Validation of a LC/MSMS method for simultaneous quantification of 9 nucleotides in biological matrices

Melissa Cortese, Cédric Delporte, Damien Dufour, Caroline Noyon, Martin Chaumont, Benjamin De Becker, Florence Reye, Alexandre Rousseau, Eker Omer, Jean Nève, Michael Piagnerelli, Karim Zouaoui Boudjeltia, Bernard Robaye, Pierre Van Antwerpen

www.elsevier.com/locate/talanta

PII: S0039-9140(18)31035-X
DOI: <https://doi.org/10.1016/j.talanta.2018.10.003>
Reference: TAL19119

To appear in: *Talanta*

Received date: 28 May 2018
Revised date: 25 September 2018
Accepted date: 1 October 2018

Cite this article as: Melissa Cortese, Cédric Delporte, Damien Dufour, Caroline Noyon, Martin Chaumont, Benjamin De Becker, Florence Reye, Alexandre Rousseau, Eker Omer, Jean Nève, Michael Piagnerelli, Karim Zouaoui Boudjeltia, Bernard Robaye and Pierre Van Antwerpen, Validation of a LC/MSMS method for simultaneous quantification of 9 nucleotides in biological matrices, *Talanta*, <https://doi.org/10.1016/j.talanta.2018.10.003>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Validation of a LC/MSMS method for simultaneous quantification of 9 nucleotides in biological matrices

Melissa Cortese^{a,b}, Cédric Delporte^{a,b}, Damien Dufour^a, Caroline Noyon^a, Martin Chaumont^c, Benjamin De Becker^c, Florence Reye^a, Alexandre Rousseau^d, Eker Omer^e, Jean Nève^a, Michael Piagnerelli^d, Karim Zouaoui Boudjeltia^d, Bernard Robaye^f, Pierre Van Antwerpen^{a,b}

^a RD3 – Pharmacognosy, Bioanalysis and Drug Development, Faculty of Pharmacy, Université Libre de Bruxelles, Campus Plaine CP 205/05, Boulevard du Triomphe, B-1050 Bruxelles, Belgium

^b Analytical Platform of the Faculty of Pharmacy, Université Libre de Bruxelles, Campus Plaine CP 205/05, Boulevard du Triomphe, B-1050 Bruxelles, Belgium

^c Department of Cardiology, Erasme University Hospital, Brussels, Route de Lennik 808, B-1070 Bruxelles, Belgium

^d Laboratory of Experimental Medicine, CHU de Charleroi, A. Vésale Hospital, Université Libre de Bruxelles, Route de Gozée 706, B-6110 Montigny-le-Tilleul, Belgium

^e Service de neuroradiologie interventionnelle, Hospices Civils de Lyon, Hôpital Neurologique Pierre Wertheimer, Bron, France

^f Institute of Interdisciplinary Research in human and molecular Biology, Université Libre de Bruxelles, Campus de Charleroi - Gosselies (Biopark), CP300, rue des Professeurs Jeener et Brachet 12, B-6041 Charleroi, Belgium

Abstract

Nucleotides play a role in inflammation processes: cAMP and cGMP in the endothelial barrier function, ADP in platelet aggregation, ATP and UTP in vasodilatation and/or vasoconstriction of blood vessels, UDP in macrophages activation. The aim of this study is to develop and validate a LC/MS-MS method able to quantify simultaneously nine nucleotides (AMP, cAMP, ADP, ATP, GMP, cGMP, UMP, UDP and UTP) in biological matrixes (cells and plasma). The method we developed, has lower LOQ's than others and has the main advantage to quantify all nucleotides within one single injection in less than 10 minutes. The measured nucleotides concentrations obtained with this method are similar to those obtained with assay kits commercially available. Analysis of plasma and red blood cells from healthy donors permits to estimate the physiological concentration of those nucleotides in human plasma and red blood cells, such information being poorly available in the literature. Furthermore, the protocol presented in this paper allowed us to observe that AMP, ADP, ATP concentrations are modified in human red blood cells and plasma after a venous stasis of 4 minutes compared to physiological blood circulation. Therefore, this specific method enables future studies on nucleotides implications in chronic inflammatory diseases but also in other pathologies where nucleotides are implicated in.

Graphical abstract:

A) Concentration of AMP in plasma of each volunteer before and after venous stasis; B) Concentration of ADP in plasma of each volunteer before and after venous stasis; C) Concentration of ATP in plasma of each volunteer before and after venous stasis

A) Concentration of ADP in red blood cells of each volunteer before and after venous stasis; B) Concentration of ATP in red blood cells of each volunteer before and after venous stasis.

1. Introduction

Intracellular as well as extracellular nucleotides play multiple and diverse physiological roles but are pathological ones: they are involved in inflammation [1], cell differentiation [2], apoptotic cell clearance [3], nociception [4]... Our laboratory is particularly interested in the processes of inflammation, vascular tone and sepsis in which the nucleotides play significant roles. It is well known that cAMP and cGMP can regulate endothelial barrier function and in this context, the balance cAMP/AMP and cGMP/GMP were studied during endothelial dysfunction and sepsis [5–7]. Moreover, the release of ATP from the perivascular sympathetic nerves and its action on smooth

muscle cells can cause high blood pressure [8]; an increase of the extracellular ADP concentration leads to platelets aggregation through the P2 receptors and both phenomena are involved in atherosclerosis and aneurysm [9,10]. Furthermore, ATP and UTP are also released by blood cells in stress condition as hypoxia with subsequent vasodilatation through the activation of endothelial nitric oxide synthase [11,12]. Finally, UTP, when released in extracellular fluids, is metabolized in UDP which plays a role in macrophage activation through P2Y₆ receptor [13]. From these examples, it is obvious that the measure of variations of nucleotide concentration in biological fluids should greatly help to understand how many nucleotides play role in inflammatory syndromes. We therefore aim to develop a new method based on a LC/MSMS method allowing us to quantify nine nucleotides (AMP, ADP, ATP, GMP, UMP, UDP, UTP, cAMP and cGMP) rapidly, simultaneously, safely and reliably. Nucleotides quantification methods already exist : ATP from plasma can be quantified with bioluminescence as AMP and ADP after transformation in ATP [14] but this method has limitations such as the standard addition method which must be used for each sample in order to convert luminescence measurements into ATP content and this method is not suitable for others nucleotides. Adenine nucleotides can also be quantified by LC/MSMS from liver samples [15] but the method has not been validated for other nucleotides. A LC/MS method for the quantification of 12 nucleotides has been described [16] and it included quantification of AMP, ADP, ATP, UMP, UDP, UTP and GMP. However, cAMP and cGMP were not included and nucleotides were extracted from rat hearts and not from plasma. A LC/MSMS method for ATP and UTP quantification in human blood was described in 2015 [17] but the limit of quantification (LOQ) of ATP and UTP was too high for our purpose. A LC/MSMS method for cyclic nucleotides quantification in mammalian tissues and cellular systems was performed in 2014 [18] but this method matches only with cyclic nucleotides. Recently, Zhu et al. [19] developed a method to quantify 20 nucleosides (including the nine nucleotides quantified in this method) but the analysis was realized by using counter-ion which required the daily cleaning of the mass spectrometer. A lot of other methods can be found in literature but none of them combined all the metabolites we needed. All these reasons reinforce the necessity to develop and validate a method for the quantification of the nine nucleotides that we wanted to study in chronic inflammatory syndromes.

2. Material and methods

2.1. Materials and reagents

NH₄ bicarbonate, formic acid, NH₄ formate, NH₄ acetate, acetonitrile (ACN), ethanol (EtOH), perchloric acid (PCA), methanol (MeOH), RIPA buffer, forskoline, AMP, cAMP, ADP, ATP, GMP, cGMP, UMP, UDP, UTP, ¹³C₁₀¹⁵N₅-AMP, Br-cAMP, ¹⁵N₅-ADP, Br-ATP, ¹³C₁₀¹⁵N₅-GMP, Br-cGMP, ¹³C₉¹⁵N₂-UMP

and $^{13}\text{C}_9^{15}\text{N}_2$ -UTP, ADP Assay Kit, ATP Bioluminescent Assay Kit and cAMP Enzyme Immunoassay Kit were purchased from Sigma-Aldrich (Steinheim, Germany). Sterile 12-well plates were from Greiner Bio-One (Kremsmünster, Austria). Endothelial cells EA.hy926 were an immortalized endothelial cell line derived from human umbilical veins HUVEC. Plasma and red blood cells were from healthy donors.

2.2. Instrumentation and conditions LC/MSMS analysis

The analyses were performed using a LC/MSMS system from Agilent Technologies (Santa Clara, CA, USA): an Agilent 1290 Infinity Binary – UHPLC system connected to a mass spectrometer Agilent Jet Stream electrospray ionization source (AJS) Triple Quadrupole (QqQ) 6490 series. After testing several separative columns as Zorbax Carbohydrate (4.6 x 250 mm – 5 μm , Agilent Technologies, Santa Clara, CA, USA), Zic[®]-Hilic (2.1 x 150 mm – 3.5 μm , Merck KGaA, Darmstadt, Germany), Zorbax Eclipse XDB-Phenyl, Zorbax Hilic Plus (4.6 x 100 mm – 3.5 μm , Agilent Technologies, Santa Clara, CA, USA), Zorbax RRHD (4.6 x 100 mm – 3.5 μm , Agilent Technologies, Santa Clara, CA, USA) or XBridge[®] BEH Amide (2.1 x 150 mm – 2.5 μm , Waters Corporation, Milford, MA, USA) and different mobile phases as formic acid, NH_4 formate, acetate or bicarbonate in combination with MeOH or ACN, the different nucleotides were finally separated thanks to a XBridge[®] BEH Amide, 2.1 x 150 mm, 2.5 μm column XP from Waters (Milford, MA, USA), using an ammonium bicarbonate 50 mM in water pH 6 / acetonitrile gradient (see supplementary data for details, **Table S1**). Source, ionization mode and Ion funnel (iFunnel) parameters were tested and finally the optimized parameters were : positive ion mode; gas temperature of 100°C; gas flow of 17 L/min; nebulizer pressure of 50 psi; sheath gas heater of 350°C; sheath gas flow of 12 L/min; capillary voltage of 3500 V; high pressure RF (iFunnel) voltage of 160 V and low pressure RF (iFunnel) voltage of 90 V. Agilent Mass Hunter Workstation[®] (version B.07.01, Agilent Technologies) was used for system control, data acquisition, fragment optimizer and data processing. Dynamic multiple reaction monitoring (dMRM) was performed to quantify nucleotides, using the following transitions (precursor ion $\rightarrow$ quantitative product ion, qualitative product ion): m/z 348.1 $\rightarrow$ 136.1, 97.0 for AMP, 363.1 $\rightarrow$ 146.1, 102.0 for $^{13}\text{C}_{10}^{15}\text{N}_5$ AMP, 330.1 $\rightarrow$ 136.1, 118.9 for cAMP, 408.0 $\rightarrow$ 214.0, 97.0 for Br-cAMP, 428.0 $\rightarrow$ 136.1, 118.9 for ADP, 433.0 $\rightarrow$ 141.0, 353.0 for $^{15}\text{N}_5$ ADP, 508.0 $\rightarrow$ 136.1, 97.0 for ATP, 585.9 $\rightarrow$ 213.9, 97.0 for Br-ATP, 346.1 $\rightarrow$ 152.1, 135.1 for cGMP, 423.9 $\rightarrow$ 229.8, 212.9 for Br-cGMP, 364.1 $\rightarrow$ 152.1, 97.0 for GMP, 379.1 $\rightarrow$ 162.1, 144.0 for $^{13}\text{C}_{10}^{15}\text{N}_5$ GMP, 325.0 $\rightarrow$ 97.0, 113.0 for UMP, 336.1 $\rightarrow$ 119.0, 101.9 for $^{13}\text{C}_9^{15}\text{N}_2$ UMP, 405.0 $\rightarrow$ 97.0, 69.0 for UDP, 485.0 $\rightarrow$ 97.0, 227.0 for UTP and 496.0 $\rightarrow$ 101.9, 73.0 for $^{13}\text{C}_9^{15}\text{N}_2$ UTP. All transitions were optimized by MassHunter Optimizer.

2.3. Sample handling

Different methods of extraction and evaporation were tested. For the extraction, MeOH, ACN and EtOH were used while for the evaporation, SpeedVac™, drying under nitrogen flow and lyophilisation were compared. First results were obtained with EtOH extraction and evaporation by lyophilisation (freezing: shelf temperature = -30 °C, sublimation: shelf temperature = 5 °C, vacuum pressure = 1.650 mbar, safety pressure = 2.170 mbar). Later, simple precipitation by PCA was tested (to improve the chromatographic pic areas), first without sample neutralization, then with neutralization by NH₄OH 30 % to protect the chromatographic column. Each sample of the cultured cells was divided in two aliquots, one for the LC/MSMS system, and the other for the quantification by the commercially available kits.

2.3.1. Treatment of extracellular pool from cultured cells :

EA.Hy926 endothelial cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % of decompemented foetal bovine serum, 2 % of Hypoxanthine Aminopterin Thymidine (HAT), 1 % of glutamine, 1% of penicillin – streptomycin and 1 % of non-essential amino acids in 12 wells-plates (from Greiner) and incubated at 37 °C under 5 % of CO₂ until 100% confluence point (1.10⁶ of cells/well).

2.3.2. Treatment of cytoplasmic pool from cultured cells :

To analyse free nucleotides within cells, EA.Hy926 endothelial cells removed from their supernatant were washed twice with Hank's Balanced Salt solution (HBSS) and cytoplasm was extracted from cells using radioimmunoprecipitation assay buffer (RIPA buffer) (200 µL/1.10⁶ cells, containing a protease inhibitor) which lyses cellular membranes or HCl 0.1 M (200 µL/1.10⁶ cells). Then lysates were collected and centrifuged for 10 min at 200 g to remove cellular debris.

2.3.3. Treatment of plasma and red blood cells

Blood samples were collected from healthy donors before and after 4 minutes of venous stasis to mimic aneurysm post stenting. The arterial pressure of each volunteer was taken before the venous stasis. A first blood sample was taken in the right arm with a normal tourniquet and for the second blood sample, the tourniquet was placed on the left arm at a pressure between the systole and diastole of each volunteer to cause a venous stasis and the blood sample was taken after 4 minutes. The samples were then centrifuged 10 minutes at 4°C and 200 g to separate plasma and red blood cells. Red blood cells were directly frozen at - 80 °C to haemolyse them. For the analysis, red blood

cells were diluted 10 times in milliQ water to ensure the total lysis of the erythrocytes in order to analyse their content. After centrifugation, the supernatant was picked up and analysed by the PCA method (see section 2.3.)

2.3.4. Nucleotides extraction from samples

EtOH/lyophilisation: 100 μL of supernatants, lysates or plasma were precipitated by adding EtOH (150 μL) and H_2O milliQ containing ISTD (50 μL) and centrifuged for 20 minutes at 11,960 g. Supernatant (200 μL) was retrieved, before evaporating by lyophilisation. Thereafter, samples were dissolved in 400 μL of H_2O milliQ, prior to injection of 5 μL into the LC/MSMS system. Red blood cells were not analysed with the EtOH/lyophilisation method.

PCA without neutralization: 150 μL of supernatants, lysates or red blood cells or 15 μL of plasma were precipitated by adding PCA 8% (100 μL) and H_2O milliQ containing ISTD (40 μL for supernatants, lysates and red blood cells, 175 μL for plasma) then centrifuged for 20 minutes at 11,960 g. Supernatant (250 μL) was retrieved and 5 μL were injected into the LC/MSMS system.

PCA with neutralization: 157.5 μL of supernatants, lysates or red blood cells or 15.75 μL of plasma were precipitated by adding PCA 8% (100 μL) and H_2O milliQ containing ISTD (42.5 μL for supernatants, lysates and red blood cells, 184.25 μL for plasma) then centrifuged for 20 minutes at 11,960 g. Supernatant (250 μL) was retrieved and 12.5 μL of NH_4OH 30% were added to neutralize the sample prior to injection of 5 μL into the LC/MSMS system.

2.4. Validation procedure

The validation of this method was done with precipitation by EtOH/lyophilisation and PCA. The method has been validated on plasma and applied to plasma, supernatant from red blood cell and cell lysis. All the criteria of the validation procedure were done in accordance to the ICH Q2(R1) guidelines.

2.4.1. Selectivity

The selectivity of the method was tested injecting the nine nucleotides separately with different collision energies then injecting different concentrations of the nine nucleotides separately and all together to ensure the chromatographic separation of the nine nucleotides.

2.4.2. Samples stability

The stability of the samples was tested injecting plasma samples spiked with 10 μM of each nucleotide in triplicate and analysing results after 0, 6, 12, 18 and 24 hours or after 1, 2, 3, 4 and 5 freezing-thawing cycles.

2.4.3. Calibration curves for the validation of nucleotides quantification

The range of the calibration curves for the validation of the quantification of the nucleotides was carried out on 8 levels of standard over concentrations 12.5-10.000 nM for GMP, UMP, UDP and UTP and on 11 levels of standard over concentrations 1-10.000 nM for AMP, cAMP, cGMP, ADP and ATP.

2.4.4. Validation of nucleotides quantification : Accuracy, Precision and Recovery

The validation was carried out on plasma samples. Four series of samples were prepared in triplicate during 3 days, according to sample handling: the first series consisted of unspiked plasma samples while the others were spiked with respectively 1, 5 and 10 μM of each nucleotide. Accuracy was achieved by the assessment of the recovery of the different spiked samples and by the within- and between-day coefficients of variation during the three days of analysis.

2.4.5. Limit of quantification

Limit of quantification (LOQ) was estimated thanks to calibration curves and signal to noise ratios (S/N). The range of these curves was carried out on 8 levels of standards over concentrations 0.01-1,000 nM for cAMP, cGMP, AMP, ADP and ATP, on 7 levels of standards over concentrations 0.1-1,000 nM for GMP and on 6 levels of standards over concentrations 12.5-1,000 nM for UMP, UDP and UTP. Then, samples composed of all nucleotides at their respective LOQ were prepared and injected 6 times.

2.4.6. Quantification of ADP, ATP and cAMP by commercially available kits

To reinforce the validation of this method, the results obtained with the LC/MSMS method were compared to those obtained with different commercially available kits. The kits were performed on cytoplasmic pool and supernatant of EA.Hy926 endothelial cells.

The ADP Assay kit (Sigma-Aldrich) was used according to the manufacturer instructions. The calibration curve was done on 8 levels from 0 to 60 μM in water for the supernatant of endothelial cells and from 0 to 6 μM in RIPA for the cytoplasmic pool. In a tube, 90.0 μL of ADP reagent were added to 10.0 μL of standard solution or sample. After 10 minutes of incubation at room temperature, the luminescence was read (RLU_{ATP}) and 5.0 μL of ADP reagent were then added to the

tube. After 2 minutes of incubation at room temperature, the luminescence was read again (RLU_{ADP}). The ADP concentration was calculated with the formula below.

$$ADP (\mu M) = \frac{RLU_{ADP} - RLU_{ATP}}{\text{slope}}$$

The ATP Bioluminescent Assay Kit (Sigma-Aldrich) was operated according to the manufacturer instructions. A calibration curve was done on 8 levels from 0 to 80 μM in water but also in RIPA for the cytoplasmic pool of the endothelial cells. First, 100.0 μL of assay mix was placed in a tube. After 3 minutes of incubation at room temperature, 100.0 μL of standard solution of sample was added to the tube and the luminescence was read immediately. The value of light was proportional to the amount of ATP in the samples.

The EIA cyclic AMP kit (Sigma-Aldrich) was performed according to the manufacturer instructions. On a multiwell plate, 100.0 μL of Assay Buffer 2 were pipetted into the NSB (nonspecific binding) and the Bo (0 nM standard) wells, 100.0 μL of standards (5 levels from 0.78 to 200 nM) and 100.0 μL of the samples were pipetted into the appropriate wells. Subsequently, 50.0 μL of Assay Buffer 2 were added in the NSB well, 50.0 μL of blue cAMP-Alkaline Phosphatase Conjugate were added into each well except the TA (total activity) and Blank wells and 50.0 μL of yellow cAMP EIA Antibody were added into each well, except the Blank, TA and NSB wells. After 2 hours of incubation at room temperature on a plate shaker (~500 rpm), the content of the wells was emptied and washed by adding 200.0 μL of 3x Wash Buffer to each well. After the final wash, the wells were emptied or aspirated. Then, 5.0 μL of the blue cAMP-Alkaline Phosphatase Conjugate were added to the TA wells and 200.0 μL of the p-Nitrophenyl Phosphate Substrate Solution were added to every well. After 1 hour of incubation at room temperature without shaking, 50.0 μL of Stop Solution were added to every well. The optical density of the plate should be read immediately at 405 nm. The intensity of the yellow colour was inversely proportional to the concentration of cAMP in either the standards or the samples. The measured optical density was used to calculate the concentration of cAMP.

2.5. Statistical analysis

Values are presented as mean $\pm$ SD. The level of statistical significance was set at $P < 0.05$. Paired Student's t-tests were applied for endothelial cells analysis with QqQ method and commercially available kits. Bland and Altman tests were used to compare the results obtained with both methods

in order to calculate the relative error and non-parametric Wilcoxon tests were used to compare the results of the blood samples before and after venous stasis for each volunteer. All the statistic tests were done with GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA, US).

3. Results

3.1. Separation by liquid chromatography

To develop the chromatographic separation of the nine nucleotides, different columns were tested. The first one was a reverse phase RRHD column but no nucleotides were retained on the column. The same observations were done for the XDB-Phenyl column. A Zic-Hilic column was then tested and showed a good resolution but with peak tailing. With the Hilic column, there was also tailing and the di- and trinucleotides were barely observed. A carbohydrate column was also tried but the intensity of the peaks was low. The last column tested and kept was the BEH Amide XBridge®, which had good resolution. Several mobile phases (formic acid, NH₄ formate, acetate or bicarbonate in combination with MeOH or ACN) and pH (5 and 6) were also evaluated to obtain less tailing and a better separation of the nine nucleotides. Best results were obtained with bicarbonate ammonium 50 mM at pH 6 (see chromatogram in supplementary data **Fig.S1**).

3.2. Mass spectrometry

The source optimizer of the Agilent Mass Hunter Workstation® (version B.07.01, Agilent Technologies) was used to determine gas temperature, gas flow, nebulizer pressure, sheath gas heater, sheath gas flow, capillary voltage, high pressure RF (iFunnel) voltage and low pressure RF (iFunnel) voltage in positive and in negative mode separately. Both modes showed equivalent areas for uridine nucleotides but for the other nucleotides, the best results were obtained with the positive mode (areas between 3,000 and 70,000 in negative mode and between 15,000 and 500,000 for positive mode according the different nucleotides for a concentration of 500 nM of each nucleotide). The signal to noise data in negative mode were better for monophosphate nucleotides (from 460 to 19,000 for negative mode compare to a signal to noise from 300 to 12,000 for positive mode) but the positive mode showed better signal to noise data for the other nucleotides (from 35 to 50,000 for positive mode compare to a signal to noise from 4 to 13,200 for negative mode). The optimisation of fragmentation was then carried out for each nucleotide thanks to the optimizer of the Agilent Mass Hunter Workstation® (version B.07.01, Agilent Technologies). Precursors, product ions, collision energy for each transition were optimized (Table S2).

3.3. Validation characteristics

3.3.1. Selectivity

Figure 1 illustrates a chromatograph of sample containing the nine nucleotides. Standards containing one nucleotide comparing to standards containing all of them did not show any difference in peak areas, calculated concentrations or retention time.

3.3.2. Samples stability

After 5 freezing-thawing cycles, no degradation of the samples was observed for ADP, ATP, cGMP and UTP. A degradation of 7 % was observed for GMP, 4 % for AMP, 3 % for UMP and UDP and 2 % for cAMP. After 24 hours, no degradation of the samples was observed for AMP, ATP, cAMP, UMP and UDP. A degradation of 6 % was observed for GMP, 5 % for cGMP, 4 % for UTP and 3 % for ADP.

3.3.3. Accuracy, precision and recovery

The calibration curves were evaluated on 9 levels of standards over the physiological concentrations: 1 – 10,000 nM. A quadratic regression was obtained for all the nucleotides in this concentration range when a weighting factor of log was used (see in supplementary data, **Fig. S2**). Repeatability (within-day variances), precision (between-day variances) and trueness were performed to draw up method performance (accuracy) and were estimated from 3 batches of samples in triplicate, as previously described. Repeatability and precision were expressed as coefficient of variations (CV, %). trueness was expressed as the recovery (%), thanks to spiked samples. For the EtOH/lyophilisation method, the repeatability was ranged between 2 % and 13 %. The corresponding values for precision were ranged between 1 % and 9 %. Recoveries were ranged between 94 % and 105 % and are illustrated in accuracy profiles (see in supplementary data, **Fig. S3**). For the PCA method, the repeatability ranged between 1 % and 10 %. The corresponding values for precision ranged between 3 % and 10 %. Recoveries ranged between 93 % and 106 % and are illustrated in accuracy profiles (see in supplementary data, **Fig. S4**). Detailed results regarding above characteristics are shown for each nucleotide in supplementary data (**Table S3**).

3.3.4. Matrix effect

Nucleotides were extracted from plasma and red blood cells with EtOH or PCA 8 % and water containing ISTD. With the EtOH method, the results show a decrease of intensity for each nucleotide in plasma compared to results obtained with ISTD in water (from 50 to 70 % of decrease in intensity) but the ISTD normalized the plasma curves without any problem except for AMP and cAMP where a correction factor was needed with the EtOH/lyophilisation method (see in supplementary data, **Fig.**

S5). For the PCA method no correction factor was needed as all nucleotides were properly recovered with the ISTD and the increase in intensity for each nucleotide in plasma compared to results obtained with ISTD in water was lower than with the EtOH method (from 20 to 35 % of decrease in intensity).

3.3.5. Limit of quantification

EtOH/lyophilisation: Detailed results for each nucleotide are shown in **Table 1**. LOQ's were between 5 fmol and 5 pmol, based on the quality requirement of $S/N \geq 10:1$. Indeed at the LOQ, S/N ratios were ranged between 14:1 and 42:1.

PCA: Detailed results for each nucleotide are shown in **Table 2**. LOQ's were between 0.1 and 500 fmol, based on the quality requirement of $S/N \geq 10:1$. Indeed at the LOQ, S/N ratios were ranged between 10:1 and 1067:1. For cAMP it was possible to decrease the LOQ even more but in this project it was not necessary.

3.3.6. Comparison with commercially available assay kits

To validate the method, the results obtained with the LC/MSMS method were compared to those obtained with different commercially available kits: a cAMP Enzyme Immunoassay kit, an ATP Bioluminescent Assay kit and an ADP assay kit. The cAMP enzyme immunoassay kit was performed on the cytoplasmic pool of endothelial cells EA.Hy926 according to the kit protocol. The cytoplasmic pool was extracted with HCl 0.1 M. The lysate of the cytoplasmic pool of endothelial cells analysed with the kit showed a mean $\pm$ SD cAMP concentration of 89 ± 5 nM compared to 95 ± 3 nM for the LC/MSMS method with a p value = 0.0059 and a relative error of 6.9 %.

Comparison of the results obtained with the QqQ vs results obtained with the cAMP Enzyme Immunoassay kit volunteer per volunteer is illustrated in supplementary data, **Fig.S5**.

A correction factor was used to convert results in concentration in the cytoplasmic pool and the results showed a mean $\pm$ SD cAMP concentration of 5.1 ± 0.3 μ M compared to 5.4 ± 0.2 μ M for the LC/MSMS method with a p value = 0.0059 and a relative error of 6.9 %.

The ATP bioluminescent assay kit was performed on supernatant and cytoplasmic pool of endothelial cells EA.Hy926 according to the kit protocol. The cytoplasmic pool was extracted with RIPA buffer. The supernatants of endothelial cells analysed with the kit showed a mean $\pm$ SD ATP concentration of 351 ± 71 nM compared to 311 ± 46 nM for the LC/MSMS method with a p value = 0.0211 and a relative error of 11,7 %.

Comparison of the results obtained with the QqQ vs results obtained with the ATP Bioluminescent kit volunteer per volunteer in supernatant of endothelial cells is illustrated in supplementary data, **Fig.S6**.

The lysate of the cytoplasmic pool obtained with the kit showed a mean $\pm$ SD ATP concentration of 301 ± 17 nM compared to 12 ± 1 μ M for the LC/MSMS method (relative error of 4017 %). This discrepancy between methods could be explained since RIPA buffer seemed to interfere with the luciferase and decreased the intensity of the luminescence, a calibration curve in RIPA buffer was performed (see supplementary data **Fig. S6**). The results obtained for the lysate of the cytoplasmic pool with the calibration curve in RIPA buffer showed a mean $\pm$ SD ATP concentration of 13.3 ± 0.8 μ M compared to 12 ± 1 μ M for the LC/MSMS method with a p value = 0.0689 and a relative error of 6.2 %.

Comparison of the results obtained with the QqQ vs results obtained with the ATP Bioluminescent kit volunteer per volunteer in cytoplasmic pool of endothelial cells with RIPA calibration curve is illustrated in supplementary data, **Fig.S7**.

A correction factor was used to convert results in concentration in the cytoplasmic pool and the results showed a mean $\pm$ SD ATP concentration of 0.761 ± 0.044 mM compared to 0.709 ± 0.061 mM for the LC/MSMS method with a p value = 0.0689 and a relative error of 6.2 %.

The ADP bioluminescent assay was performed on supernatant and cytoplasmic pool of endothelial cells EA.Hy926 according to the kit protocol. The cytoplasmic pool was extracted with RIPA buffer. The calibration curve of the assay kit was also performed in RIPA buffer to take into account its interference as observed with the ATP Bioluminescent Kit. The supernatant of endothelial cells analysed with the kit showed a mean $\pm$ SD ADP concentration of 183 ± 15 nM compared to 174 ± 22 nM for the LC/MSMS method with a p value = 0.0916 and a relative error of 5.6 %.

Comparison of the results obtained with the QqQ vs results obtained with the ADP assay kit volunteer per volunteer in supernatant of endothelial cells is illustrated in supplementary data, **Fig.S8**.

The lysate of the cytoplasmic pool showed a mean $\pm$ SD ADP concentration of 1.38 ± 0.23 μ M compared to 1.42 ± 0.23 μ M for the LC/MSMS method with a p value = 0.6384 and a relative error of 2.3 %.

Comparison of the results obtained with the QqQ vs results obtained with the ADP assay kit volunteer per volunteer in cytoplasmic pool of endothelial cells lysed with RIPA is illustrated in supplementary data, **Fig.S9**.

A correction factor was used to convert results in concentration in the cytoplasmic pool and the results showed a mean $\pm$ SD ATP concentration of $79 \pm 10 \mu\text{M}$ compared to $81 \pm 13 \mu\text{M}$ for the LC/MSMS method with a p value = 0.6384 and a relative error of 2.3 %.

3.4. Nucleotides concentration in biological matrices

After the validation and the comparison with commercially available kits, the method was used to quantify nucleotides in cultured endothelial cells, human plasma and red blood cells. For human plasma and red blood cells, two blood samples were taken on each volunteer, one before venous stasis and the other after a 4 minutes venous stasis to mimic aneurysm post stenting. The precipitation of the samples was done with the PCA method because the intensities of the signals were higher than those obtained with EtOH/lyophilisation (areas between 400 and 3,000,000 for EtOH/lyophilisation and between 4,000 and 6,000,000 for PCA according to the different nucleotides for a concentration of $10 \mu\text{M}$ of each nucleotide). The direct consequence was the capacity of the method to quantify lower concentrations in samples. Moreover, the PCA method was quicker and more convenient than the EtOH/lyophilisation method.

3.4.1. Plasma analysis

Based on the PCA precipitation method, analysis of plasma from healthy donors before venous stasis showed the presence of mean $\pm$ SD AMP ($0.5 \pm 0.2 \mu\text{M}$), cAMP ($3.5 \pm 0.1 \mu\text{M}$), ADP ($0.7 \pm 0.2 \mu\text{M}$), ATP ($5 \pm 1 \mu\text{M}$), and cGMP ($2.42 \pm 0.03 \mu\text{M}$). The analyses did not show the presence of GMP, UMP, UDP and UTP in human plasma. Analysis of plasma from healthy donors after venous stasis showed the presence of mean $\pm$ SD AMP ($0.6 \pm 0.2 \mu\text{M}$), cAMP ($3.6 \pm 0.2 \mu\text{M}$), ADP ($0.7 \pm 0.1 \mu\text{M}$), ATP ($4.6 \pm 0.8 \mu\text{M}$), and cGMP ($2.44 \pm 0.07 \mu\text{M}$). The analyses did not show the presence of GMP, UMP, UDP and UTP in human plasma.

3.4.2. Blood samples analysis

Analysis of red blood cells from healthy donors before venous stasis showed the presence of mean $\pm$ SD AMP ($1443 \pm 317 \mu\text{M}$), cAMP ($4.8 \pm 0.2 \mu\text{M}$), ADP ($1038 \pm 235 \mu\text{M}$), ATP ($268 \pm 68 \mu\text{M}$), GMP ($150 \pm 27 \mu\text{M}$), cGMP ($2.2 \pm 0.2 \mu\text{M}$) and UDP ($60 \pm 18 \mu\text{M}$). The analyses did not show the presence of UMP and UTP in human red blood cells. Analysis of red blood cells from healthy donors after venous stasis showed the presence of mean $\pm$ SD AMP ($1582 \pm 430 \mu\text{M}$), cAMP ($4.7 \pm 0.3 \mu\text{M}$), ADP ($1256 \pm$

235 μM), ATP ($340 \pm 98 \mu\text{M}$), GMP ($177 \pm 48 \mu\text{M}$), cGMP ($2.2 \pm 0.1 \mu\text{M}$) and UDP ($67 \pm 17 \mu\text{M}$). The analyses did not show the presence of UMP and UTP in human red blood cells. The blood samples before and after venous stasis showed significant differences for some nucleotides: in plasma, the concentrations of ATP and ADP decreased ($p < 0.01$ for ATP and $p < 0.05$ for ADP) whereas the concentration of AMP increased ($p < 0.05$) (**Fig.6**); in red blood cells, the concentration of ADP and ATP after venous stasis increased ($p < 0.05$ for ATP and ADP) (**Fig.7**).

3.4.3. Endothelial cells analysis

The analysis of the cytoplasmic pool of cultured endothelial cells EA.Hy926 lysed by RIPA buffer showed the presence of AMP, cAMP, ADP, ATP, GMP, cGMP, UMP, UDP and UTP (Table 3). The analysis of the supernatant of cultured endothelial cells EA.Hy926 showed the presence of AMP, cAMP, ADP, ATP, GMP, cGMP, UMP and UDP but no presence of UTP.

4. Discussion

Nucleotides, as second messenger (cAMP, cGMP) or extracellular nucleotide receptors agonists (ADP, ADP, ATP, UDP, UTP) play several roles in physiological and pathological conditions, including inflammatory processes. Their quantification could therefore be important to improve our knowledge in pathologies which are not fully understood. In this project, we developed and validated a new LC/MSMS method able to quantify rapidly and easily nine nucleotides in biological extracellular fluids as well as in cytoplasmic preparation of cells. This method turned out to be sensitive and accurate (LOQ between 0.1 and 500 fmol, and accuracy between 93 and 106 %) and the comparison with the commercially kits for cAMP, ADP and ATP proved its robustness and the better throughput of the LC/MSMS method (maximally 11.7% of relative error). Moreover, this method does not require counter-ion and so avoid the daily cleaning of the mass spectrometer. The statistics for the cytosolic pool of the endothelial cells analysed with the cAMP kit and for the supernatant of endothelial cells analysed with the ATP kit compared to the LC/MSMS method showed p values < 0.05 but the relative errors stayed under 12 %. According to **Table 3a** and **Table 3b**, most of the nucleotides quantified in human red blood cells and plasma were in the ranges described in literature except for cyclic nucleotides. The differences between this new method and those described in literature are that citrate tubes were used for blood samples instead of heparinised tubes and that the precipitation was done with PCA and not MeOH or ACN. Cyclic nucleotides are rapidly metabolized in human blood at room temperature through enzymatic processes and our approach probably stabilized better cAMP and cGMP.

The quantification of nucleotides in endothelial cells is poorly described in the literature. Therefore, it is difficult to compare our results with literature. The quantification of some nucleotides in other cells like tumorous cells, monocytes, lymphocytes, neutrophils or eosinophils are available in the literature but they are not comparable to our results.

According to the published data, those nine nucleotides have variable concentrations in plasma and erythrocytes. It depends on gender, age, health and disease... As a consequence, it is very difficult to compare those results. However, some of the nucleotides are in the range founded in the literature (see **Table 4 and Table 5**) and plasma results demonstrate that we are able to better recover cyclic nucleotides compared to other methods in the literature. More interesting is the variation of nucleotide concentration in different conditions in the same volunteer or patient such as before and after exercise, stenting, dialyse, inflammation, sepsis With the purpose to illustrate changes of nucleotides rates in different conditions, a venous stasis was used as a model to observed hemorheological changes induced by prolonged tourniquet [20]. Results demonstrated a significant increase of AMP with a decrease of ADP and ATP in plasma, while ADP and ATP concentrations increased in red blood cells. ATP is known to be released by red blood cells during ischemia or hypoxia[21] which should involve an increase of the extracellular concentration of ATP but ADP can also activate a negative feedback pathway for ATP release from erythrocytes via P2Y₁₃ receptors [12]. Moreover, after being released by red blood cells, ATP binds immediately to purinergic receptors covering the increase of ATP concentration that should be observed [22]. ADP is known to bind the P2Y₁ receptors while ATP binds the P2Y₂ receptors to induce vasodilation in case of ischemia, hypoxia, platelet aggregation, sympathetic nerve stimulation, or cellular damage and this can explain the decreasing concentration of both nucleotides after the venous stasis [23–25]. Moreover, extracellular ATP can be quickly degraded in ADP and then in AMP by ATPase's that are associated with erythrocytes membranes, which could also explain the phenomenon associated with an increase of AMP concentration post stasis [24,26]. ATP can also be sequestered in erythrocytes which could explain the increasing of ATP concentration in red blood cells after venous stasis. Upon signals (chemical, mechanical, or physiological), ATP can be released but several stimuli are needed to induce deformability of erythrocytes inducing the release of ATP and that may explain why the results did not show an increase in the plasmatic concentration of ATP [27,28]. As ATP is quickly degraded in ADP by ATPase, this could also explain the increasing in ADP concentration in erythrocytes after the stasis. More samples have to be analysed to support and confirm the mechanistic underlying the described nucleotide variations.

5. Conclusion

A LC/MSMS method was developed and validated to quantify AMP, cAMP, ADP, ATP, GMP, cGMP, UMP, UDP and UTP in plasma and cells. Good sensitivity compatible with the very low concentration of some nucleotides was achieved. All nucleotides which seem to be implicated in cells signalling during many conditions (inflammatory syndromes, cells differentiation...) can be quantified simultaneously within less than 10 minutes, without counter-ion. The results obtained with this method are similar to those obtained with commercially available assay kits for three of the nine nucleotides (cAMP, ADP and ATP). Furthermore, the analysis of endothelial cells, human plasma and red blood cells permitted to have an idea of the concentration of those nucleotides in normal conditions. The analysis of human red blood cells and plasma after a venous stasis of 4 minutes showed an interesting modification in AMP, ADP and ATP rate. This study allows new investigations for the implication of nucleotides in inflammatory conditions (as atherosclerosis, aneurysm or sepsis), cell differentiation, apoptotic cell clearance, etc., in order to better understand these not fully understood phenomena.

Acknowledgments

This study was supported by a PDR from the Belgian Fund for Scientific Research (FRS-FNRS, PDR T.0136.13) and David and Alice van Buuren Funds. M. Cortese is research fellow of the FRS-FNRS, C. Delporte is a postdoctoral researcher funded by the FRS-FNRS.

References

- [1] M. Idzko, D. Ferrari, A. Riegel, H.K. Eltzschig, Extracellular nucleotide and nucleoside signaling in vascular and blood disease, *Blood* 124 (2014) 1029–1037. doi:10.1182/blood-2013-09-402560.
- [2] K. Mujoo, J.S. Krumenacker, F. Murad, Nitric oxide-cyclic GMP signaling in stem cell differentiation, *Free Radic. Biol. Med.* 51 (2011) 2150–2157. doi:10.1016/j.freeradbiomed.2011.09.037.
- [3] F.B. Chekeni, K.S. Ravichandran, The role of nucleotides in apoptotic cell clearance: Implications for disease pathogenesis, *J. Mol. Med.* 89 (2011) 13–23. doi:10.1007/s00109-010-0673-7.

- [4] B. a Chizh, P. Illes, P2X receptors and nociception., *Pharmacol. Rev.* 53 (2001) 553–68. <http://www.ncbi.nlm.nih.gov/pubmed/11734618>.
- [5] C. Schudt, A. Hatzelmann, R. Beume, H. Tenor, Phosphodiesterases as drug targets, 2011. doi:10.1007/978-3-642-17969-3.
- [6] T.J. Chong, G.P. Victorino, Cyclic nucleotide second messengers (cAMP and cGMP) play a central role in signal transduction and regulation of mesenteric postcapillary fluid leak, *J.Trauma.* 59 (2005) 302–306. doi:10.1097/01.ta.0000180385.23675.98.
- [7] J. Surapisitchat, K.I. Jeon, C. Yan, J.A. Beavo, Differential regulation of endothelial cell permeability by cGMP via phosphodiesterases 2 and 3, *Circ Res.* 101 (2007) 811–818. doi:10.1161/CIRCRESAHA.107.154229.
- [8] G. Burnstock, Local control of blood pressure by purines, (1987) 24:156–160.
- [9] J. Lecka, S. Molski, M. Komoszynski, Extracellular-purine metabolism in blood vessels (part I). Extracellular-purine level in blood of patients with abdominal aortic aneurysm, *Nucleosides Nucleotides Nucleic Acids.* 29 (2010) 647–657. doi:10.1080/15257770.2010.502164.
- [10] J. Lecka, E. Bloch-Boguslawska, S. Molski, M. Komoszynski, Extracellular purine metabolism in blood vessels (Part II): Activity of ecto-enzymes in blood vessels of patients with abdominal aortic aneurysm., *Clin. Appl. Thromb. Hemost.* 16 (2010) 650–7. doi:10.1177/1076029609354329.
- [11] O. Gidlöf, R. Sathanoori, M. Magistri, M.A. Faghihi, C. Wahlestedt, B. Olde, D. Erlinge, Extracellular uridine triphosphate and adenosine triphosphate attenuate endothelial Inflammation through miR-22-mediated ICAM-1 inhibition, *J. Vasc. Res.* (2015) 71–80. doi:10.1159/000431367.
- [12] L. Wang, G. Olivecrona, M. Götberg, M.L. Olsson, M.S. Winzell, D. Erlinge, ADP acting on P2Y13 receptors is a negative feedback pathway for ATP release from human red blood cells, *Circ. Res.* 96 (2005) 189–196. doi:10.1161/01.RES.0000153670.07559.E4.
- [13] T. Kimura, S. Kobayashi, F. Hanihara-Tatsuzawa, A. Sayama, T. MaruYama, T. Muta, Responses of macrophages to the danger signals released from necrotic cells, *Int. Immunol.* 26 (2014) 697–704. doi:10.1093/intimm/dxu080.
- [14] M.W. Gorman, D.R. Marble, K. Ogimoto, E.O. Feigl, Measurement of adenine nucleotides in plasma, *Luminescence.* 18 (2003) 173–181. doi:10.1002/bio.721.

- [15] Y. Jiang, C. Sun, X. Ding, D. Yuan, K. Chen, B. Gao, Y. Chen, A. Sun, Simultaneous determination of adenine nucleotides, creatine phosphate and creatine in rat liver by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry, *J. Pharm. Biomed. Anal.* 66 (2012) 258–263. doi:10.1016/j.jpba.2012.03.027.
- [16] J. Mei Wang, Y. Chu, W. Li, X. Yang Wang, J. Hua Guo, L. Lu Yan, X. Hui Ma, Y. Li Ma, Q. Hui Yin, C. Xiao Liu, Simultaneous determination of creatine phosphate, creatine and 12 nucleotides in rat heart by LC-MS/MS, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 958 (2014) 96–101. doi:10.1016/j.jchromb.2014.03.008.
- [17] T. Kamčeva, T. Bjånes, A. Svardal, B. Riedel, J. Schjøtt, T. Eide, Liquid chromatography/tandem mass spectrometry method for simultaneous quantification of eight endogenous nucleotides and the intracellular gemcitabine metabolite dFdCTP in human peripheral blood mononuclear cells, *J. Chromatogr. B.* 1001 (2015) 212–220. doi:10.1016/j.jchromb.2015.07.041.
- [18] X. Jia, B.M. Fontaine, F. Strobel, E.E. Weinert, A facile and sensitive method for quantification of cyclic nucleotide monophosphates in mammalian organs: basal levels of eight cNMPs and identification of 2',3'-cIMP., *Biomolecules.* 4 (2014) 1070–92. doi:10.3390/biom4041070.
- [19] B. Zhu, H. Wei, Q. Wang, F. Li, J. Dai, C. Yan, Y. Cheng, A simultaneously quantitative method to profiling twenty endogenous nucleosides and nucleotides in cancer cells using UHPLC-MS/MS, *Talanta.* 179 (2018) 615–623. doi:10.1016/j.talanta.2017.11.054.
- [20] S. Forconi, Hemorheological changes during venous stasis as result of tourniquet application, *Clin. Hemorheol Microcirc.* 20 (1999) 197–199.
- [21] A.W. Lohman, M. Billaud, B.E. Isakson, Mechanisms of ATP release and signalling in the blood vessel wall, *Cardiovasc. Res.* 95 (2012) 269–280. doi:10.1093/cvr/cvs187.
- [22] M.L. Ellsworth, C.G. Ellis, R.S. Sprague, P. Science, Microvascular oxygen supply in skeletal muscle, 216 (2017) 265–276. doi:10.1111/apha.12596.Role.
- [23] M.W. Gorman, E.O. Feigl, C.W. Buffington, Human plasma ATP concentration, *Clin. Chem.* 53 (2007) 318–325. doi:10.1373/clinchem.2006.076364.
- [24] R. Sluyter, P2X and P2Y receptor signaling in red blood cells, *Front. Mol. Biosci.* 2 (2015) 1–7. doi:10.3389/fmolb.2015.00060.
- [25] R.M. Bateman, M.D. Sharpe, M. Singer, C.G. Ellis, The effect of sepsis on the erythrocyte, *Int. J. Mol. Sci.* 18 (2017). doi:10.3390/ijms18091932.

- [26] W.G. O'Brien, H.S. Ling, Z. Zhao, C.C. Lee, New insights on the regulation of the adenine nucleotide pool of erythrocytes in mouse models, PLoS One. 12 (2017) 1–20.
doi:10.1371/journal.pone.0180948.
- [27] E. Rapaport, A. Salikhova, E.H. Abraham, Continuous intravenous infusion of ATP in humans yields large expansions of erythrocyte ATP pools but extracellular ATP pools are elevated only at the start followed by rapid declines, Purinergic Signal. 11 (2015) 251–262.
doi:10.1007/s11302-015-9450-y.
- [28] J. González-Alonso, ATP as a mediator of erythrocyte-dependent regulation of skeletal muscle blood flow and oxygen delivery in humans, J. Physiol. 590 (2012) 5001–5013.
doi:10.1113/jphysiol.2012.235002.

Fig. 1 Chromatograph of a sample containing the nine nucleotides standards

Fig. 2 Comparison of the mean of the results obtained with the QqQ vs mean of the results obtained with the cAMP Enzyme Immunoassay kit.

Fig. 3 Comparison of the mean of the results obtained with the QqQ vs mean of the results obtained with the ATP bioluminescent – supernatant of endothelial cells.

Fig. 4 Comparison of the mean of the results obtained with the QqQ vs mean of the results obtained with the ATP bioluminescent kit – cytoplasmic pool of endothelial cells with RIPA calibration curve.

Fig. 5 Comparison of the mean of the results obtained with the QqQ vs mean of the results obtained with the ADP assay kit – supernatant of endothelial cells

Fig. 6 Comparison of the mean of the results obtained with the QqQ vs mean of the results obtained with the ADP assay kit – cytoplasmic pool of endothelial cells lysed with RIPA.

Fig. 7 : A) Concentration of AMP in plasma of each volunteer before and after venous stasis; B) Concentration of ADP in plasma of each volunteer before and after venous stasis; C) Concentration of ATP in plasma of each volunteer before and after venous stasis

Fig. 8 : A) Concentration of ADP in red blood cells of each volunteer before and after venous stasis; B) Concentration of ATP in red blood cells of each volunteer before and after venous stasis.

Table 1: Limit of quantification of the nine nucleotides with EtOH/lyophilisation method

	cAMP	cGMP	AMP	GMP	ADP	ATP	UMP	UDP	UTP
LOQ (fmol)	5	12.5	25	125	150	750	1250	2500	5000
LOQ (pg)	8	212	43	227	320	1902	2026	5052	12103
S/N (mean)	14	22	33	11	42	40	22	13	27

Table 2: Limit of quantification of the nine nucleotides with PCA method

	cAMP	cGMP	AMP	GMP	ADP	ATP	UMP	UDP	UTP
LOQ (fmol)	0.1	0.2	0.25	62.5	0.9	12.5	250	500	25
LOQ (pg)	0.2	0.3	0.4	113	1.9	32	405	1010	60
S/N (mean)	1067	60	14	25	11	21	25	22	10

Table 3: Concentration in nucleotides the cytoplasmic pool of cultured endothelial cells EA.Hy926 lysed by RIPA buffer and in the supernatant of cultured endothelial cells EA.Hy926

Nucleotides	Cytoplasmic pool of endo cells (μM)	Supernatant of endo cells (μM)
AMP	18 ± 1	0.024 ± 0.007
cAMP	5.4 ± 0.2	0.253 ± 0.007
ADP	81 ± 13	0.17 ± 0.02
ATP	709 ± 61	0.31 ± 0.05
GMP	2.1 ± 0.2	0.043 ± 0.005
cGMP	5.6 ± 0.4	$0.092 \pm .009$
UMP	1.9 ± 0.2	0.5 ± 0.1
UDP	51 ± 6	0.5 ± 0.1
UTP	120 ± 11	/

Table 4: Comparison between published and measured data for nucleotides concentrations in human red blood cells. *ND is for Not Detectable.*

	Published data	Measured data
AMP	0.001 – 50 μ M[19–22]	0.1 μ M
cAMP	4.43 nM[23]	0.5 μ M
GMP	0 – 17 μ M[19,22]	15 μ M
cGMP	3 – 44 nM[23,24]	0.2 μ M
ADP	83 – 390 μ M[19–22]	104 μ M
ATP	102 nM – 2.1 mM[19–22]	27 μ M
UMP	0[22]	ND
UDP	0[19]	6 μ M
UTP	0 – 2.9 μ M[22]	ND

Table 5: Comparison between published and measured data for nucleotides concentration in human plasma. *ND is for Not Detectable, NF for Not Found in the literature*

	Published data	Measured data
AMP	1.5 μ M[25]	0.5 μ M
cAMP	14-55 nM[26–28]	3.5 μ M
GMP	NF	ND
cGMP	0.9 – 15 nM[26,27,29]	2.42 μ M
ADP	0.9 – 14.8 μ M[30]	0.8 μ M
ATP	1 – 13.2 μ M[30]	5 μ M
UMP	NF	ND
UDP	NF	ND
UTP	14 nM[31]	ND

Highlights

- A high throughput method for nucleotide quantification has been developed
- This method allows the quantification of 9 nucleotides in biological samples
- A better sensitivity of the method was observed.
- A better recovery for cyclic nucleotides was obtained
- It allows the quantification of uridine nucleotides in endothelial cells