.5 min 98% B, 6.5–6.6 min 98–0% B, 6.6–12.5 min 0% B. The primary flow was 500 μl/min and it was split to 100 μl/min by using electronic flow control. The total chromatographic run time was 12.5 min.

2.3. Mass-spectrometric analysis

The detection of Ap_nAs was achieved in negative ion mode for all analytes by using a HCT mass spectrometer (Bruker Daltonic, Bremen, Germany), equipped with an electrospray ionization interface. The ionization parameters of nebulizer gas, dry gas and dry temperature were 25 psi, 9 l/min, and 300 °C respectively. The ion spray voltage was set to 3000 V. The maximal accumulation time was set to 200 ms. Extracted ion chromatograms were used for quantification of Ap_3A: m/z 755.0 ± 0.2, Ap_4A: m/z 835.0 ± 0.2, Ap_5A: m/z 915.0 ± 0.2 and Ap_6A: m/z 994.9 ± 0.2. To evaluate the identity of the Ap_nAs an aliquot of 2 μl was injected and MS/MS ESI-fragment spectra were acquired. All data were acquired and processed using "Compass 1.3 Software" (Bruker Daltonik, Bremen, Germany). Calculations including calibration curve regressions, sample concentrations values and statistics were performed by using "GraphPad Prism 5.0" software (GraphPad Software, San Diego, USA).

2.4. Immobilization of phosphodiesterase-I

Phosphodiesterase-I (10 units) was diluted in 1000 μl of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3) for immobilization of the enzyme. Cyanogen-bromide activated sepharose resin (500 μl) was swollen in 10 ml of 1 mM cold HCl for 1 h. The resin was than washed 3 times with 10 ml of 1 mM HCl and 3 times with 10 ml of coupling buffer and the diluted phosphodiesterase I was immediately transferred to 500 μl of the washed resin. The immobilization was carried out for 1 h at room temperature. After the immobilization the unbound substrate was washed out 3 times using 1.5 ml of blocking buffer (1 M NaCl, 0.05 M glycine, pH 3.5). The supernatant was removed and the unbound reactive group of the sepharose resin were blocked using blocking buffer incubated for 2 h at room temperature. After the blocking procedure the resin was washed alternately using 1.5 ml of washing buffer 1 (0.5 M NaCl, 0.1 M $NaCH_3COO$, pH 4) and 1.5 ml of washing buffer 2 (0.5 M NaCl, 0.1 M Tris, pH 8). The immobilized phosphodiesterase-I was stored in PBS containing 0.02% NaN_3 at 4 °C until use. Prior to use the immobilized phosphodiesterase-I was washed 5 times using 1 ml PBS.

2.5. Preparation of diadenosine polyphosphate free plasma

To degrade endogenous diadenosine polyphosphates an aliquot of plasma (500 μl) was incubated with immobilized phosphodiesterase-I (100 μl resin) for 24 h at 37 °C. An aliquot of