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A Novel Method for Measuring the ATP-Related Compounds in Human Erythrocytes

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The ATP-related compounds in whole blood or red blood cells have been used to evaluate the energy status of erythrocytes and the degradation level of the phosphorylated compounds under various conditions, such as chronic renal failure, drug monitoring, cancer, exposure to environmental toxics, and organ preservation. The complete interpretation of the energetic homeostasis of erythrocytes is only performed using the compounds involved in the degradation pathway for adenine nucleotides alongside the uric acid value. For the first time, we report a liquid chromatographic method using a diode array detector that measures all of these compounds in a small human whole blood sample (125 μL) within an acceptable time of 20 min. The stability was evaluated for all of the compounds and ranged from 96.3 to 105.1% versus the day zero values. The measurement had an adequate sensitivity for the ATP-related compounds (detection limits from 0.001 to 0.097 μmol/L and quantification limits from 0.004 to 0.294 μmol/L). This method is particularly useful for measuring inosine monophosphate, inosine, hypoxanthine, and uric acid. Moreover, this assay had acceptable linearity (r > 0.990), precision (coefficients of variation ranged from 0.1 to 2.0%), specificity (similar retention times and spectra in all samples) and recoveries (ranged from 89.2 to 104.9%). The newly developed method is invaluable for assessing the energetic homeostasis of red blood cells under diverse conditions, such as in vitro experiments and clinical settings.

Keywords: adenosine triphosphate; energetic homeostasis; erythrocytes; liquid chromatography; whole blood sample
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Introduction

The ATP-related compounds in whole blood or red blood cells (RBCs) have commonly been used to evaluate the energy status of erythrocytes and the degradation level of the phosphorylated compounds under conditions such as chronic renal failure, drug monitoring, cancer, exposure to environmental toxics, streptozotocin induced diabetes, organ preservation, and experimental hypertension (Smoleński and Marlewski 1990; Smoleńska et al. 1999; Bolzonella et al. 2001; Baranowska-Bosiacka and Hlynczack 2003; Dudzinska and Hlynczack 2004; Domański et al. 2007; Yeung et al. 2008). These evaluations help to define the energetic homeostasis of erythrocytes because the cell can maintain their vital functions when have enough energy (Schellenberger et al. 1981; van Wijk and van Solinge 2005).

The complete interpretation of the energetic homeostasis of RBCs under stressful conditions might be performed with a liquid chromatographic (LC) method using ultraviolet (UV) detection that measures all the compounds involved in the degradation pathway for adenine nucleotides alongside the uric acid (Anderson and Murphy 1976; Schweinsberg and Loo 1980; Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Bontemps et al. 1986; Werner et al. 1987; Maessen et al. 1988; Tekkanat and Fox 1988; Smolenski et al. 1990; Nishikawa et al. 1991; Guieu et al. 1994; Smoleńska et al. 1999; Caruso et al. 2004; Taniai et al. 2006; Coolen et al. 2008; Yeung et al. 2008; Contreras-Sanz et al. 2012). The commercial kits (ab65313, abcam; K255-200, BioVision; TB288, Promega; FL-AA, Sigma-Aldrich) and LC methods using fluorometric detection or mass spectrometry (Levitt et al. 1984; Ramos-Salazar and Baines 1985; Fujimori et al. 1990; Kawamoto et al. 1998; Katayama et al. 2001; Tuytten et al. 2002; Xing et al. 2004; Klawitter et al. 2007; Wang et al. 2009; Birkler et al. 2010; Pabst et al. 2010; Jiang et al. 2012) can only detect some of these compounds simultaneously.

To conserve the adenine nucleotides in RBCs, these

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residues should be immediately measured in whole blood (Stocchi et al. 1987). Nevertheless, some LC-UV methods have only successfully quantified ATP-related compounds in whole blood samples (Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Tekkanat and Fox 1988; Nishikawa et al. 1991; Caruso et al. 2004; Coolen et al. 2008). These methods may measure from three to eight ATP-related compounds; however, the eight-compound methods did not measure the inosine monophosphate (IMP) (Coolen et al. 2008) or uric acid (Stocchi et al. 1985, 1987). In addition, the specificity of a LC method is less reliable using UV detection (Épshtein 2004). Hence, diode-array technology can be employed to enhance the quality of the specificity (Gilliard and Ritter 1997; Épshtein 2004; International Conference on Harmonization 2005).

The purpose of this work is to report a novel LC method using a diode array detector (DAD) that measures the IMP and uric acid alongside the rest of the ATP-related compounds involved in the degradation pathway of adenine nucleotides using human whole blood samples. The experimental data support the advantages of the present technique due to its smaller volume of blood, higher specificity, total stability data, superior sensitivity and complete picture of the energetic homeostasis assay compared to previous methods.

Materials and Methods

Chemicals

ATP-related compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and 2-propanol were of LC grade while the rest of reagents were of analytical-grade. These reagents were supplied by J.T. Baker (Mallinckrodt Baker Inc., Mexico City, Mexico). Ultra-pure water (18.2 MΩ; Millipore Milli-QUF Plus, Millipore S.A.S, Molsheim, France) was used in every procedure.

Sampling

Six healthy male volunteers above 20 years old with normal hematological parameters were included in this study (Van den Bossche et al. 2002; Díaz et al. 2012). Eight milliliters of blood were collected in two EDTA tubes (Vacutainer, Becton-Dickinson, NJ, USA) using venipuncture from the antecubital vein of each volunteer. This study with human subjects and materials was approved by the Research Ethics Committee from our institution.

Sample preparation

Immediately after collection, 125 μL of whole blood was added to an equal volume of ice-cold physiological saline solution (154 mM NaCl, 0.02 mM $NaHCO_3$, pH = 7.4) and mixed with 250 μL of ice-cold 8.0% $HClO_4$. Subsequently, the mixture was centrifuged at 12,100 rpm for 10 min at 4°C using a Sorvall RMC 14 Refrigerated Microcentrifuge (Thermo Fisher Scientific, Waltham, MA, USA). A sample of supernatant (308 μL) was aggregated in 17 μL of ice-cold 7.0 M KOH. This mixture was centrifuged at 12,100 rpm for 10 min at 4°C. One hundred eighty microliters of supernatant (final sample, FS) was separated and 50 μL of the FS was added to 350 μL of ice-cold water. Finally, 180 μL of this final dilution (FD) was placed in a vial insert inside an amber glass HPLC vial (part numbers: 5181-1270 and 5188-6535, Agilent Technologies, Palo Alto, CA, USA) for chromatographic analysis.

Stock solutions and working standards

The individual 4.0 mM stock solutions were prepared in ice-cold 7.0 mM KH_2PO_4 at a pH of 4.5 (ATP, ADP, AMP, cAMP and IMP), water (adenosine, inosine and hypoxanthine) or 0.1 M NaOH (uric acid). Each solution was freshly prepared once a week and immediately frozen at −80°C until use. During the workday, the stock solution was added to ice-cold water to prepare working standards. Afterwards, 250 μL of the standard was mixed with an equal volume of ice-cold 8.0% $HClO_4$ and processed as outlined in the sample preparation.

Working conditions

Adenosine, inosine, hypoxanthine and uric acid stock solutions, blood sampling and alkaline evaluations were performed at room temperature (24 ± 2°C). Meanwhile, the remainder of stock solutions, sample and standard preparation and chromatography were performed at 14 ± 1°C. In addition, the stock solutions, standards and whole blood samples were handled on ice.

Equipment and software

A 1100 series Agilent HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a quaternary pump with a degasser having a continuous seal wash option (G1311A), an autosampler (G1313A), a DAD (G1315A), and a thermostated column compartment (G1316A) was used for the chromatographic analysis. The data collection, integration and purity evaluation were performed using the Agilent ChemStation software for LC 3D systems. The mobile phase components were filtered through a glass filter holder with 0.45-μm PVDF Durapore Membranes (Merck Millipore, Billerica, MA, USA) before use. The hematological parameters as well as the pH values of the blood and solutions were determined with an ADVIA® 60 Hematology System (Bayer, Leverkusen, Germany), a RAPIDLab® 1265 Series (Siemens Medical solutions, Malvern, PA, USA) and an Accumet® AR15 pH Meter (Fisher Scientific, Pittsburgh, PA, USA), respectively.

Separation

The separation was carried out using a 5-μm particle size Zorbax SB-C18 column (250 mm × 4.6 mm i.d.; Agilent Technologies, Palo Alto, CA, USA) at 24°C. The mobile phase contained 430.0 μM H_3PO_4, methanol and 80.0 mM KH_2PO_4 at a pH of 6.0 in a composition dictated by the separation system in Table 1A. The pH of the potassium dihydrogen phosphate solution was adjusted using 20.0% KOH. The injection volume was 50 μL of the FD. The eluate was monitored at 260 nm, and the spectra were directly drawn by the DAD from 190 to 400 nm. The 20-min run time included the elution of the ATP-related compounds and the re-equilibration of the column for the next injection.

Column washing system

At the end of the workday, the column was eluted with 100% of 80.0 mM KH_2PO_4 (pH = 6.0) at a rate of 1.2 ml/min and 40°C. Subsequently, the column was eluted with 98% of 80.0 mM KH_2PO_4 (pH = 6.0) plus 2% of methanol for 20 min, and the washing system in Table 1B was utilized. After the washing protocol was performed, the column was eluted with 98% water plus 2% methanol before uti-

Table 1. Conditions for the LC-DAD method.

A. Separation system					B. Washing system				
Time (min)	Mobile phase composition (%)			Flow (ml/min)	Duration (min)	Mobile phase composition (%)			Flow (ml/min)
	a	b	c			d	b	e	
0.0	0.0	0.0	100.0	1.2	20.0	98.0	2.0	0.0	1.0
2.0	0.0	0.0	100.0	1.2	60.0	97.0	3.0	0.0	0.4
10.0	0.0	10.0	90.0	1.2	30.0	0.0	100.0	0.0	0.4
10.3	90.0	10.0	0.0	1.2	90.0	0.0	0.0	100.0	0.4
12.0	80.0	20.0	0.0	1.2	30.0	0.0	100.0	0.0	0.4
15.0	91.0	9.0	0.0	1.5	90.0	0.0	0.0	100.0	0.4
15.3	0.0	8.0	92.0	1.5	30.0	0.0	100.0	0.0	0.4
17.0	0.0	0.0	100.0	1.5					
18.0	0.0	0.0	100.0	1.2					
20.0	0.0	0.0	100.0	1.2					

The components include 430.0 μM H_3PO_4 (a), methanol (b), 80.0 mM KH_2PO_4 at a pH of 6.0 (c), water (d) and 2-propanol (e). The column temperature for the wash system is 40°C.

lizing the separation system. The wash cycles were performed in accordance with the cleaning recommendations (Majors 2003; Zhang and Thurbide 2006).

Assay validation

The specificity for each ATP-related compound included the identification and purity evaluation. The calibration curve for each compound was obtained using the average of three duplicated curves on three consecutive days; each curve was plotted using six different concentrations of the working standards. The concentration range for each compound was from 0.5 to 250 μmol/L in the FD. The precision was evaluated using the working standards in six replicated runs within one day (within-day precision) and measuring duplicates on three consecutive days (between-day precision) for every ATP-related compound. The limits of detection (DLs) and quantification (QLs) were determined using the mathematical equations recommended by the International Conference on Harmonization (ICH) guidelines (ICH 2005). For the limits of each compound, we used three curves near the detection limit, and each curve was plotted using five different concentrations of the working standards.

Mixtures of whole blood spiked with a stock solution were processed for assessing the recoveries. The FD had a theoretical spiked concentration of 90 μmol/L of the ATP-related compound. The recovery percentages were calculated using the values from spiked and unspiked blood. To assess the stability, mixtures of whole blood spiked with every stock solution were processed, but each FS was divided into three equal volumes (60 μL). The first FS aliquot was immediately processed (d = 0) and this FD injected again after 12 h (d = 0.5). The second and third FS aliquots were immediately frozen at −80°C and were evaluated on days 2 (d = 2) and 4 (d = 4), respectively. The final dilutions had a theoretical spiked concentration of 23 μmol/L of each compound. The stability percentage was obtained to compare each d value with respect to d = 0.

Application

After validation, the method was used to evaluate the energetic homeostasis of RBCs under basal and alkaline conditions. A physiological saline solution (154 mM NaCl, 0.02 mM $NaHCO_3$, pH = 7.4) was used under basal conditions, while an alkaline saline solution (106 mM NaCl, 295 mM $NaHCO_3$, pH = 8.5) was utilized for alkaline conditions. These saline solutions were freshly prepared each week and stored at −80°C until use. On the day of the assay, the frozen saline solutions were thawed at room temperature and placed in a water bath at 37°C.

Under basal conditions, 125 μL of whole blood and an equal volume of the saline solution were gently mixed; this mixture was used to measure the hematocrit, RBC count or pH. The basal measurements of the ATP-related compounds were performed as described in the sample preparation. Under alkaline conditions, 125 μL of whole blood and an equal volume of saline solution were gently mixed, and the mixture was incubated for 1 h in a 37°C water bath. The incubated samples were used to measure the hematocrit, RBC count or pH, while the samples used to quantify the ATP-related compounds were mixed with 250 μL of ice-cold 8.0% $HClO_4$ and processed as described in the sample preparation.

The ATP, ADP, AMP, cAMP and IMP contents in the erythrocytes (μmol/L RBCs) were obtained from the FD values (μmol/L) multiplied by the dilution factors of the sample preparation and the hematocrits. The total adenylate nucleotides (TAN) and the adenylate energy charge (AEC) as well as the ATP/ADP and ADP/AMP ratios were calculated using the amounts of these compounds in the RBCs (Dudzinska et al. 2010). The adenosine, inosine, hypoxanthine and uric acid contents were obtained as nucleotide amounts, but the hematocrit corrections were not applied for these compounds because they were reported in μmol/L of whole blood (Coolen et al. 2008; Dudzinska et al. 2010).

The data obtained from the healthy volunteers under each set of conditions are presented as the mean ± standard deviation. Normality was evaluated using the Shapiro-Wilk test. The data of the two conditions were analyzed by Mann Whitney test. A P value of < 0.05 was considered statistically significant. GraphPad Prism 6 software (San Diego, CA, USA) was used for the statistical analysis.

Results

Specificity (chromatograms and 3D spectrograms)

The retention times were 4.1 min (uric acid), 5.0 min (ATP), 5.5 min (IMP), 6.4 min (ADP), 8.1 min (hypoxanthine), 9.2 min (AMP), 12.7 min (inosine), 14.9 min (cAMP) and 15.2 min (adenosine) in the chromatograms from all samples (Fig. 1A-C); hence, the compounds were identified in the blood matrix. We measured nine ATP-related compounds in 20 min, including IMP and uric acid. In addition, the ATP, IMP, ADP, AMP, hypoxanthine and uric acid peaks were observed in whole blood samples from healthy volunteers (Fig. 1C). The wavelength absorption maxima (λ_{max}) values were 290 nm (uric acid), 260 nm (ATP), 248 nm (IMP), 260 nm (ADP), 248 nm (hypoxanthine), 260 nm (AMP), 248 nm (inosine), 258 nm (cAMP) and 258 nm (adenosine) in the 3D spectra from all the samples (vertical arrows in Figs. 2 and 3). The 3D spectra and λ_{max} values agreed between all samples including the water and basal data (data not shown). Consequently, the identities of the compounds were confirmed in the whole blood samples. However, the ADP spectra in whole blood samples had an additional peak (λ_{max} at 222 nm) versus the water standards (horizontal arrows in Figs. 2 and 3).

Linearity, precision, sensitivity, recovery, specificity (purity factor) and stability

Table 2 lists the values obtained for the rest of parameters of validation for each compound. The r values were ≥ 0.990 and the regression equations for ATP, ADP, AMP, cAMP, IMP, adenosine, inosine, hypoxanthine and uric acid were $y = 12.86x - 1.23$, $y = 14.41x - 2.43$, $y = 13.17x + 14.16$, $y = 13.32x + 9.95$, $y = 6.57x + 9.41$, $y = 13.71x + 27.77$, $y = 8.30x + 12.00$, $y = 7.91x + 3.55$, $y = 3.38x + 1.50$ (x in μmol/L; y in peak area), respectively. The coefficients of variation (CVs) for the within-day precision ranged from

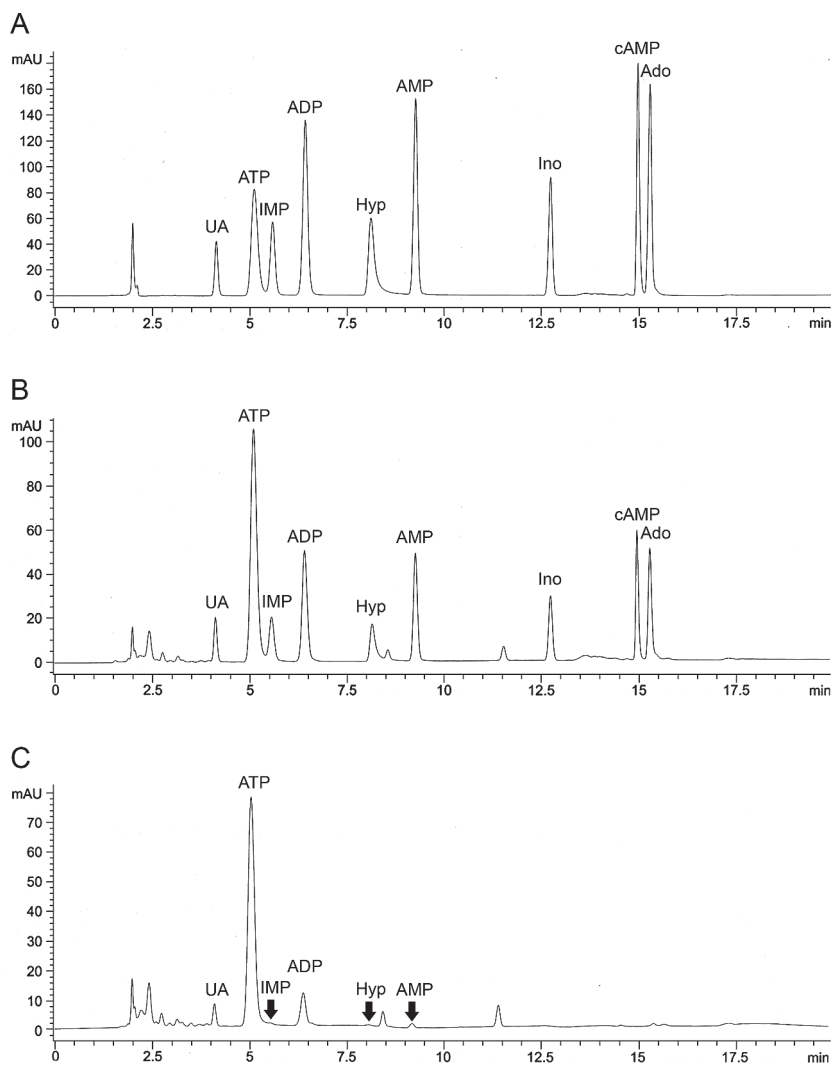

Fig. 1. Representative chromatograms of the ATP-related compounds in samples obtained from a standard mixture in water (A), spiked whole blood (B) and the whole blood of a healthy volunteer under basal conditions (C).
UA, uric acid; Hyp, hypoxanthine; Ino, inosine; Ado, adenosine.

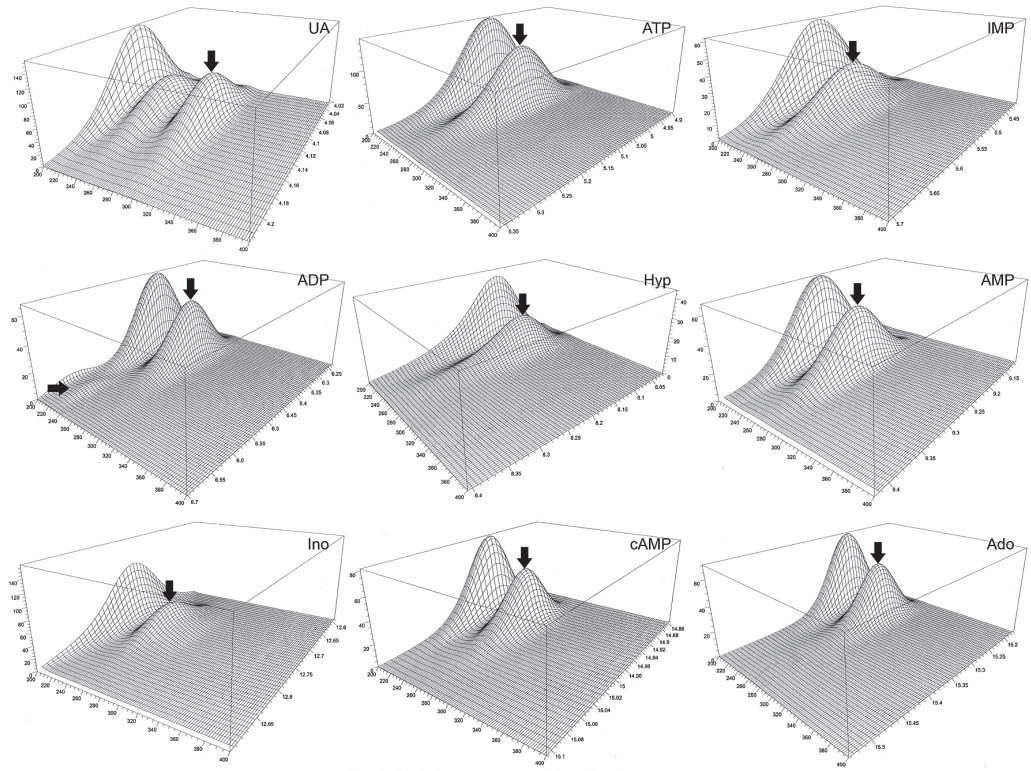

Fig. 2. Representative spectra of the ATP-related compounds in samples obtained from spiked whole blood. In each spectrogram, the absorbance (mAU) on the Y-axis, the wavelength (nm) on the X-axis and the time (min) on the Z-axis are plotted.
UA, uric acid; Hyp, hypoxanthine; Ino, inosine; Ado, adenosine.

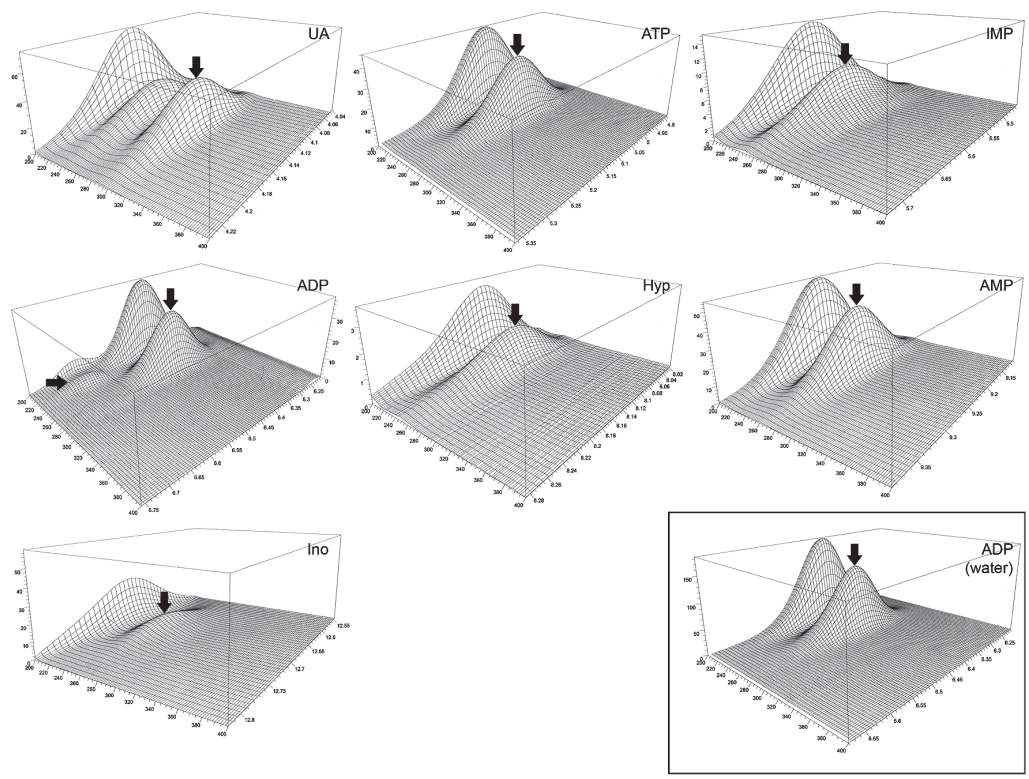

Fig. 3. Representative spectra of the ATP-related compounds in samples obtained from alkaline experiments. The ADP spectrum from water standards is showed within the box. In each spectrogram, the absorbance (mAU) on the Y-axis, the wavelength (nm) on the X-axis and the time (min) on the Z-axis are plotted.
UA, uric acid; Hyp, hypoxanthine; Ino, inosine.

Table 2. Parameters of validation for the ATP-related compounds.

Compound	Linearity (r)	Precision, CV (%) (80 μmol/L)		DL (μmol/L)	QL (μmol/L)	Purity factors				Recovery (%)	Stability (%)		
						Water	Whole blood						
							Spiked	Unspiked					
		Within-day	Between-day					Basal	Alkaline		12 h	2 d	4 d
ATP	0.999	0.5	0.7	0.097	0.294	997.5	994.8	997.6	965.4	100.7	98.7	100.2	101.3
ADP	0.999	0.4	0.9	0.058	0.174	998.2	891.9	877.5	770.9	99.6	103.7	101.5	101.3
AMP	0.999	0.7	2.0	0.054	0.164	999.7	987.0	927.1	991.7	104.9	101.0	99.6	101.2
cAMP	0.999	0.5	1.7	0.009	0.028	998.4	980.1	-	-	98.2	100.4	99.0	99.8
IMP	0.999	0.6	1.8	0.001	0.004	998.8	999.5	-	996.8	103.0	101.0	97.1	102.4
Ado	0.999	0.8	1.0	0.039	0.119	999.6	998.6	-	-	101.1	99.9	96.6	99.7
Ino	0.999	0.3	0.6	0.018	0.056	965.1	964.2	-	982.4	101.7	100.5	96.3	100.9
Hyp	0.999	0.1	1.7	0.031	0.093	998.0	993.4	-	962.4	89.2	101.2	100.7	101.2
UA	0.999	0.2	0.2	0.082	0.249	999.5	999.3	995.5	997.9	90.6	99.5	98.9	105.1

The r values were obtained using the average of three replicated curves. CV is the coefficient of variation (N = 6). The DL and QL were calculated from curves near the DL. Each purity factor is the average of the values in water (N = 3), spiked whole blood (N = 3) and unspiked whole blood under basal (N = 4) and alkaline (N = 2) conditions. Each recovery and stability value is the mean (N = 5).

DL, detection limit; QL, quantification limit; r, correlation coefficient; h, hours; d, days; Ado, adenosine; Ino, inosine; Hyp, hypoxanthine; UA, uric acid.

0.1 to 0.8%, while the between-day precision values ranged from 0.2 to 2.0%. The DLs, QLs and recoveries ranged from 0.001 to 0.097 μmol/L, from 0.004 to 0.294 μmol/L and from 89.2 to 104.9%, respectively. The purity factors for ATP, ADP (water), AMP, cAMP, IMP, adenosine, inosine, hypoxanthine and uric acid ranged from 924.8 to 999.7. Meanwhile, the ADP purity factors were between 770.9 and 891.9 in whole blood samples. The stability of the ATP-related compounds relative to the day zero values ranged from 98.7 to 103.7% in samples stored for 12 h, from 96.3 to 101.5% in samples frozen for 2 days and from 99.7 to 105.1% in samples frozen for 4 days.

Application

Table 3 displays the sample values for the pH, RBCs count, ATP-related compounds and energetic state parameters in whole blood samples under basal and alkaline conditions from healthy subjects as well as the respective normal range for each compound. The basal values for the pH, ADP, AMP, cAMP, adenosine, inosine, uric acid, TAN, ATP/ADP and ATP/AMP ratios agreed with the normal intervals. However, the basal values for the RBCs count and hypoxanthine were outside the normal intervals while those for ATP, IMP and AEC were slightly outside the normal ranges. Our alkaline conditions resulted in unchanged values for the RBCs count and uric acid ($P > 0.6$), reduced values for ATP, ATP/ADP, ATP/AMP and AEC (P = 0.0022), a slightly diminished value for TAN (P = 0.0411), and increased values for the pH, ADP, AMP, IMP, inosine and hypoxanthine (P from 0.0004 to 0.0022) relative to the basal values. Under alkaline conditions, cAMP and adenosine were not detected, similar to under the basal conditions. The alkaline values for the pH, ADP, AMP, inosine, hypoxanthine, degradation ratios and AEC were outside the normal ranges while those for ATP and IMP were slightly outside the normal intervals.

Discussion

This novel quantitative method was able to evaluate nine ATP-related compounds using a smaller volume of whole blood (125 μL) compared to previous measurements of these compounds, which employed 500 to 2,000 μL of whole blood (Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Formato et al. 1990; Nishikawa et al. 1991; Smoleńska et al. 1999; Baranowska-Bosiacka and Hlynczak 2004; Dudzinska and Hlynczak 2004; Domański et al. 2007; Coolen et al. 2008; Dudzinska et al. 2010; Suska and Skotnicka 2010). Consequently, blood from a single sample can be used for more tests.

Our method was more efficient than the preceding techniques because the seven compounds from the adenine nucleotide pathway in RBCs plus the uric acid were measured within 20 min (Fig. 1B). Previous methods can only measure three and seven of these compounds in 35 min and 19 min, respectively. Additionally, this is the first assay measuring both IMP and uric acid in whole blood from a same sample (Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Tekkanat and Fox 1988; Nishikawa et al. 1991; Caruso et al. 2004; Coolen et al. 2008).

The r values indicated a good linearity according to the ICH guidelines (Épshtein 2004), and the ranges of CVs were within the precision in the literature for water based solutions because the previous data showed CVs up to 9.1% (Tekkanat and Fox 1988; Maessen et al. 1988; Taniai et al. 2006; Contreras-Sanz et al. 2012). The present limits sug-

Table 3. Using the LC-DAD method with whole blood samples from healthy subjects.

Parameter	Unit	Normal range	Condition		
			Basal	Alkaline	*P* value
pH	–	7.35 - 7.45	7.39 ± 0.03	7.95 ± 0.04	0.0022
RBCs count	$10^6/\mu$L	3.98 - 6.10	2.56 ± 0.29	2.50 ± 0.25	0.8528
ATP	μmol/L RBCs	1,140 - 2,130	2,028.44 ± 140.40	1,034.88 ± 128.60	0.0022
ADP	μmol/L RBCs	83.0 - 390	223.39 ± 35.54	627.55 ± 65.67	0.0022
AMP	μmol/L RBCs	10.6 - 50	17.42 ± 5.76	358.78 ± 41.12	0.0022
cAMP	μmol/L RBCs	ND	ND	ND	–
IMP	μmol/L RBCs	5 - 70	1.85 ± 1.82	95.36 ± 38.77	0.0022
Ado	μmol/L WB	ND - 0.6	ND	ND	–
Ino	μmol/L WB	ND - 0.2	ND	56.92 ± 16.35	0.0004
Hyp	μmol/L WB	ND - 2.20	4.09 ± 2.27	28.08 ± 5.12	0.0022
UA	μmol/L WB	252.2	221.04 ± 33.72	205.29 ± 36.84	0.6753
TAN	μmol/L RBCs	1,726.0 - 2,450	2,269.26 ± 141.96	2,021.21 ± 222.45	0.0411
ATP/ADP	–	4.32 - 18.0	9.29 ± 1.77	1.65 ± 0.09	0.0022
ATP/AMP	–	44.9 - 190.0	128.26 ± 44.34	2.89 ± 0.28	0.0022
AEC	–	0.897 - 0.945	0.943 ± 0.010	0.667 ± 0.014	0.0022

Each normal range is described using the upper and lower means from the literature data; however, cAMP and uric acid were mentioned only once. Every value includes the mean and standard deviation ($N = 6$).

RBCs, red blood cells; ND, not detectable; Ado, adenosine; WB, whole blood; Ino, inosine; Hyp, hypoxanthine; UA, uric acid.

gest that our method had an appropriate sensitivity for measuring ATP-related compounds in human whole blood samples, including improved sensitivities for IMP, inosine, hypoxanthine and uric acid with respect to prior measurements (Anderson and Murphy 1976; Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Tekkanat and Fox 1988; Maessen et al. 1988; Smolenski et al. 1990; Caruso et al. 2004; Taniai et al. 2006; Coolen et al. 2008; Yeung et al. 2008; Contreras-Sanz et al. 2012). Moreover, our recoveries were satisfactory because they were within the literature values ranging 58.6 to 108.2% in the blood matrix (Harmsen et al. 1981; Crescentini and Stocchi 1984; Stocchi et al. 1985, 1987; Tekkanat and Fox 1988; Caruso et al. 2004; Coolen et al. 2008). The stability ranges at 12 h were in accordance with the conservation of ATP, ADP and AMP in the blood extracts on ice over 12 h (Stocchi et al. 1985). Likewise, the stability ranges for 2 and 4 days agreed with the 13 day stabilities of ATP, ADP and AMP in neutralized supernatants from whole blood at low temperatures (Caruso et al. 2004). Therefore, we completed the stability data for the ATP-related compounds in whole blood samples because only ATP, ADP and AMP were previously evaluated.

The purity factors were higher than 900 confirming the peak purities in all of the samples. However, the ADP peak in blood samples might be eluted with peptides because purity factors were < 900, their spectra had an additional peak and the peptide bond typically absorbs from 180 to 240 nm in the UV region (Kelly and Price 2000). This impure ADP peak was not reported in other methods because this is the first time that the ATP-related compounds were evaluated using the 3D spectra and purity factors (Scholar et al. 1973; Anderson and Murphy 1976; Schweinsberg and Loo 1980; Harmsen et al. 1981; Ericson et al. 1983; Crescentini and Stocchi 1984; de Korte et al. 1985; Stocchi et al. 1985, 1987; Werner et al. 1987; Tekkanat and Fox 1988; Maessen et al. 1988; Smolenski et al. 1990; Formato et al. 1990; Nishikawa et al. 1991; Guieu et al. 1994; Caruso et al. 2004; Taniai et al. 2006; Coolen et al. 2008; Yeung et al. 2008; Contreras-Sanz et al. 2012).

Moreover, the ranges in Table 3 were established using the normal values from human samples of whole blood and RBCs (Scholar et al. 1973; Rapoport et al. 1976; Harmsen et al. 1981; Ericson et al. 1983; Crescentini and Stocchi 1984; de Korte et al. 1985; Stocchi et al. 1985, 1987; Werner et al. 1987; Trulock 1990; Özer et al. 2000; Van den Bossche et al. 2002; Coolen et al. 2008; Dudzinska et al. 2010; Díaz et al. 2012); however, some normal amounts of nucleotides were not included in these ranges because these values were not reported per volume of RBCs (Beutler et al. 1983; Crescentini and Stocchi 1984; de Korte et al. 1985; Tekkanat and Fox 1988; Coolen et al. 2008) or the units did not agree with the amounts (Schweinsberg and Loo 1980; Formato et al. 1990; Buoncristiani et al. 1996; Bolzonella et al. 2001).

The basal data from healthy subjects were within the normal intervals except the RBCs count, hypoxanthine, ATP, IMP and AEC values. The dilution with a saline solution during the sample preparation reduced the basal RBCs count. Our basal hypoxanthine expressed per volume of RBCs (8.61 ± 4.81 μmol/L RBCs) was similar to normal human values of 8.0 ± 6.2 μmol/L RBCs and 8.2 ± 1.3

μmol/L RBCs (Boulieu et al. 1983; Werner et al. 1987). Our basal ATP and IMP values might indicate a lower activity of AMP-deaminase via decreased oxidative stress than previous normal measurements because diminished H_2O_2 concentrations caused a reduction in the AMP-deaminase activity resulting in higher ATP and lower IMP values than outcomes from isolated human erythrocytes with larger H_2O_2 amounts (Tavazzi et al. 2000). In addition, the present basal AEC suggest that the current process provided a stable metabolic state in RBCs because the decreased energy charge was associated with the degradation of nucleotides (Atkinson 1968; Matsumoto et al. 1979; de Atauri et al. 2006). The data for the basal evaluation suggested that the energetic homeostasis was maintained in the RBCs under the present method.

The present alkaline information agreed with the changes in the RBCs in alkaline media except the IMP, uric acid and AEC values (Minakami and Yoshikawa 1966; Tsuda et al. 1975; Bontemps et al. 1986; Berman et al. 1988). The IMP value in alkaline samples differed from previous reports because the alkaline evaluations did not observed changes or showed a reduction of IMP. Our difference was likely produced by the low pO_2 during incubation because the reduction of oxygen increased the IMP in RBCs (Berman et al. 1988). The alkaline values for the uric acid and hypoxanthine might indicate an appropriate re-synthesis of purines in the RBCs (Smolénska et al. 1999; Baranowska-Bosiacka et al. 2004). However, this observation cannot be compared with other studies because uric acid was not evaluated. The AEC in the alkaline samples was lower than the basal amount; however, it was not previously reported. This excessive reduction suggested there was cellular dysfunction in the RBCs because AEC coordinates many metabolic reactions (Matsumoto et al. 1979; Schuster and Holzhütter 1995). The RBCs count in the alkaline samples demonstrated the maintenance of the number of these cells during incubation because a reduction in the number of erythrocytes is detected using the RBCs count or other parameters (McGrath 1993). Additionally, alkaline shifts versus basal amounts were confirmed by information in the literature. The data from the alkaline evaluation suggested that the energetic homeostasis of erythrocytes was lost due to the intense degradation of the phosphorylated compounds and the inclusion of the reduction of AEC for first time. However, this imbalance did not lead to the inhibition of purine re-synthesis or the destruction of erythrocytes.

The data from basal and alkaline samples support the advantage of our method in comparison with others techniques due to that provide more information of the energetic homeostasis of erythrocytes in a test only.

In conclusion, ATP-related compounds were efficiently measured in samples generated from a small volume of human whole blood using the present LC-DAD method, including IMP and uric acid together for first time. The specificity was improved using the diode-array technology, and this assay successfully completed a stability evaluation and had a superior sensitivity for IMP, inosine, hypoxanthine and uric acid while the rest of ATP-related compounds had similar limits to previous methods. Additionally, this measurement technique provided a complete picture of the energetic homeostasis of RBCs under basal and stressed conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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