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Simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection

Erik J.C.M. Coolen [a,b,*], Ilja C.W. Arts [a], Els L.R. Swennen [a,b], Aalt Bast [b], Martien A. Cohen Stuart [c], Pieter C. Dagnelie [a]

[a] *Department of Epidemiology, NUTRIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands*
[b] *Department of Pharmacology and Toxicology, NUTRIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands*
[c] *Department of Agrotechnology and Food Sciences, Laboratory of Physical Chemistry and Colloid Science, Wageningen University, P.O. Box 8038, 6700 EK Wageningen, The Netherlands*

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Abstract

To obtain insight in mechanisms of action of extracellular adenosine triphosphate (ATP) and adenosine, a simple HPLC method has been optimized and applied to investigate ATP metabolism in human whole blood ex vivo. This method provided good chromatographic resolution and peak shape for all eight compounds within a 19 min run time. The baseline was clean, the lower limit of quantification was below 0.3 μmol/L for all adenine nucleotides and the method demonstrated good linearity. Within-day precision ranged from 0.7 to 5.9% and between-days from 2.6 to 15.3%. Simplicity and simultaneous detection of ATP and its metabolites make this method suitable for clinical pharmacokinetic studies.

Keywords: ATP; Blood; HPLC; Adenosine

1. Introduction

The nucleotide profile of blood is relatively simple compared to that of nucleated cells. Because no DNA synthesis occurs, only ribonucleotides are present. The adenine ribonucleotide pool, which in metabolically normal erythrocytes mainly consists of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), is much larger than that of other nucleotides, such as the guanine ribonucleotides (GTP, GDP, and GMP) [1]. Generally, ADP and AMP concentrations are only 12.5–20% and 1–2%, respectively, of the ATP concentrations [2]. Apart from its role as an intracellular energy carrier, extracellular ATP is involved in processes such as neurotransmission, mechanosensory transduction, secretory functions and vasodilatation, and long-term (chronic) signalling functions in development and regeneration of cells [3]. Moreover, once ATP is released, for example through cell lysis during organ injury, it can mediate several inflammatory responses, including the release of cytokines, tumor necrosis factor-α and interleukin-1β from monocytes and macrophages, and facilitation of leukocyte adhesion to the endothelium [4,5]. Proliferation of various in vitro tumor cell lines has been shown to be suppressed after exposure to ATP [6–8]. The pharmacological use of ATP has received increasing attention following reports of its benefit in pain, vascular disease and cancer [9]. Given our interest in the metabolism of ATP and its behaviour after ATP supplementation in humans, we needed a quick and reliable method for the simultaneous measurement of purine nucleotides, nucleosides, and bases in human blood. Given the complexity of the nucleotide breakdown cascade, which involves various enzymes, special care had to be taken to suppress unwanted nucleotide degeneration in fresh samples.

Several methods have been published to measure nucleotides, including an enzyme assay [10], bioluminescence, ^{31}P nuclear magnetic resonance spectroscopy (NMR), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), ion-pair reversed-phase HPLC [11–16], gradient HPLC [17–19], and ion-exchange methods [20]. RP-HPLC lacks some of the drawbacks of other methods, like, for ion-exchange HPLC, the

* Corresponding author at: Department of Epidemiology, NUTRIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Tel.: +31 43 3881343; fax: +31 43 3884149.
E-mail address: erik.coolen@epid.unimaas.nl (E.J.C.M. Coolen).

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need for highly concentrated elution buffers and long analysis times. An advantage of the method is that it allows simultaneous detection of nucleotides, nucleosides and nucleobases in a single run with short times between injections. Given the high concentrations of adenine nucleotides in human blood (1500–1900 μmol/L) [21], there was no need for measurement in the lower nanomolar range, which is for instance achievable by bioluminescence kits.

In the present paper we report a reversed-phase HPLC method for the simultaneous analysis of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, and uric acid in human blood. Sample collection and processing methods were optimized. The application of the method is illustrated by an experiment in which the concentrations of adenine nucleotides are monitored after incubation of human blood samples with ATP, a model used in our lab to study anti-inflammatory properties of ATP and its breakdown products.

2. Materials and methods

2.1. Chemicals

Adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate disodium salt (ADP), adenosine 5′-monophosphate sodium salt (AMP), adenine, inosine, hypoxanthine and uric acid were purchased from Sigma Chemical Co., St. Louis, USA. Adenosine was obtained from Bufa B.V., Uitgeest, The Netherlands. Perchloric acid 70% solution in water (PCA) was purchased from Sigma–Aldrich, Steinheim, Germany. Potassium hydroxide (KOH), potassium dihydrogen phosphate (KH_2PO_4), potassium carbonate (K_2CO_3), di-potassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$) and sodium hydroxide (NaOH) were obtained from Merck, Darmstadt, Germany. 0.9% saline was purchased from Braun, Melsungen, Germany. RPMI 1640 (order number 21875) medium-containing L-glutamine was obtained from Gibco, Paisly, UK.

2.2. Sample preparation

Blood was collected from healthy volunteers by venipuncture in EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). Five hundred microliters of blood was vortex-mixed for 5 s with 500 μL of ice-cold 8% PCA in a 1.5 mL Eppendorf tube. After precipitation of the protein fraction (at 12,000 × *g*, 10 min, 4 °C), insoluble perchlorate was formed by addition of 40 μL 2 M K_2CO_3 in 6 M KOH to 650 μL of supernatant, sufficient to neutralize the pH of the samples. Following centrifugal removal of the perchlorate (12,000 × *g*, 10 min, 4 °C), 40 μL supernatant was mixed with 160 μL of mobile phase A (see Section 2.4 for its composition) in HPLC microvials (Agilent Technologies, Palo Alto, CA, USA).

2.3. Equipment

ATP and its metabolites were quantified using a series 1100 Agilent HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a quaternary gradient pump, a variable wavelength detector (set at 254 nm) and a solvent degasser system. Separation was achieved with a 3 μm particle size Hypersil ODS C18 RP column (150 mm × 4.6 mm i.d.; Thermo Electron Corp., USA) with pore diameter 120 Å, protected by a 5 μm Hypersil C18 guard column (10 mm × 4 mm i.d.; Thermo Electron Corp).

2.4. Separation

A 50 mM phosphate buffer (pH 6.0) (mobile phase A), 100% methanol (mobile phase B) and a flow of 1 mL/min were employed to separate the compounds of interest. Buffers were prepared by diluting 50 mL of a stock solution of 0.6 M K_2HPO_4/0.4 M KH_2PO_4, to 1 L of MilliQ water. The pH was adjusted to 6.0 with concentrated phosphoric acid. From 0 to 2 min, the eluent consisted of 100% mobile phase A. Between 2 and 10 min the amount of mobile phase B increased linearly to 12.5%, and then stayed like that for 2 min. Finally, between 12 and 17 min, the gradient returned linearly to 100% mobile phase A. The column was equilibrated between injections for 2 min, leading to a total run time of 19 min. Twenty-five microliters of the sample was injected. The autosampler temperature was set at 4 (±2) °C. The column was kept at room temperature (20 ± 2 °C).

2.5. Standard solutions

All purine standards were treated in a manner similar to the blood samples. First, the standards were individually dissolved in mobile phase A to a concentration of 4 mM (ATP: 8 mM). Uric acid was dissolved in 0.1 M NaOH instead of mobile phase A. Next, equal volumes of four compounds were mixed and added to 8% PCA in a final ratio of mobile phase A to 8% PCA of 1:1. This resulted in two mixtures with concentrations of 500 μM of each compound (ATP: 1000 μM). The mixtures were subsequently prepared as described in Section 2.2 and finally diluted with mobile phase A to acquire a standard range which reflects pre-preparation concentrations of 10–500 μM (ATP: 20–1000 μM). Concentrations of ATP and its metabolites in blood were determined by comparing peak areas to appropriate standards using Chemstation software (Version A.09.03; Agilent, Palo Alto, CA, USA). A mathematical adjustment is applied to correct for the experimentally determined 20% increase in metabolite concentration measured in the supernatant, which occurs due to protein precipitation in blood samples. The stability of ATP was determined using 500 μM ATP solutions in mobile phase A that were (a) stored at −20 °C for 2 or 7 days, (b) stored at 4 °C for 1 day, or (c) incubated at 37 °C for 1 h. The percentage of degradation products formed was used as a measure for the amount of degradation.

2.6. Assay validation

Linearity of the assay was assessed using three calibration curves analyzed on separate days. The curves were constructed by plotting the peak areas against the concentration of the sample.

The within-day coefficient of variation (CV) was determined by repeated analysis of five aliquots of a single volume of whole blood. The blood was spiked with high and low concentrations of standards. Between-day CV was determined by analyzing, on five different days, a blood sample that was collected from one subject, mixed with PCA and then stored in five aliquots at −20 °C awaiting further sample preparation and analysis. The noise was determined by Chemstation software in the time range between 10.5 and 13 min after injection of 10 separate whole blood samples. The limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated by multiplying the SD of the noise by 3 and 10, respectively. The values for all compounds are presented as concentrations in whole blood, in order to present the actual limits that are achievable by our method.

Recovery data of all eight compounds were obtained in duplicate by adding known amounts of nucleotide standards (individually dissolved in mobile phase A (uric acid in 0.1 M NaOH)) or a blank solution (mobile phase (A) to blood samples immediately after collection, at high and low spike concentrations. Preparation then continued as described above for both these samples and for samples containing spikes in buffer. Recovery was calculated as

$$\text{Recovery}\,(\%) = 100 \times ([\text{blood} + \text{spike}] - [\text{blood} + \text{blank}]) \times ([\text{spike}])^{-1}$$

2.7. Optimization

2.7.1. Sample handling

By using the method of Schweinsberg and Loo [2] as a starting point, optimizations were performed with blood samples obtained from healthy volunteers. The optimization of sample preparation included varying (a) the anticoagulant in the blood collection tube (EDTA or Li-Heparin), (b) the timing of protein precipitation by PCA after blood collection (directly after blood collection or after storage at −80 °C), (c) thawing conditions after cold storage (at room temperature or on ice), (d) the precipitation of PCA (separate from, or simultaneous with pH neutralization by KOH), and (e) the sample pH (pH range 3–6). The effects of changing these parameters were rated optically based on peak shape, yield and compound separation on the HPLC chromatogram.

2.7.2. Chromatographic conditions

The parameters varied for optimization of the chromatographic conditions were: (a) elution buffer pH (range 3–6) and phosphate concentration (range 50–100 mM), (b) timing of the elution gradient, and (c) injection volume.

2.8. Application

After optimization, we applied the method to investigate the metabolism of ATP added to whole blood ex vivo, and more specifically, the effects of dilution of the blood with saline or RPMI 1640 medium on the concentration of ATP. This experiment was a follow-up to an earlier study by Swennen et al. [22], in which the ex vivo whole blood model was used to investigate the immunoregulatory effects of ATP. In this model, whole blood was diluted fourfold with RPMI 1640 medium, resulting in an approximate ATP half-life of 2 h. We set out to determine the effect of the dilution factor and type of dilution medium on the ATP metabolism, since literature reports half times of 15 min in undiluted set-ups [23].

Blood from a healthy volunteer was collected in heparin-containing vacutainer tubes and directly put on ice (Vacutainer, 170 IU). Use of EDTA as an anticoagulant was avoided, given our interest in ATP breakdown. Next, appropriate volumes of blood were put in 6- or 24-well plates and spiked with a known volume of 6 mM ATP in MilliQ, saline or medium. Next, the spiked blood was used either undiluted or diluted two- or fourfold by mixing gently with saline or medium. The final concentration of ATP was 300 μM in each well. Samples of 450 μL were taken at 0, 2, 4, 8, 15, 30 min and 2, 4, and 24 h after addition of the ATP spike. Plates were incubated at 5% CO_2 and 37 °C between sampling. Upon collection, samples were centrifuged (3000 × *g*, 10 min, 4 °C), 100 μL of cell-free supernatant was transferred to a clean Eppendorf tube and stored at −80 °C. ATP and its degradation products were determined as described above. Standard solutions were prepared, as described in Section 2.5, and used to calculate the concentrations of ATP and its degradation products. This resulted in time-dependent degradation profiles for undiluted, and two- and fourfold diluted blood.

3. Results

3.1. Optimization of the sample handling

The goal of optimizing sample handling was to prevent loss of adenosine nucleotides, which are normally rapidly degraded by ecto-enzymes located on the plasma membrane. Lower concentrations of nucleotides were present when blood collection tubes containing the anticoagulant Li-heparin were used instead of EDTA-containing tubes (data not shown). Furthermore, the precipitation of proteins by mixing blood with PCA was found to be most effective in retaining endogenous nucleotides when done directly following blood collection. This in comparison to the situation in which the whole blood was mixed with PCA after it had been stored at −80 °C and then thawed. The ATP concentration was lowered on average by 8% in the latter case. The samples of blood mixed with PCA could either be stored at −80 °C or processed further for direct HPLC measurement without affecting the nucleotide concentration. After cold storage, equal levels of adenine nucleotides were found, regardless of whether the samples were thawed on ice or at room temperature (data not shown).

During sample preparation, it was found that precipitation of PCA in the samples with K_2CO_3 could be performed together with neutralization by KOH without loss of nucleotides. Optimal yield and peak shapes were obtained when all samples were neutralized with buffer to pH 6.0. At pH 3, 4 and 5, peak shapes notably worsened, affecting separation and yield of the various

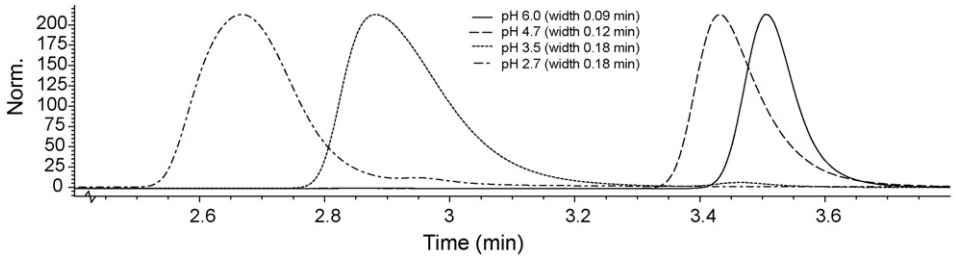

Fig. 1. Chromatogram showing the effects of buffer pH on peak shape and retention time after separate injections of ATP (100 μmol/L). Peaks are overlaid on the same time-axis, and normalized to equal height on the *Y*-axis. Only the relevant part of the *X*-axis is shown. Widths reported were taken at half height of the peaks.

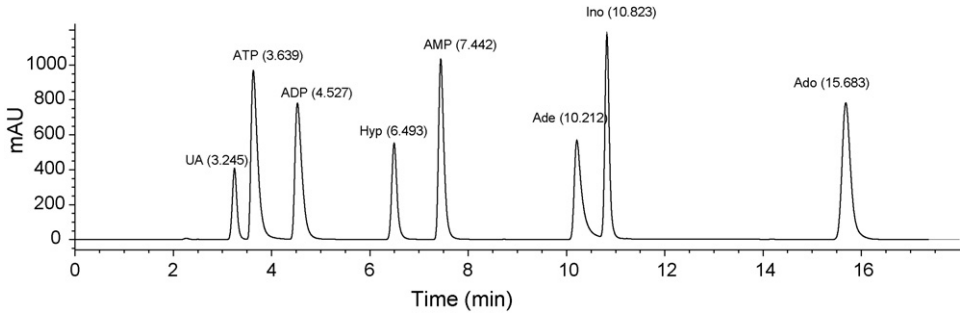

Fig. 2. Chromatogram of 500 μmol/L of uric acid (UA), ATP, ADP, hypoxanthine (Hyp), AMP, Adenine (Ade), inosine (Ino) and adenosine (Ado). Conditions of the separation are as described in Section 2.3.

compounds. An example of the effects of buffer pH on the ATP peak is displayed in Fig. 1.

3.2. Optimization of chromatographic conditions

Peak shape and compound separation benefited from a buffer pH of 6.0 and a phosphate concentration of 50 mM (data not shown). The elution gradient used was a compromise between a fast analysis time and sufficient separation of the peaks. Higher methanol concentrations resulted in overlapping peaks of the compounds of interest. By returning the methanol concentration to 0–12 min after injection, the amount of time needed for column equilibration was limited to 2 min. Finally, the sample injection volume was set at 25 μL, since higher loading volumes resulted in worse separation of ATP and uric acid, especially in blood samples, in which both are the most abundant compounds. A representative chromatogram of the separation of eight purine standards in one run is shown in Fig. 2.

3.3. Assay validation

Chromatograms of unspiked and spiked whole blood samples are shown in Fig. 3. Calibration curves were calculated using peak areas at six standard concentrations, the range of which was proportional to the concentration of analyte in the prepared whole blood samples. The concentration range was linear from 1 to 500 μmol/L for all analytes. The regression coefficient for all calibration curves was greater than 0.999 (Table 1).

Within-day CV (range 0.7–5.9%) and between-day CV (2.6–15.3%), LOD and LLOQ are presented for every compound in Table 2. The concentrations reported represent the actual LOD and LLOQ of compounds in blood, since dilution of the blood necessary for sample preparation is taken into account. The LOD were all below 0.15 μmol/L and the LLOQ below 0.5 μmol/L.

The recovery of the method after addition of known amounts of compounds to whole blood samples ranged between 58 and 108% as presented in Table 3. The concentrations of ATP, ADP, and AMP found in the whole blood were 1217 ± 75, 95 ± 15, and

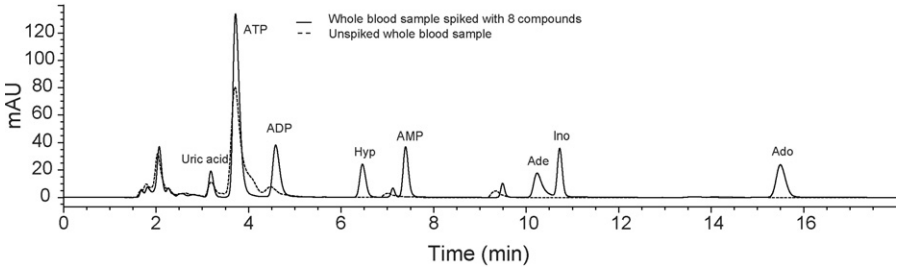

Fig. 3. Chromatogram of two whole blood samples. One sample was spiked with a mix of eight compounds (solid line) followed by immediate sample preparation as described in Section 2.2. In the fresh unspiked whole blood sample, only uric acid, ATP, ADP and AMP are detected (dashed line).

Table 1
Summary of linearity (range, slope, r^2, and intercept values) and analyte retention times

Analyte	Linearity				Retention time (min)
	Range (μmol/L)	r^2	Slope ± SD	Intercept ± SD	
ATP	1–500	0.999	9.59 ± 0.01	17.27 ± 7.27	3.6
ADP	1–500	0.999	8.25 ± 0.01	8.93 ± 6.62	4.5
AMP	1–500	0.999	8.54 ± 0.01	17.02 ± 6.30	6.5
Hypoxanthine	1–500	0.999	5.55 ± 0.01	42.76 ± 4.36	7.4
Uric acid	1–500	0.999	2.91 ± 0.002	5.77 ± 1.74	3.2
Adenine	1–500	0.999	7.04 ± 0.01	14.35 ± 3.96	10.2
Adenosine	1–500	0.999	10.37 ± 0.02	14.88 ± 8.43	15.7
Inosine	1–500	0.999	8.23 ± 0.07	34.34 ± 2.79	10.8

For each analyte $N = 9$ datapoints were used.

Table 2
Summary of precision and detection limits of ATP, ADP, AMP, hypoxanthine, adenine, adenosine, inosine and uric acid (CV, coefficient of variation; LOD, limit of detection; LLOQ, lower limit of quantification)

Analyte	Within-day precision		Between-day precision		LOD (μmol/L)	LLOQ (μmol/L)
	Mean (μmol/L), $N = 5$	CV (%)	Mean (μmol/L), $N = 5$	CV (%)	$N = 10$	$N = 10$
ATP	1003.0	0.7	974.3	3.6	0.049	0.162
ADP	356.5	0.9	357.3	5.5	0.072	0.239
AMP	241.1	1.3	244.5	2.6	0.052	0.172
Hypoxanthine	169.9	5.9	206.1	15.3	0.082	0.272
Uric acid	304.9	1.6	326.4	11.5	0.121	0.405
Adenine	167.5	3.2	194.4	10.4	0.090	0.299
Adenosine	226.1	1.7	221.8	4.3	0.070	0.232
Inosine	206.0	2.1	234.8	15.0	0.059	0.197

Between-day precision of five aliquots of a single blood sample, measured on 5 different days spread over a period of 7 months. Within-day precision is calculated after repeated analysis ($N = 5$) of multiple aliquots of a single volume of whole blood, spiked with known amounts of standards. Values are the mean concentrations and the coefficient of variation (CV). The LOD and LLOQ were calculated as 3 and 10 times the SD of the noise of the UV baseline in untreated whole blood samples ($N = 10$) in the time range of 10.5–13 min after injection.

Table 3
Recovery data for ATP, ADP, AMP, Hypoxanthine, adenine, adenosine, inosine and Uric Acid in whole blood

Compound	Blood (μmol/L)	Spike (μmol/L)	Blood + spike (μmol/L)	Recovery (%)
ATP	1216.9	2271.2	3462.1	98.9
		1087.5	2393.0	108.2
ADP	94.5	583.4	664.8	97.8
		305.0	393.8	98.1
AMP	27.1	546.3	495.9	85.8
		285.7	271.0	85.4
Adenine	42.3	648.2	493.1	69.6
		381.5	277.3	61.6
Adenosine	0	588.5	483.6	82.2
		289.6	242.1	83.6
Inosine	0	569.5	479.4	84.2
		283.7	237.7	83.8
Hypoxanthine	1.6	755.9	444.2	58.6
		424.0	262.7	61.7
Uric acid	252.2	589.4	613.2	69.8
		316.0	419.3	68.8

Standard solutions (spike) were added to a whole blood sample with predetermined metabolite levels (blood). The resulting metabolite concentration of the mixture (blood + spike) was determined. The concentrations presented are averages of $N = 5$ blood samples and $N = 5$ blood + spike samples at each concentration.

27.1 ± 6 μmol/L, respectively, and the ATP/ADP and ATP/AMP ratios were 12.8 and 44.9, respectively.

The stability of ATP in mobile phase A was assessed for three storage conditions. The proportion of degradation products detected was on average 1% of ATP for storage at 4 °C for 1 day and 37 °C for 1 h, and 2% for storage and thawing at −20 °C for 7 days.

3.4. Application

In this experiment, the effects of dilution of blood with saline or RPMI 1640 medium on ATP metabolism were investigated in an ex vivo set-up. Fig. 4 displays the degradation of ATP added at $t = 0$ to the undiluted (A), and twofold (B and C) and fourfold (D and E) diluted aliquots of blood. Adenine, adenosine and inosine were not detected. Besides uric acid, none of the other compounds were present in samples taken before the addition of ATP.

In the undiluted situation, the ATP concentration declined steadily until complete degradation after 30 min. The ADP concentration doubled 15 min after incubation, followed by a decline to zero after 2 h. AMP rose between 2 and 30 min, with the strongest increase between 15 and 30 min, followed by a decline to zero after 4 h. Hypoxanthine was first formed after 30 min incubation and kept rising sharply until the final measurement after 19 h. In contrast to the other metabolites, uric acid levels were quite stable with only a minor increase at the later stages of the incubation.

In the twofold diluted situation, every compound behaved similarly, but with a time-frame that was shifted by approximately 90 min. Two notable differences are (a) the near simultaneous disappearance of ADP and ATP, instead of ATP

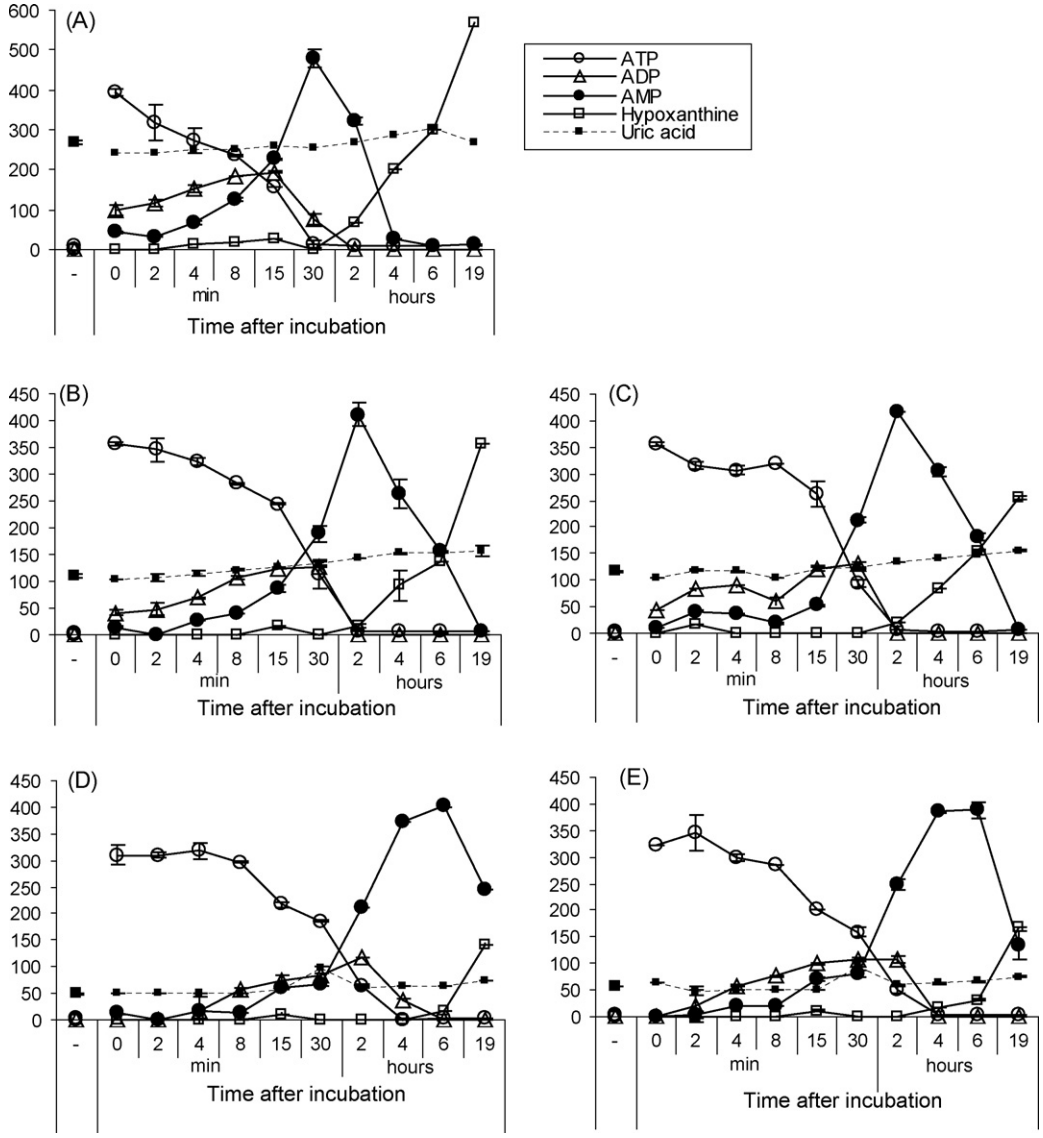

Fig. 4. Degradation profile of ATP added to blood that was (A) undiluted, (B) diluted two times with saline, (C) diluted two times with medium, (D) diluted four times with saline, or (E) diluted four times with medium. *X*-axis (not to scale) plots the time in minutes and hours after incubation at $t = 0$. Concentrations in the samples taken before addition of the ATP spike are represented by —. The legend shown in A applies to all conditions. The concentration (μM) of each of the detected compounds is plotted on the *Y*-axis. Curves represent means and error bars represent standard deviation ($n = 2$).

preceding ADP and (b) the smaller rise in hypoxanthine concentration after 19 h.

Compared to the undiluted situation, the shift in time in the fourfold diluted situation was approximately 3.5 h. One difference is that the ATP concentration does not decline directly after the start of the incubation, but only after 15 min (Fig. 4D) or 4 min (Fig. 4E). Finally, the increase in hypoxanthine after 19 h was even smaller, compared to the twofold diluted situation.

No remarkable differences between medium and saline were found. In the fourfold diluted situation, complete degradation of ADP took slightly longer in saline than in medium. Finally, as remarked in the previous paragraph, ATP seems to start degrading somewhat later when diluted in saline rather than medium.

4. Discussion

In the present paper we have optimized and applied a simple and rapid reversed-phase HPLC method for the simultaneous analysis of ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, and uric acid in human whole blood. Crucial steps in the collection, handling and preparation of the blood samples include collection in EDTA-tubes, immediate PCA addition, and neutralization before HPLC analysis at pH 6. The faster ATP breakdown in heparin-containing tubes, compared to EDTA-tubes, can be explained by EDTA's Ca^{2+}-chelating activities that inactivate the ATP-degrading enzymes [24]. Addition of PCA serves the same purpose, since PCA precipitates all proteins and thus inactivates enzymes as well. For sufficient inactivation of the enzymes, PCA had to be thoroughly mixed with the blood before freezing the samples. The enzymes were properly inactivated, as shown by the observation that no significant loss of ATP occurred when samples were thawed on ice or at room temperature. Further preparation was not necessary, thus making this method suitable for clinical applications (bedside). Chromatographic separation can be difficult for compounds with multiple pK_a's. Neutralization of the samples with KOH was necessary before HPLC analysis, and eluent pH values below six resulted in worse shape and separation of the peaks. Finally, the stability of the samples and the standard compounds was such that large batches of samples could be analyzed in one run, without risking degradation.

The recovery levels ranged between 82 and 108% for ATP, ADP, AMP, adenosine and inosine, but were below 70% for uric acid, adenine and hypoxanthine. These low recovery levels may be caused by coprecipitation of these metabolites together with proteins during the centrifugation step of the acid extraction preparation of the samples or with the perchlorate precipitate after neutralization [25,26]. In whole blood, precipitate volumes are large, and the 20% adjustment of concentration to correct for this volume change may have been an overcorrection for more apolar metabolites, such as uric acid, adenine and hypoxanthine, resulting in lower recoveries. Recovery levels reported by others for methods using perchloric acid for the extraction of nucleotides from cells or tissues range between 75 and 120.5% [27–30]. The recoveries did not differ substantially whether the nucleotides were spiked at high or low concentrations. The whole blood concentrations of ATP, ADP, AMP and uric acid reported in Table 3 correlate well with those reported by others [2,31].

The LOD (below 0.15 μmol/L for all compounds) and LLOQ (all below 0.5 μmol/L) were more than adequate for the purpose for which this method was developed: the quantification of ATP and its metabolites in whole blood. Concentrations of ATP in whole blood are extremely high compared to plasma concentrations, because ATP present at millimolar concentrations inside the erythrocytes is released before measuring whole blood. Erythrocyte ATP concentrations exceed plasma ATP concentrations, which are in the nanomolar range, by over 10,000-fold [32]. A frequently used method to measure ATP is the luciferin-luciferase assay [33,34]. This method has the sensitivity needed for the detection of low plasma ATP levels. A recent similar method is presented by Farthing et al. [35]. For measurement of ATP in whole blood, extreme dilutions are necessary. However, its main disadvantage compared to our method is that only ATP can be determined, whereas we can quantify the complete adenylate pool in one run. Ion-exchange methods often require more extensive pretreatment of samples and have difficulties in separating nucleobases, nucleosides and nucleotides in one run, given the charged nature of the nucleotides in the operating pH range (pH 2–7) [33,36–39].

Adenosine and inosine concentrations in whole blood were below the LOD. This is in line with results of studies investigating the adenine nucleotide content in human whole blood and erythrocytes [40,41]. Our within-day CV (range 0.7–5.9%) and between-day CV (2.6–15.3%) correlate well with the findings of others [42].

In the application of the method, we found that ATP added to whole blood ex vivo was converted into several intermediate products, which, together, created a typical degradation profile. The 30 min degradation period of ATP in the undiluted sample is consistent with the literature data [23,43]. In a similar set-up, Heptinstall et al. [23] found that the degradation of ATP to AMP in plasma can be attributed mainly to leukocytes, since these possess a high ectonucleotidase activity. When comparing the whole blood model to the in vivo situation, the main difference is the absence of vascular endothelial cells in the model. The ectonucleotidases that are active on the luminal surface of endothelial cells, shorten the ATP half-life to seconds or less in vivo [44]. In an ex vivo set-up, but with endothelial factors present, the half-life of ATP is longer (5–10 min), probably due to the static nature of this set-up, in comparison to the in vivo situation in microvasculature [45–47].

In addition to ATP, the degradation profiles of the other compounds also showed similarities to those reported before in the literature [23], even though we employed a longer incubation period and started at a higher ATP concentration. First, as was also reported by Coade and Pearson [45], this profile indicates the sequential catabolism of ATP to ADP and AMP. ADP formation coincided with ATP degradation, whereas the formation of AMP began 4 min later. The latter indicates that ADP, while still being formed as a degradation product from its precursor ATP, is simultaneously being degraded into AMP. This also becomes clear from the observation that, starting 30 min after incubation, the decline in ADP concentration coincides with the complete

depletion of its precursor ATP. The combination of both processes leads to a strong rise in AMP concentration between 30 min and 2 h after incubation. Second, the profile shows a strong increase in hypoxanthine concentrations between 2 and 19 h, after which the experiment was terminated. The catabolism of adenine nucleotides beyond AMP has been shown to involve the enzyme 5′-nucleotidase (CD73) to yield inosine monophosphate (IMP), inosine and hypoxanthine [48]. Heptinstall et al. [23,45,49] reported that this enzyme acts independently from leukocytes or erythrocytes, since breakdown of AMP added to cell-free plasma was similar to that in whole blood. In line with other studies [23], no other intermediate products (adenosine and inosine) were detected. Studies with the uptake inhibitor dipyridamole revealed that adenosine is taken up efficiently by the erythrocytes through equilibrative nucleoside transporters after which rapid sequential conversion into hypoxanthine occurs intracellularly, with hypoxanthine finally being distributed outside of the cell, resulting in the observed increased hypoxanthine levels [23,50].

In our experiment, hypoxanthine was the final degradation product and we observed only a very small rise in the concentration of uric acid, which is the endproduct in vivo [51]. This could be explained by the probable absence of xanthine oxidase (EC 1.2.3.2) in our model, the enzyme responsible for the further degradation of hypoxanthine to xanthine and finally uric acid. The highest activity of xanthine oxidase is reportedly found in the liver and intestinal mucosa [52,53], but it is also present in various organs and vascular endothelial cells [54,55]. Both liver and endothelial cells are absent in our set-up, which explains the lack of change in uric acid levels.

Besides interest in the breakdown profile of ATP added to whole blood, we were also interested in the effects of dilution of the whole blood with two different media: either saline of RPMI 1640 medium. The RPMI 1640 medium is used by Swennen et al. [22] in the whole blood model to more closely resemble the in vivo situation. We therefore hypothesized that addition of this medium would help stabilize ATP more than would a similar dilution with saline. However, degradation profiles in blood diluted with either saline or medium were quite similar. This result indicates that the medium does not contain any substance that might delay the degradation of ATP.

In contrast, the extent of dilution does influence the degradation profile of ATP added to the blood. First, the delay in ATP breakdown got more pronounced with increasing dilution of the whole blood (Fig. 4). For instance, whereas in undiluted blood, ATP completely degraded in 30 min, this process can take up to 2 and 4 h in two- and fourfold diluted blood, respectively. Second, other features of the degradation profile were delayed as well, such as the decline in ADP combined with the sharp rise in AMP that coincides with ATP depletion at 2 h. Furthermore, the maximum level of AMP was reached after incubation of 2 h (twofold diluted) or after incubation of 4–6 h (fourfold diluted).

5. Conclusion

The HPLC procedure described in this paper allows separation of the main metabolites of adenine nucleotide metabolism found in human whole blood samples. Compared to previous methods, it is a simple and rapid procedure, which has a short run time, good peak shape and high sensitivity. The method has been validated with respect to accuracy, precision, linearity and limit of detection, recovery and stability. The method is suitable for purine analysis in vivo or in an ex vivo set-up, enabling nucleotide metabolic processes to be followed in time. It has been reported for long that lowered blood ATP levels have been associated with acute disease states. Decreased levels of adenine nucleotides in erythrocytes were, for instance, observed in patients with various malignancies [56,57]. The method described here may be used to monitor changes that occur in these patients and after therapeutic administration of ATP.

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References

[1] I. Baranowska-Bosiacka, A.J. Hlynczak, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 134 (2003) 403.
[2] P.D. Schweinsberg, T.L. Loo, J. Chromatogr. 181 (1980) 103.
[3] G. Burnstock, Pharmacol. Rev. 58 (2006) 58.
[4] F. Di Virgilio, D. Ferrari, M. Idzko, E. Panther, J. Norgauer, A. La Sala, G. Girolomoni, Drug Dev. Res. 59 (2003) 171.
[5] M.J. Bours, E.L. Swennen, F. Di Virgilio, B.N. Cronstein, P.C. Dagnelie, Pharmacol. Ther. 112 (2006) 358.
[6] H.J. Agteresch, M.H.C. van Rooijen, J.W.O. van den Berg, G.J. Minderman-Voortman, J.H.P. Wilson, P.C. Dagnelie, Drug Dev. Res. 60 (2003) 196.
[7] T. Yamada, F. Okajima, M. Akbar, H. Tomura, T. Narita, T. Yamada, S. Ohwada, Y. Morishita, Y. Kondo, Oncol. Rep. 9 (2002) 113.
[8] A.D. Conigrave, L. van der Weyden, L. Holt, L. Jiang, P. Wilson, R.I. Christopherson, M.B. Morris, Biochem. Pharmacol. 60 (2000) 1585.
[9] H.J. Agteresch, P.C. Dagnelie, J.W. van den Berg, J.H. Wilson, Drugs 58 (1999) 211.
[10] D.J. Hearse, Cardiovasc. Res. 18 (1984) 384.
[11] K.F. Childs, X.H. Ning, S.F. Bolling, J. Chromatogr. B Biomed. Appl. 678 (1996) 181.
[12] B. Pelzmann, S. Hallstrom, P. Schaffer, P. Lang, K. Nadlinger, G.D. Birkmayer, K. Vrecko, G. Reibnegger, B. Koidl, Br. J. Pharmacol. 139 (2003) 749.
[13] G.A. Cordis, R.M. Engelman, D.K. Das, J. Chromatogr. 386 (1987) 283.
[14] A. Jahraus, M. Egeberg, B. Hinner, A. Habermann, E. Sackman, A. Pralle, H. Faulstich, V. Rybin, H. Defacque, G. Griffiths, Mol. Biol. Cell 12 (2001) 155.
[15] P. Peveri, P.G. Heyworth, J.T. Curnutte, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 2494.
[16] V. Reichelova, F. Albertioni, J. Liliemark, J. Chromatogr. B Biomed. Appl. 682 (1996) 115.
[17] P.R. Brown, J. Chromatogr. 52 (1970) 257.
[18] E. Harmsen, P.P. de Tombe, J.W. de Jong, J. Chromatogr. 230 (1982) 131.
[19] D.R. Webster, G.D. Boston, D.M. Paton, J. Pharmacol. Methods 13 (1985) 339.
[20] N.G. Anderson, Anal. Biochem. 4 (1962) 269.
[21] H.J. Agteresch, P.C. Dagnelie, T. Rietveld, J.W. van den Berg, A.H. Danser, J.H. Wilson, Eur. J. Clin. Pharmacol. 56 (2000) 49.
[22] E.L. Swennen, A. Bast, P.C. Dagnelie, Eur. J. Immunol. 35 (2005) 852.

[23] S. Heptinstall, A. Johnson, J.R. Glenn, A.E. White, J. Thromb. Haemost. 3 (2005) 2331.
[24] H. Holmsen, I. Holmsen, A. Bernhardsen, Anal. Biochem. 17 (1966) 456.
[25] J.F. Huertas-Perez, M. Heger, H. Dekker, H. Krabbe, J. Lankelma, F. Ariese, J. Chromatogr. A 1157 (2007) 142.
[26] A. Werner, J. Chromatogr. 618 (1993) 3.
[27] D. Huang, Y. Zhang, X. Chen, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 784 (2003) 101.
[28] T. Grune, W.G. Siems, J. Chromatogr. 618 (1993) 15.
[29] K.K. Tekkanat, I.H. Fox, Clin. Chem. 34 (1988) 925.
[30] R.T. Smolenski, D.R. Lachno, S.J. Ledingham, M.H. Yacoub, J. Chromatogr. 527 (1990) 414.
[31] G. Crescentini, V. Stocchi, J. Chromatogr. 290 (1984) 393.
[32] R.A. Harkness, S.B. Coade, A.D. Webster, Clin. Chim. Acta 143 (1984) 91.
[33] E.H. Abraham, A. Salikhova, E.B. Hug, Drug Dev. Res. 59 (2003) 152.
[34] M.W. Gorman, E.O. Feigl, C.W. Buffington, Clin. Chem. 53 (2007) 318.
[35] D. Farthing, D. Sica, T. Gehr, B. Wilson, I. Fakhry, T. Larus, C. Farthing, H.T. Karnes, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 854 (2007) 158.
[36] H. Daxecker, M. Raab, M. Cichna, P. Markl, M.M. Muller, Clin. Chim. Acta 310 (2001) 81.
[37] J.D. Torrance, D. Whittaker, Br. J. Haematol. 43 (1979) 423.
[38] D. de Korte, W.A. Haverkort, A.H. van Gennip, D. Roos, Anal. Biochem. 147 (1985) 197.
[39] A. Werner, W. Siems, H. Schmidt, I. Rapoport, G. Gerber, R.T. Toguzov, Y.V. Tikhonov, A.M. Pimenov, J. Chromatogr. 421 (1987) 257.
[40] R. Caruso, J. Campolo, C. Dellanoce, R. Mariele, O. Parodi, R. Accinni, Anal. Biochem. 330 (2004) 43.
[41] V. Stocchi, L. Cucchiarini, F. Canestrari, M.P. Piacentini, G. Fornaini, Anal. Biochem. 167 (1987) 181.
[42] N. Cooper, R. Khosravan, C. Erdmann, J. Fiene, J.W. Lee, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 837 (2006) 1.
[43] S. Heptinstall, J.R. Glenn, A. Johnson, B. Myers, A.E. White, L. Zhao, Arterioscler. Thromb. Vasc. Biol. 26 (2006) e22.
[44] J.W. Ryan, U. Smith, Trans. Assoc. Am. Physicians 134 (1971) 297.
[45] S.B. Coade, J.D. Pearson, Circ. Res. 65 (1989) 531.
[46] J.D. Pearson, J.L. Gordon, Annu. Rev. Physiol. 47 (1985) 617.
[47] A.K. Price, D.J. Fischer, R.S. Martin, D.M. Spence, Anal. Chem. 76 (2004) 4849.
[48] A.C. Newby, Biochem. J. 186 (1980) 907.
[49] H. Zimmermann, Naunyn Schmiedebergs, Arch. Pharmacol. 362 (2000) 299.
[50] D.A. Griffith, S.M. Jarvis, Biochim. Biophys. Acta 1286 (1996) 153.
[51] H. Zimmermann, Drug Dev. Res. 39 (1996) 337.
[52] U.A. Al-Khalidi, T.H. Chaglassian, Biochem. J. 97 (1965) 318.
[53] R.W. Watts, J.E. Watts, J.E. Seegmiller, J. Lab. Clin. Med. 66 (1965) 688.
[54] E.D. Jarasch, C. Grund, G. Bruder, H.W. Heid, T.W. Keenan, W.W. Franke, Cell 25 (1981) 67.
[55] Y. Moriwaki, T. Yamamoto, M. Suda, Y. Nasako, S. Takahashi, O.E. Agbedana, T. Hada, K. Higashino, Biochim. Biophys. Acta. 1164 (1993) 327.
[56] J. Laciak, S. Witkowski, Otolaryngol. Pol. 20 (1966) 269.
[57] H. Wand, K. Rieche, Dtsch Gesundheitsw 27 (1972) 1072.