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Short Communication

Rapid separation of creatine, phosphocreatine and adenosine metabolites by ion-pair reversed-phase high-performance liquid chromatography in plasma and cardiac tissue

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ABSTRACT

A rapid ion-pair reversed-phase high-performance liquid chromatographic method has been developed for the simultaneous detection of creatine, phosphocreatine, hypoxanthine, inosine, adenosine, AMP, ADP, ATP, 8-azaguanine, 2-chloroadenosine, and 2'-O-methyladenosine. This method has proven useful for measuring changes in nucleotide concentrations in both heart tissue and plasma samples. Separation of the compounds of interest is achieved in less than 8 min with re-equilibration in 7 min, making the total run time 15 min. Separation is performed on a 3-μm Ultrasphere ODS column employing tetrabutylammonium phosphate as the ion-pair agent and dipotassium hydrogenphosphate as the counter ion. The accuracy, rapid separation, and re-equilibration time make this method particularly useful for the routine analysis of a large number of samples.

INTRODUCTION

Adenosine and its phosphorylated forms have a wide variety of physiological effects and functions both inter- and extracellularly. Extracellular adenosine has been shown to be a potent vasodilator, involved in neurotransmission, and a modulator of inflammatory activity through its action on leukocytes. Extracellular adenosine and adenine nucleotide receptors have been identified and, whereas adenosine receptors appear linked to adenylate cyclase, nucleotide receptors seem to control ion fluxes. The development of adenosine analogues have had a significant impact on our knowledge of these receptor functions. Therapeutic uses of intravascularly administered adenosine and its phosphorylated forms are a future possibility [1,2]. The involvement of adenosine in biological processes has usually been monitored by the measurement of adenosine and its metabolites in biological fluids. Due to the extremely rapid metabolism of adenosine and related compounds, a sample must be collected in the presence of enzymatic inhibitors (such as the analogue 8-azaguanine) to accurately measure levels of the metabolites as they existed under biological conditions [3,4]. Tissue samples are collected with rapid freezing. The meta-

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bolic enzymes are later inactivated by protein precipitation prior to analysis.

The phosphorylated forms of adenosine and creatine are major intracellular forms of energy storage and transfer. Various models of disease states include periods of inadequate oxygen supply and/or reduced substrate delivery to tissues resulting in depletion of intracellular energy stores. Evaluation of conditions that preserve tissue levels of phosphorylated adenosine and creatine are of vital interest [5,6]. Therefore, a rapid means of analyzing tissue levels of adenosine metabolites is of great interest. The use of analogues such as 2-chloroadenosine or 2′-O-methyladenosine as an internal standard provides a means of determining recovery of these compounds throughout the extraction procedure and the analysis.

Several recent reports describe the separation of high-energy adenine nucleotides [7–10]. The methods that analyze phosphocreatine in addition to the adenine nucleotides [8–10] either have a relatively long run time [8], use a large initial sample size [10], measure at only one wavelength [8,9], employ an undesirable mobile phase [8], or use a cumbersome step gradient [10]. Our method employs the best of these protocols with the unique feature of the ability to separate adenosine analogues that can be used as internal standards in as little as 15 min.

This paper describes a technique which employs an ion-pair reversed-phase high-performance liquid chromatographic (HPLC) column with a dual-wavelength detector. A computer-driven data acquisition system facilitates the integration and analysis of the detected response obtained from the metabolites of adenosine and creatine. This method rapidly separates and quantitates creatine, phosphocreatine, hypoxanthine, inosine, adenosine, 2′-O-methyladenosine, 8-azaguanine, 2-chloroadenosine, AMP, ADP, and ATP. We have verified the method by evaluating the distribution of these compounds in both biological fluid (blood) and organ tissue (cardiac muscle) under differing experimental conditions. Techniques for extraction of plasma and tissue samples including measurement of recovery are described.

EXPERIMENTAL

Materials

Tetrabutylammonium phosphate, anhydrous dipotassium hydrogenphosphate, creatine, phosphocreatine, 8-azaguanine, adenosine, and adenosine metabolites and derivatives were obtained from Sigma (St. Louis, MO, USA). HPLC-grade water and methanol were acquired from Fisher Scientific (St. Louis, MO, USA). All buffers and solutions used for HPLC analysis were carefully degassed and filtered through a 0.45-μm filter (Schleicher and Schull, Kenne, NH, USA).

Instrumentation and chromatographic conditions

Analysis was performed on a Model 338 HPLC system (Beckman, Arlington Heights, IL, USA) consisting of two 110B pumps, a 210A sample injection valve (50 μl), and a 167 dual-wavelength detector. Solvent programming, data collection, and integration were accomplished on-line with Beckman software. Separation was achieved on a 3-μm Ultrasphere XL ODS (70 mm × 4.6 mm I.D.) column (Beckman) by a two-component gradient mobile phase. The initial mobile phase (A) consisted of 0.1 *M* anhydrous dipotassium hydrogenphosphate and 0.01 m*M* tetrabutylammonium phosphate in HPLC-grade water. The pH was adjusted to 6.5 with 6 *M* hydrochloric acid. Mobile phase B was prepared as described above, except the reagents were dissolved in 40% methanol.

Before assays were performed, the column was equilibrated with mobile phase A until response factors for successive analyses of the reference solution differed by less than 1% (usually obtained by the second reference analysis). With the injection of a 50-μl sample, solvent flow was initiated by employing a 3-min linear gradient from 0 to 50% mobile phase B at a flow-rate of 1.0 ml/min. These conditions were maintained for an additional 4 min before the gradient was returned to 100% mobile phase A over a 4-min interval. The column was equilibrated with mobile phase A for 4 min before the next injection, making the run a total of 15 min. The eluent was monitored at 254 and 214 nm. Concentrations were calculat-

ed using the previously computed response factors (μmol/l per unit area) at 214 nm for creatine and phosphocreatine and 254 nm for all other compounds. Peak identities were confirmed by coelution with standards and relative absorbancies at 214/254 nm.

Standards

Reference solutions of ATP, ADP, AMP, adenosine, 2-chloroadenosine, 2'-O-methyladenosine, hypoxanthine, inosine, 8-azaguanine, creatine, and phosphocreatine were prepared by individually dissolving the compounds in mobile phase A to a concentration of 1 mg/ml. The standard mixture was freshly prepared by combining equal volumes of each compound.

Biological fluid preparation: plasma samples

Blood was collected into pre-weighed, chilled syringes containing 4 m*M* 8-azaguanine, 100 μ*M* dipyridamole, and 4 m*M* EDTA. Immediately following collection, the blood was separated (1000 *g* for 15 min at 4°C) into its cellular and plasma components. Plasma (500 μl) was deproteinized by ultrafiltration in a Centricon 10 microconcentrator (Amicon Division, W. R. Grace, Danvers, MA, USA) and centrifuged at 5000 *g* for 3 h at 4°C. A 50-μl volume of the ultrafiltrate obtained was analyzed directly by HPLC.

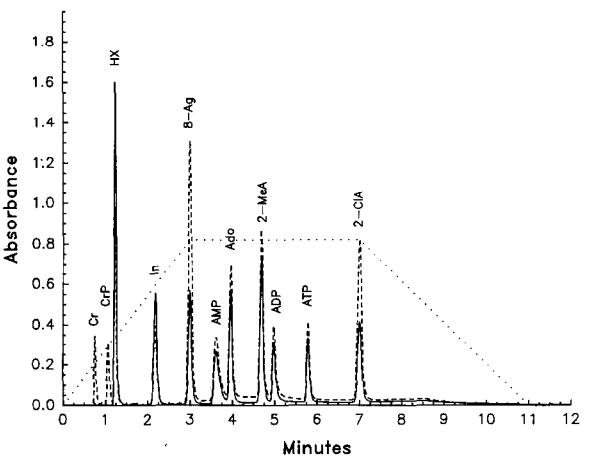

Fig. 1. Ion-pair reversed-phase HPLC on a 3-μm (70 mm × 4.6 mm I.D.) Ultrasphere XL ODS column of a standard mixture using chromatographic conditions reported under Experimental. The chromatogram was obtained with a 50-μl injection of the standard mixture. The compounds were monitored at 254 nm (——) and 214 nm (– – –).

Tissue sample preparation: heart tissue

Frozen myocardial tissue, weighing between 5 and 10 mg, was placed into a glass grinder containing 0.4 ml of cold (4°C) 12% (w/v) trichloroacetic acid (TCA) and 50 μl of 0.95 mg/ml 2-chloroadenosine. Extraction was conducted at 4°C for 30 min with periodic grinding producing a homogenous slurry. After centrifugation at 4°C for 2 min at 11 000 *g*, the supernatant was added to 800 μl of a 3:1 mixture of freon and tri-*n*-octylamine and vortex-mixed for 1 min [11]. Centrifugation at 11 000 *g* for 2 min at 4°C provided phase separation. The aqueous layer was analyzed directly on the HPLC system. Chromatographic values (μmol/l) were converted to μmol/g of total protein. The amount of total protein was ascertained by resuspending the protein pellet (precipitated during the acid extraction) in 1.0 ml of 0.5 *M* sodium hydroxide overnight at 4°C. The protein content of the resuspended pellet was determined by a modified Lowry assay (Sigma). Recoveries were determined as described for plasma procedures.

RESULTS

Retention times, wavelength ratios, and scan comparisons were used to determine peak purity and peak identification. Fig. 1 demonstrates the separation of the standards using the method stated above. This method proved valuable for the routine measurement of creatine, phosphocreatine, and/or adenosine and its associated forms in both plasma and cardiac tissue. The chromatogram of a cardiac biopsy extract is shown in Fig. 2. The ability of the method to detect the depletion of ATP to hypoxanthine and inosine in canine cardiac tissue is shown in Fig. 3. Determined values are comparable to those reported by others [5]. Fig. 4 demonstrates the conservation of the adenine backbone in canine tissue during a 5-min *in vitro* incubation within a closed system where blood flow cannot remove nucleotides and metabolites. The data from Fig.

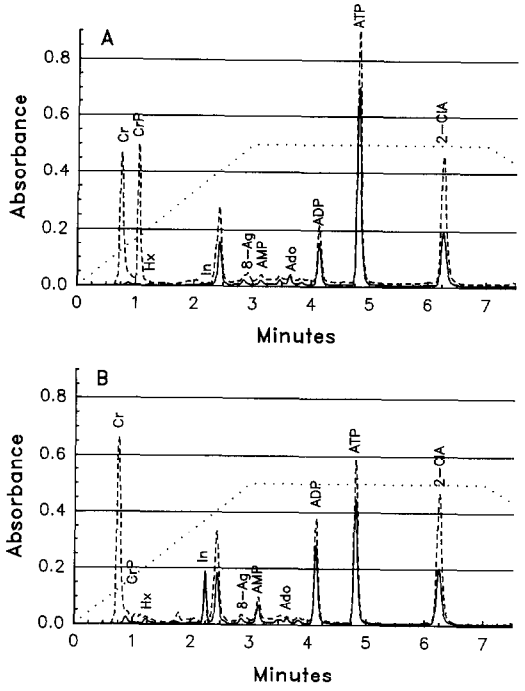

Fig. 2. Dog heart tissue separated by ion-pair reversed-phase HPLC on a 3-μm (70 mm × 4.6 mm I.D.) Ultrasphere XL ODS column using chromatographic conditions reported under Experimental to measure adenosine metabolites in tissue. This chromatogram of ischemia (A) or reperfusion (B) was obtained with a 50-μl injection and the eluent monitored at 254 nm (——) and 214 nm (– –). Peaks: Cr = creatine; CrP = phosphocreatine; Hx = hypoxanthine; In = inosine; 8-Ag = 8-azaguanine; AMP = adenosine monophosphate; Ado = adenosine; ADP = adenosine diphosphate; ATP = adenosine triphosphate; 2-ClA = 2-chloroadenosine.

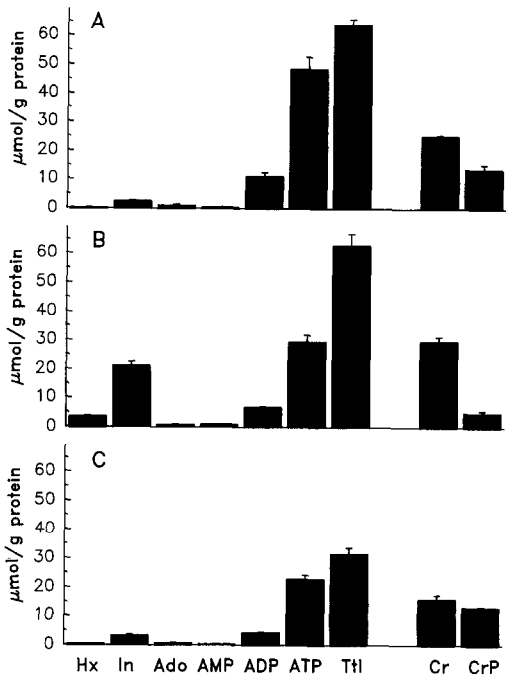

Fig. 3. Concentration of creatine and adenosine precursors and degradation products in canine cardiac tissue. Cardiac biopsies were performed at 37°C immediately prior to initiating cardiopulmonary bypass (A), 30 min after aortic cross-clamping (B), and after 30 min of reperfusion (C). Harvested tissue was immediately immersed in liquid nitrogen and subsequently divided. Each biopsy was prepared according to tissue preparation procedures as described. Hx = hypoxanthine; In = inosine; Ado = adenosine; AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; Ttl = total adenine pool; Cr = creatine; CrP = phosphocreatine. Reperfusion significantly decreased total adenine nucleotide degradation products, suggesting a washout of soluble precursors during reperfusion. The results support the concept of a reperfusion injury.

4 illustrate that the differences observed in the cardiac tissue in Fig. 3 were not due to technique. The ability to quantitate the total adenine pool (Figs. 3 and 4) has allowed the assessment of the degree of adenosine transport and deamination blockade, along with the re-phosphorylation potential of myocardial adenosine during reperfusion. The results of a representative experiment investigating the clearance of adenosine from plasma are shown in Fig. 5. The data are consistent with that reported by others [12] and demonstrate the regulation of plasma adenosine by red blood cells.

The experimental recoveries determined by adding a known amount of standard to the tissue extracts were 72–87% for plasma sample extractions and 96–101% for cardiac muscle preparations. To assess the linearity of the described method, increasing concentrations of the individual standards were analyzed. The linear relationships of peak area to concentration were defined by the following ranges: creatine and phosphocreatine, 10 nM to 1 mM; hypoxanthine, inosine, 8-azaguanine, adenosine, 2′-O-methyladenosine, 2-chloroadenosine, AMP, ADP, and ATP, 50 nM to 0.5 mM. The correlation coefficients de-

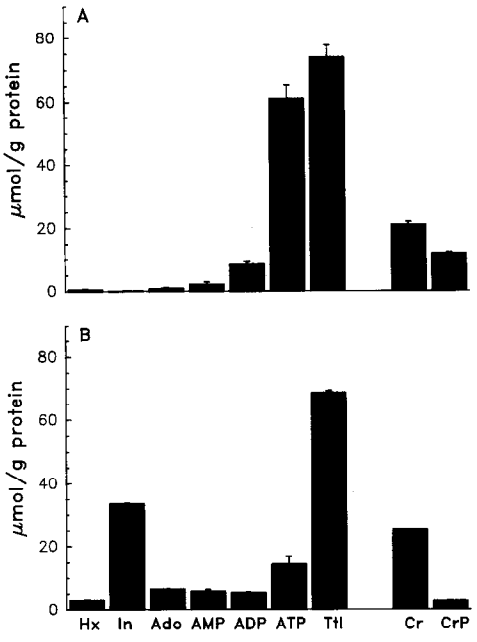

Fig. 4. *In vitro* incubation of canine cardiac tissue illustrating the use of this method to determine adenine backbone conservation (total adenine pool) during ATP metabolism. (A) Baseline/pre-incubation; (B) *in vitro* incubation for 5 min at 37°C. A and B were prepared according to tissue sample preparation procedures as described. Hx = hypoxanthine; In = inosine; Ado = adenosine; AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; Ttl = total adenine pool; Cr = creatine; CrP = phosphocreatine.

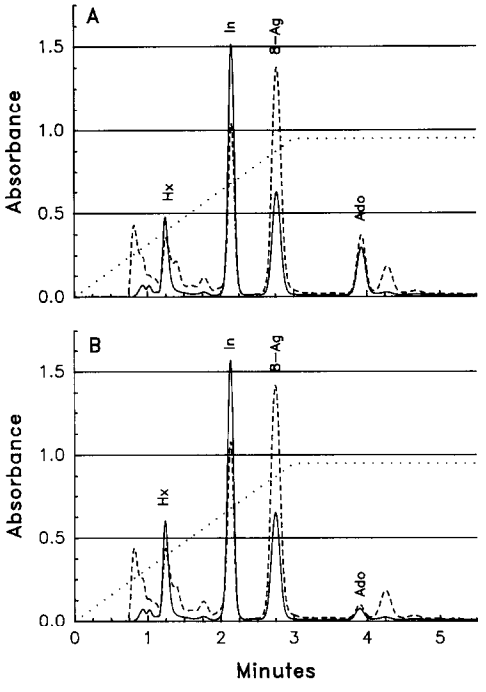

Fig. 5. Rabbit blood separated by ion-pair reversed-phase HPLC on a 3-μm (70 mm × 4.6 mm I.D.) Ultrasphere XL ODS column using chromatographic conditions reported under Experimental to measure adenosine metabolites in plasma. These chromatograms of adenosine degradation in rabbit blood were obtained with 50-μl injections and the eluents monitored at 254 nm (——) and 214 nm (– – –). In these experiments, blood samples were collected into weighed syringes containing 4 m*M* 8-azaguanine, 100 μ*M* dipyridamole, and 4 m*M* EDTA at 10 s (A) and 60 s (B) after 500 μg of adenosine in 1.0 ml of saline was added to 5.0 ml of rabbit blood tonometered (Model 237, Instrumentation Labs.) at 37°C. A and B were prepared according to biological fluid extraction procedures as described. Due to the rapid adenosine uptake by red blood cells and significant adenosine deaminase activity which is characteristic of rabbit blood, adenosine could be recovered only in the presence of adenosine deaminase inhibition by 8-azaguanine in the sample collection syringe. Over the 50-s incubation interval between chromatograms A and B, the decrease in plasma adenosine concentration was accompanied by a concomitant rise in hypoxanthine and inosine. Peaks: Hx = hypoxanthine; In = inosine; 8-Ag = 8-azaguanine; Ado = adenosine.

scribing these linear relationships, for all compounds examined, were ≥ 0.998.

DISCUSSION

The aim of the present work was to develop a rapid, accurate and reproducible HPLC method which would allow analysis of adenosine, its high-energy precursors, degradation products (inosine and hypoxanthine) and analogues (2-chloroadenosine, 8-azaguanine, and 2′-O-methyladenosine) in various biological fluids and organ tissues. The HPLC method described in this paper will separate all of the adenine nucleotides and the analogues described. With regard to guanosine derivatives, GMP and GDP are not totally separable from inosine and adenosine, respectively. In both normoxic and ischemic tissues guanosine derivatives have been reported to be 1–4% of the adenosine metabolite concentrations [13,14]. This was supported by our sample analyses. The wavelength ratios of the sample peaks for inosine and adenosine demonstrated that the guanosine derivatives are in quantities below the

level of detectability and therefore do not interfere with the analyses described.

This method is attractive when considering its rapid separation and re-equilibration with minimal change in retention time and steady baseline with baseline separation of the compounds of interest. Further, the dual-wavelength detection employed affords the simultaneous quantification of creatine and phosphocreatine with adenosine compounds while providing a second determination for ratio purposes. A 5-mg amount of heart tissue has proven to be an adequate sample size which is an important consideration where multiple biopsies are to be taken during an experiment, particularly in small species. The method is tolerant of sample injectate derived from various biological fluids and tissues and extraction procedures, eliminating the need for preliminary purification processes. This method is also capable of separating analogues and other related compounds. Not only does 8-azaguanine (commonly used to uncouple oxidative phosphorylation and inhibit adenosine deaminase) not interfere with analysis but its concentration can be determined. So also with the adenosine receptor analogues 2′-O-methyladenosine and 2-chloroadenosine. Thus, 8-azaguanine, 2′-O-methyladenosine, and 2-chloroadenosine provide a selection of compounds that can be used as internal standards, providing percent recoveries for each sample. The short time of analysis, the reasonably high sensitivity, the reproducibility of the system, and the accurate evaluation of the compounds under investigation make this method particularly useful.

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