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HPLC assay with UV detection for determination of RBC purine nucleotide concentrations and application for biomarker study *in vivo*☆

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Abstract

ATP and other purine nucleotides are important biomarkers for ischemia and may have considerable potential as targets for management of ischemic heart disease and stroke. The main objective of the study is to develop a rapid HPLC assay, which has adequate sensitivity and specificity for measuring concentrations of ATP, ADP, AMP, GTP, GDP and GMP in erythrocytes (RBC). The assays used ion-pair chromatography coupled with ultraviolet detection at 254 nm to separate and detect the purine nucleotides. Using 50–100 μL of RBC lysate as blank biologic matrix, the assay was linear from 100 to 2000 μg/mL for ATP and ADP, and 20–400 μg/mL for AMP, GTP, and GDP with coefficients of determination (r^2) >0.99. GDP and GMP were not measurable in the study because of low concentrations and interference from endogenous materials, respectively. The intra-assay and inter-assay variations over a period of 1 year were less than 10% and 20%, respectively for most of the nucleotides. The assay was successfully applied to two pilot biomarker studies to measure RBC concentrations of the purine nucleotides in rats under restraining and exercise conditions. Preliminary results showed that the RBC concentrations of ATP and GTP were higher in the spontaneously hypertensive rats (SHR) compared to the Sprague–Dawley (SD) rats, and that exercise increased RBC concentrations of ATP in rats treated with the calcium channel blocker diltiazem.

Keywords: HPLC; ATP; RBC; Ion-paired; Biomarkers

1. Introduction

The importance of adenosine and adenosine 5′-triphosphate (ATP) in regulating many biological functions has long been recognized, especially for their effects on the cardiovascular system [1–8]. It is known that adenosine and ATP play an important role in the regulation of coronary blood flow [5,9–11], inhibiting platelet aggregation [12], protection of myocardium [13–15], neuromodulation [16–18], modulating tissue necrosis [7,19], ischemic preconditioning [20–24], immunomodulation [25], energy metabolism [26–28], and perhaps other functions as well (e.g. pain mediation) which maintain the homeostasis of the cardiovascular system.

The activity of adenosine is very short lived because it is rapidly taken up by myocardial and endothelial cells, erythrocytes (RBC), and also rapidly metabolized to the oxypurine metabolites (e.g. hypoxanthine and uric acid) and adenine nucleotides [1,9,29,30]. Extracellular ATP is broken down rapidly to adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) and finally to adenosine by 5′-nucleotidase. These metabolic events are known to occur in the myocardium as well as in erythrocytes (RBC), but it is not clear whether or not they are regulated by similar mechanisms [31,32]. Chronic myocardial ischemia is often treated clinically with anti-ischemic drugs such as the calcium channel blockers (CCBs), β-blockers, ACE inhibitors (ACEIs) and the angiotensin II receptor blockers (ARBs), which act either directly or indirectly on the adenosine and ATP systems [20,27,33,34]. We

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have previously shown that the calcium channel blocker diltiazem (DTZ) alters oxidative metabolism of adenosine in both healthy volunteers and patients with effort angina [30], and that it potentiates the hemodynamic effect of adenosine *in vivo* [35]. There are also studies which indicate that RBC concentrations of ATP may be used as biomarkers for primary pulmonary hypertension [36], chronic renal failure [37], diabetes mellitus and aging [38], metabolic syndrome [39,40], and has the potential as biomarkers for ischemia and a potential target for cardiovascular agents [41]. This paper describes development and qualification of a HPLC assay for measuring the concentrations of ATP, ADP, AMP and the guanine nucleotides including guanosine 5′-triphosphate (GTP) and guanosine 5′-diphosphate (GDP), and guanosine 5′-monophosphate (GMP) in RBC, which is applied for the first time for pilot biomarker studies of the calcium channel blocker using both normotensive Sprague–Dawley (SD) rats and spontaneously hypertensive rats (SHR) under restraining and exercise conditions *in vivo*.

2. Experimental

2.1. Chemical reagents

Adenine and guanine nucleotides (ATP, ADP, AMP, GTP, GDP and GMP), the internal standard 3,7-dimethyl uric acid (DMUA), and the ion-pair reagent tetra-butylammonium hydrogen sulfate (TBAS) were purchased from Sigma–Aldrich Chem Co. (St. Louis, MO, USA). Diltiazem hydrochloride was received as gift from Biovail Pharmaceuticals Canada (Mississauga, ON). Solid phase extraction (SPE) columns were CN bonded materials (100 mg/mL) purchased commercially (Chromosep®,[1] Chromatographic Specialties Inc., Brockville/ON, Canada). Solvents were HPLC grade, and all other chemicals were reagent grade (Fisher Scientific, ON, Canada).

2.2. HPLC system

The HPLC system consisted of a Shimadzu LC-10AT *VP* solvent delivery module (Man-Tech Associate Inc., Guelph, ON), a Rheodyne syringe loading injector (model 9725), with a 100 μL PEEK injection loop (Scientific Products & Equipment, Concord, ON, Canada), and a Spectra-Physics variable wavelength ultraviolet detector (Spectra 100, Spectra-Physics Inc./San Jose, Ca, USA). Chromatographic separation was achieved on a 250 mm × 3.0 mm i.d. Supelcosil™[2] LC-18-T column bonded to a 5 μm spherical silica packing, pore size 120 Å (Supelico Inc., Bellefonte, PA, USA) preceded with a 5 μm 4.0 mm × 4.0 mm i.d. C_{18} reversed phase cartridge guard column (LiChrocart®[3] E.M. Merck, Germany) using a mobile phase of a 0.0005 M tetra-butyl ammonium hydrogen sulfate (TBAS) solution in a 0.1 M KH_2PO_4:acetonitrile:methanol (9.6:0.3:0.1, v/v/v) with final pH 6.3. The system was operated at room temperature (23–25 °C) with a flow rate of 0.5 mL/min and an operating pressure of 11.5 mPa (ca. 1.7 kpsi). The wavelength was set at 254 nm for detection and quantification. Detector output was recorded by an integrator (Hewlett-Packard HP3395 Integrator/Palo Alto, CA, USA), and digitalized using the Peak Simple®[4] software (SRI Instruments Torrance, CA, USA).

2.3. Preparation of standard solutions

Stock solutions of the adenine nucleotides (ATP, ADP and AMP) and guanine nucleotides (GTP, GDP and GMP) were prepared at 20 mg/mL and 4 mg/mL in deionised HPLC water, respectively. One millilitre of each of the nucleotide solutions was mixed with 4 mL of phosphate buffer saline (PBS pH 7.4) to prepare an initial standard spiking solution (2 mg/mL for ATP and ADP; and 0.4 mg/mL for AMP, GTP, GDP and GMP). Serial dilution of this standard solution was made with the PBS to provide standard solutions which contained 1, 0.5, 0.25, or 0.1 mg/mL of ATP and ADP, and 0.2, 0.1, 0.05, or 0.02 mg/mL of AMP, GTP, GDP and GMP. The stock solutions were kept at −20 °C for storage and should be stable for greater than 6 months. A calibrating solution consisting of a mixture of the nucleotides and the internal standard DMUA was prepared at 1 μg/mL, and was used for system stability testing. This solution was kept at 4 °C, and should be stable for 3 months. In addition, a working internal standard solution of 0.05 mg/mL of DMUA was prepared by diluting the stock solution (1 mg/mL) with deionised HPLC water immediately prior to use.

2.4. Solid phase extraction

To each labelled conical glass culture tube (Kimble®,[5] Kimble Glass Inc., Vineland, NJ, USA) was added 0.1 mL of RBC lysate and 0.1 mL of the Spiking Solutions for quality control (QC) standard samples or PBS for QC blank and study samples. The RBC lysate was prepared by suspending RBC with equal volume of PBS and immediately lysed with equal volume (0.15–0.2 mL) of ice-cold 10% trichloroacetic acid (TCA) as described previously in the HPLC assay for adenosine [42]. The lysate was collected after centrifugation (1760 × *g*, 10 min, 4 °C), and stored at −80 °C until analysis. A 0.1 mL of the working internal standard solution (5 μg of DMUA) was added, and followed by 1.0 mL of methyl *tert*-butyl ether (MTBE). The mixture was vortex mixed for 5 min in a multi-tube mixer (IKA-VIBRAX-VX2®,[6] Janke & Kunkel GMBH & Co., IKA-Labortechnik, Staufen, Germany) to remove excess trichloroacetic acid from the lysate, centrifuged

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at 4 °C, 3000 rpm for 10 min (Beckman Model TJ-6R Beckman Instruments San Ramon, CA, USA) to enhance stability of the analytes in the residual sample. The organic layer was carefully removed, and a gentle stream of nitrogen was applied to the sample at 55 °C for 5–10 min in a Thermolyne Dri-bath (Fisher Scientific Co., ON, Canada) to remove the residual MTBE in the aqueous fraction. Pipette the remaining aqueous portion into a 100 mg CN-bonded SPE extraction column pre-conditioned with 2× 1 mL of methanol followed by 2× 1 mL of PBS. The sample was passed through the column drop wise at a constant vacuum of 130 mm of Hg, and collected into a clean culture tube. Each column was subsequently washed with 0.2 mL of 10% methanol in deionised HPLC water, and the washing was combined with the sample and stored at −20 °C until analysis. Recoveries of the nucleotides were determined by measuring the amounts (expressed as peak heights) after the SPE extraction, and compared them to the amounts added to the quality control (QC) samples.

2.5. Data analysis

Standard curves were plotted using known RBC concentrations of each of the nucleotides (*x*-axis) and the peak height ratios of the nucleotide to the internal standard DMUA (*y*-axis), from 0.1 to 2 mg/mL for ATP and ADP, and 0.02 to 0.4 mg/mL for ATP, GTP and GDP and analyzed by linear regression (Lotus 1–2–3®,[7] IBM Corporation, Armonk, New York, USA). GMP concentrations were not measurable due to interference by endogenous materials in the RBC lysate not separated from GMP. The QC samples at each concentration were performed in five replicates in the method development. Intra- and inter-assay variations were assessed from two concentrations of the QC samples (0.25 and 0.1 mg/mL for ATP and ADP; and 0.05 and 0.02 mg/mL for AMP, GTP, and GDP) for each batch analysis over a year. Sensitivity of the assay was assessed by determining the smallest amount of analytes injected on-column which result in a signal to noise ratio of greater than 3.

2.6. Biomarker study

The assay was applied to two separate preliminary biomarker studies, which were approved by the Dalhousie University Committee on Laboratory Animals using the Canadian Council of Animal Care (CCAC) guidelines. In one study, male SD rats and spontaneously hypertensive rats (SHR) ($n = 4$–5 each) weighing 300–450 g were purchased from Charles River Laboratories. Although both strains of rats are commonly used for cardiovascular research, the SHR are genetically selected and phenotyped for higher blood pressure than the SD rats, and regularly used as a preclinical model for hypertension [43]. They were placed in a restrainer and sequential blood samples were collected every hour over a period of 6 h. In another study, male SD rats ($n = 6$ in each group) were treated with normal saline (0.9% sodium chloride) or DTZ 10 mg/kg by subcutaneous injections (sc) twice daily for five doses before exercise as described previously [44]. Briefly, the exercise test was performed on a research Exercise Treadmill (Model Exer-4™,[8] Columbus Instruments International Corporation, Columbus, Ohio, U.S.A.), with speed set at 7 m/min at 3% grade. Each rat was exercised under this condition for 7 min. Blood samples were collected in both studies via an indwelling catheter during exercise or in the restrainer from the left carotid artery as described previously [45,46]. Each blood sample collected (0.3 mL) was immediately mixed with 0.05 mL of an ice-cold "Stopping Solution" made up of 26 μM EHNA, 100 mM dipyridamole, 4 mM EDTA, and a final concentration of 2 μg/mL of indomethacin in heparinized normal saline with pH adjusted to 7.4 to minimize *in vitro* degradation and production of adenosine during sample processing [30,47,48]. The mixture will be immediately mixed, centrifuged (1760 × *g*, 10 min, 4 °C) to separate the plasma from the RBC, and to determine hematocrit value for subsequent calculation of RBC concentrations. The RBC was suspended with equal volume of PBS and immediately lysed with equal volume (0.15–0.2 mL) of ice-cold 10% trichloroacetic acid (TCA). The lysate was collected after centrifugation, and stored at −80 °C until analysis as described earlier. The concentrations of the nucleotides in the lysates (mg/mL) were normalized to μmol/mL (mM) per RBC volume after correcting for dilution factor using the hematocrit value obtained for the sample. Data were analysed by two-sample *t*-test and differences between groups considered significant where $p < 0.05$ (Minitab®[9] Inc., Release 14, State College, PA, USA).

3. Results and discussion

Under the described ion-paired chromatographic conditions, the nucleotides and the internal standard DMUA are well separated from each other, with retention times ranged from 4.2 min for GMP to 17 min for ATP. GMP was not measurable in the RBC lysate due to interference from the biologic samples (Fig. 1B). The standard curves constructed at different stages of the method development over a period of 4 months were linear between 0.1 and 2 mg/mL for ATP and ADP, and between 0.02 and 0.4 mg/mL for AMP, GTP, and GDP with regression coefficients of greater than 0.99 for all the nucleotides measured. Typical standard curves are shown in Fig. 2. The recoveries of the nucleotides at the two lowest standard concentrations (0.25 and 0.1 mg/mL for ATP and ADP; and 0.02 and 0.05 mg/mL for AMP, GTP, and GDP) were greater than 70%. The sensitivity or lower limits of detection (LOD) of the assay based on absolute on-column injection of the nucleotides was about 1 ng for ATP, and lower for the other nucleotides (Fig. 1A). The intra- and inter-assay variations determined at the lower standard concentrations over 1 year were less than 10% and 20%, respectively (Table 1).

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[8] Exer-4™ is a trademark of Columbus Instruments International Corporation, Columbus, Ohio, U.S.A.

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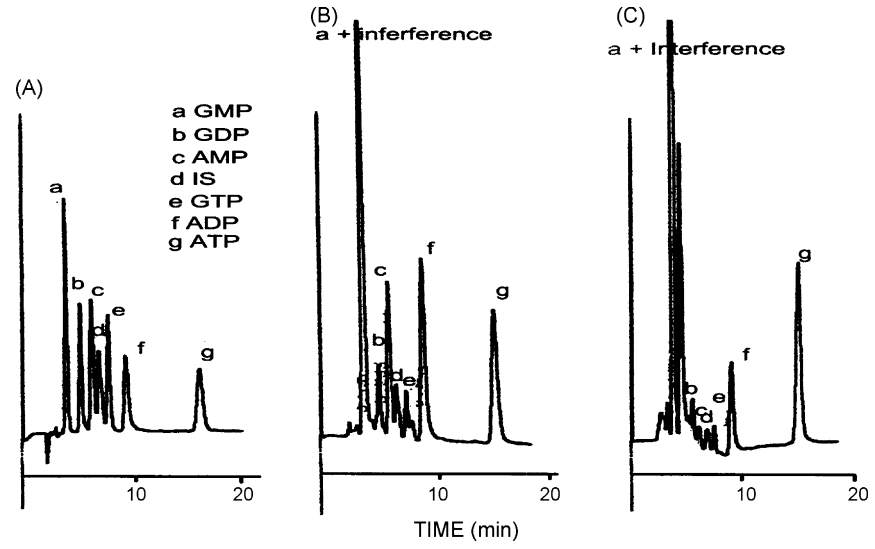

Fig. 1. HPLC–UV chromatograms of the purine nucleotides. (A) On-column injection of 5 ng of each nucleotide and the IS DMUA (5 μL injection volume); (B) quality control standard which contained 0.1 mg/mL of ATP and ADP and 0.02 mg/mL of AMP, GTP, GDP and GMP in lysate (1 μL injection volume); (C) a typical lysate sample from a SHR (2 μL injection volume). Full scale response on *y*-axis = 0.01 absorbance unit.

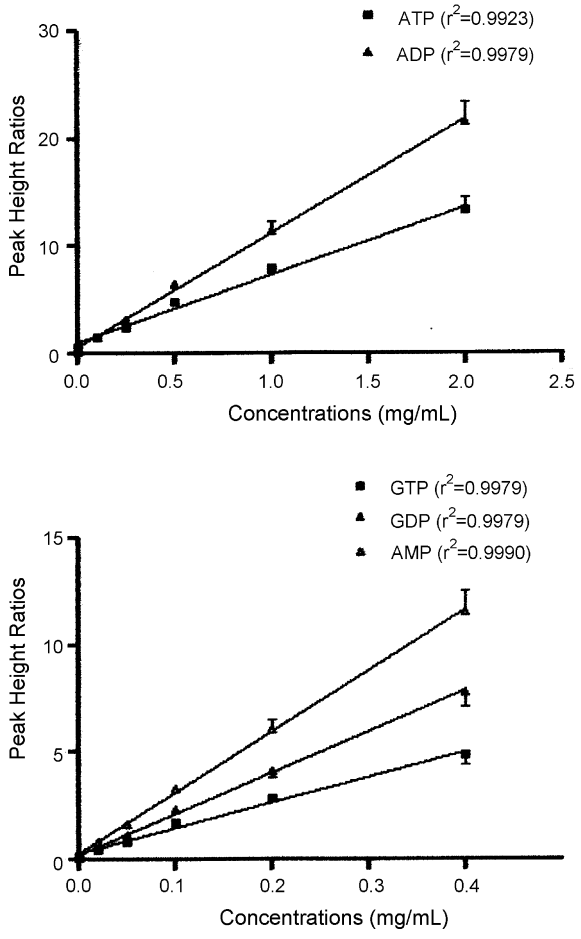

Fig. 2. Standard curves of the HPLC assay of the nucleotides after injecting 1–5 μL of the extracted QC samples. Each point represents mean ± S.D. of five replicates of the peak-height ratios of the analyte to the internal standard.

The average circulatory concentrations of the nucleotides in RBC obtained from the SD rats and SHR are shown in Table 2. The RBC concentrations of ATP and GTP were significantly higher in the SHR (4.46 ± 0.76 vs. 1.16 ± 0.59 μmol/mL for ATP and 0.44 ± 0.07 vs. 0.12 ± 0.05 μmol/mL for GTP, $p < 0.05$). The concentrations of GMP and GDP were not measurable in most of the rat samples, and thus they are not reported in this study. Exercise increased RBC concentrations of ATP by twofold and continue to increase after the exercise was completed in the rats treated with DTZ, and not in the control rats (Fig. 3). The RBC ATP concentrations measured at the end of the exercise experiments (30–60 min after exercise) were 3.8 ± 0.7 μmol/mL in the DTZ treated rats and 0.5 ± 0.2 μmol/mL in the control animals. More detailed analyses of the data for ATP and the other nucleotides are in progress and will be published elsewhere.

There have been several HPLC assays reported for ATP in RBC and other cellular materials to-date [49–54], but none of these methods was designed for pharmacodynamic and *in vivo* biomarkers studies. These methods either employed a gradient reversed phase chromatography [49], ion-paired chromatography [50] or reversed phase chromatography following derivatization with a fluorescent label [53,55]. Other HPLC-based assays, which may have considerable potential for measuring purine nucleotides are combined LC–MS method with atmospheric pressure ionisation (API) [56]. These mass spectrometry based methods, however, have not been vigorously tested for measuring the nucleotides in biomarker studies *in vivo*. The current HPLC assay employs a simple ion-paired chromatography using TBAS and operated in an isocratic condition at room temperature, which separates ATP, ADP, AMP, GTP, GDP and GMP from the internal standard DMUA (Fig. 1), although GMP was not measurable in the RBC lysate samples because of interference by endogenous substance. The assay could be optimised to separate GMP from the interfering

Table 1
Intra- and inter-assay variations over a period of 1 year

Adenine nucleotides	0.25 mg/mL		0.1 mg/mL	
	Intra-assay variation (%R.S.D.)	Inter-assay variation (%R.S.D.)	Intra-assay variation (%R.S.D.)	Inter-assay variation (%R.S.D.)
ATP	7.6	19	6.8	16
ADP	3.9	14	6.0	17

AMP and guanine nucleotides	0.05 mg/mL		0.02 mg/mL	
	Intra-assay variation (%)	Inter-assay variation (%)	Intra-assay variation (%)	Inter-assay variation (%)
AMP	6.8	12	7.7	18
GTP	6.9	17	8.7	15
GDP	8.6	13	8.2	17
GMP	Not determined due to interference			

materials by decreasing the content of methanol and slightly raising the pH, but it would compromise separation of the other nucleotides from the internal standard. The assay also includes a protein precipitation followed by a non-retentive SPE to remove the constituents included in the "Stopping Solution". This addition of "Stopping Solution" was necessary to stabilize blood samples and prevent *in vitro* degradation and production of adenosine during sample collection and manipulation [30,47,48]. The SPE procedure also removes other drugs enabling the assay to be used for *in vivo* pharmacodynamic and biomarker assessment for drug development as shown in this study.

The HPLC assay has demonstrated a good linearity ($r^2 > 0.99$) (Fig. 2) and adequate reproducibility and robustness over a 1-year period. The intra-assay variations were less than 10% for all the nucleotides measured, and the inter-assay variations were less than 20% (Table 1). The HPLC assay has also shown sufficient sensitivity (based on LOD) and specificity to measure ATP, ADP, AMP and GTP in RBC obtained from the SD and SHR rats. It should be also applicable for clinical studies as the concentrations of the adenine nucleotides in humans are in the similar range as the rat samples [37,57]. Due to relatively low concentrations (<0.01 mM) and presence of endogenous materials that interfere with measurement of GMP, the concentrations of GDP and GMP were not measurable in most of the study samples.

RBC concentrations of ATP and other adenine nucleotides have been used as biomarkers for assessing the functional integrity of RBC *in vitro* for transfusion [39,40,58–61], and for *in vivo* assessment of primary pulmonary hypertension [36], chronic renal failure [37], diabetes mellitus and aging [38], metabolic syndrome [39,40], and in a rat model of colitis [62]. The potential of RBC concentrations of GTP, GDP and GMP as biomarkers is not clear although they may have a diagnostic role in cancer and inflammatory disorders [63,64]. More recently, we have shown that ATP and adenosine concentrations in the RBC may be used as surrogate biomarkers for anti-ischemic drugs [41,65]. It is interesting to note that RBC concentrations of ATP and GTP were significantly higher in the SHR than the SD rats (Table 2), and that exercise increased RBC concentrations of ATP only in the DTZ-treated rats (Fig. 3). In contrary, the concentrations of AMP were higher in the SD rats, but the difference was not significance ($p > 0.05$). While the clinical relevance of these data is not clear at the moment, the results suggest that ATP metabolism in the RBC may be different between the SD rats and SHR, and that anti-ischemic drugs such as diltiazem may alter ATP metabolism *in vivo* particularly in respond to exercise induced hemodynamic and neurohormonal changes [44]. Further studies on the potential of RBC concentrations of the nucleotides as relevant biomarker targets for anti-ischemic drugs, and their putative roles in cardiovascular disease are warranted.

Table 2
Average circulatory concentrations of nucleotides in the RBC for restrained rats

Nucleotides/rat (μmol/mL)	SD rats ($n = 4$)	SHR ($n = 5$)
ATP	1.16 ± 0.59[a]*	4.46 ± 0.76*
ADP	1.17 ± 0.46	2.43 ± 0.53
AMP	2.20 ± 0.87	0.55 ± 0.05
GTP	0.12 ± 0.05*	0.44 ± 0.07*
GDP	Not measurable	Not measureable
GMP	Not measurable due to interference	Not measurable due to interference

*Difference $p < 0.05$ (two-sample *t*-test).
[a] Each value represents mean ± S.E.M. of 4–5 rats.

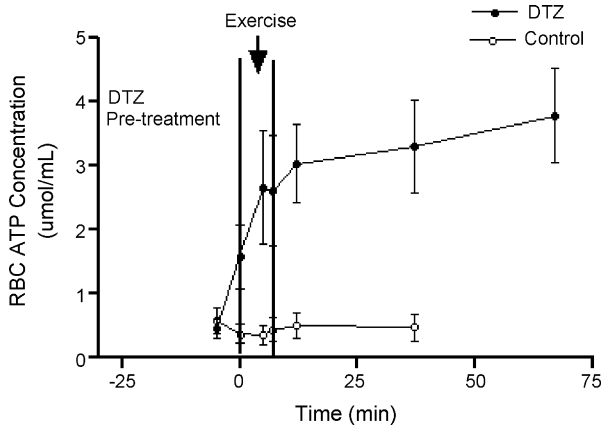

Fig. 3. RBC concentrations of ATP during exercise in rats with and without pre-treatment with DTZ (10 mg/kg sc twice daily for five doses). Each point represents mean ± S.E.M. of 5–6 SD rats.

4. Conclusion

The simple HPLC assay as described in this research article has demonstrated adequate sensitivity (based on LOD), specificity and robustness for measuring RBC concentrations of ATP, ADP, AMP, and GTP for pharmacodynamic and biomarker studies in rats *in vivo*. It supports further the potential of these purine nucleotides as surrogate bioamarkers for cardiovascular drugs and heart and stroke diseases.

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