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SIMULTANEOUS QUANTIFICATION OF MYOCARDIAL ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new ion-pair reversed-phase high-performance liquid chromatography method was applied to simultaneous measurements of porcine myocardial adenine nucleotides and creatine phosphate. The homogeneity of each desired peak was tested by the retention times of standards, chromatography of spiked samples, the absorbance ratios at various wavelengths, and the differing retention times for a number of other compounds found in porcine myocardial extracts.

INTRODUCTION

Preservation of creatine phosphate (CP) and adenosine triphosphate (ATP) levels during an ischemic insult, when there is a temporary inhibition of blood flow, is considered to be one of the most important determinants for the functional recovery of biological tissues, especially hearts. Although it is a common practice to measure the levels of CP and ATP in an ischemic myocardium, there is no method available to quantitate these two parameters by a single assay. Currently available ion-pair reversed-phase high-performance liquid chromatographic (HPLC) methods require a separate injection with a change in wavelength and mobile phase in order to measure the CP and give incomplete separation of adenosine monophosphate (AMP)[1]. The ion-exchange HPLC method of Harmsen *et al.*[2] allows a simultaneous determination, but the baseline drifts due to the gradient, and baseline separation of AMP is not achieved. In this report, we describe a rapid ion-pair reversed-phase HPLC method for the simultaneous measurements of porcine myocardial adenine nucleotides and creatine phosphate.

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EXPERIMENTAL

Materials

Nucleotides, nucleosides, nucleobases, creatine, and creatine phosphate used as chromatographic standards were purchased from Sigma (St. Louis, MO, U.S.A.). Myokinase, 5'-adenylic acid deaminase, and adenosine 5'-triphosphatase were also from Sigma. Tetrabutylammonium phosphate was purchased as PIC Reagent Low UV A from Waters Assoc. (Milford, MA, U.S.A.). UV spectrograde acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). HPLC-grade water was prepared by filtering deionized, glass-distilled water through Norganic cartridges from Waters Associates. Centrifugal 0.45 μm Nylon microfilters were from Schleicher & Schuell (Keene, NH, U.S.A.).

Equipment

A Waters HPLC system was used, consisting of a WISP Model 710B injector, Model 720 System Controller, Model 730 Data Module, Model 450 UV detector, Model 6000A pump, Model 45 pump, μBondapak C_{18} Guard-PAK precolumn, Z-Module, and a Radial-PAK μBondapak C_{18} radial compression cartridge.

Methods

Myocardial biopsies from anesthetized 20-kg pigs of either sex were obtained using a high-speed dental drill fitted with a vacuum line for rapid removal of tissue. The samples were immediately immersed in liquid nitrogen and stored at $-73°C$ until extracted. The biopsy specimens were homogenized in 6% perchloric acid using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY, U.S.A.), centrifuged, neutralized to pH 7.0 with 5 *M* potassium carbonate, and filtered for HPLC, as described previously[3].

A volume of 25 μl of a filtered, neutralized, perchloric acid extract was injected onto a Radial-PAK μBondapak C_{18} column with an initial mobile phase of 48 m*M* monobasic potassium phosphate, 2.9 m*M* tetrabutylammonium phosphate (pH 5.8, with phosphoric acid) at a flow-rate of 2 ml/min. The effluent was monitored at an absorbance of 210 nm in order to measure both the adenine nucleotides and CP. After 7 min, a linear gradient was initiated, raising the concentration of acetonitrile to 10% after 15 min, and the second mobile phase, which contained 48 m*M* monobasic potassium phosphate, 2.9 m*M* tetrabutylammonium phosphate, 10% acetonitrile (pH 5.8), was continued until ATP was eluted. The column was equilibrated with the initial mobile phase for 30 min before the next injection.

RESULTS

Chromatograms

CP, ATP, adenosine diphosphate (ADP), and AMP were separated, both as standards (Fig. 1A) and in a porcine myocardial extract (Fig. 1B), with retention times (t_R) of approximately 5.4, 18, 22, and 28 min respectively. Peaks designated as ATP, ADP, AMP, and CP were identified with authentic compounds as described below.

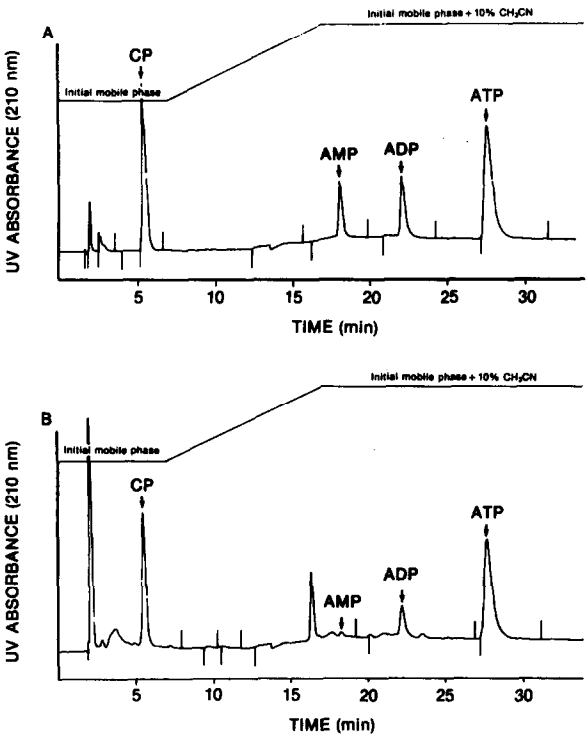

Fig. 1. Separation of AMP, ADP, ATP and CP by ion-pair reversed-phase HPLC. A volume of 25 μl of sample was injected into a C_{18} column as described in Experimental. (A) Mixture of standards containing 1 m*M* CP, 0.1 m*M* AMP, 0.15 m*M* ADP, and 0.5 m*M* ATP. (B) Porcine myocardial perchloric acid extract, filtered and neutralized, as described in Experimental.

Peak identification

The identification of various peaks was performed according to previously established methods[4–6]. Identification was principally performed by comparing retention times and by peak shift studies[1]. Although a large number of peaks appeared when pig heart extracts were subjected to HPLC, we confined our attention to the adenine nucleotides and CP.

When porcine myocardial extracts were spiked with standards, the CP and adenine nucleotide peaks increased both quantitatively and symmetrically. By injecting standards, the retention times for a number of other compounds such as creatine (t_R = 2.3 min), hypoxantine (t_R = 4.8 min), xantine (t_R = 4.0 min), uric acid (t_R = 6.8 min), adenine (t_R = 2.9 min), adenosine (t_R = 3.4 min), inosine (t_R = 9.2 min), inosine monophosphate (IMP) (t_R = 12.3 min), and nicotinamide adenine dinucleotide (NAD) (t_R = 16.4 min) were shown to differ from the retention times of the adenine nucleotides and creatine phosphate without any peak overlap. Triplicate injections of CP (t_R = 5.4 min) and adenine nucleotide (AMP, t_R = 18 min; ADP, t_R = 22 min; ATP, t_R = 28 min) standards, along with porcine myocardial extract, measured at 210 nm, 220 nm, 254 nm, and 280 nm, further showed the

TABLE I

PEAK AREA RATIOS OF STANDARDS AND PORCINE MYOCARDIAL EXTRACT AT DIFFERENT WAVELENGTHS

Both standards (creatine phosphate and adeine nucleotides) and porcine myocardial extract were injected into a C_{18} column and chromatographed as described in Experimental. The peak areas from triplicate runs were obtained at wavelengths of 210 nm, 220 nm, 254 nm, and 280 nm. The peak area ratios are expressed as mean ± standard deviation (S.D.).

Compound	λ_1/λ_2	*Standard*	*Extract*
AMP	254/280	3.94 ± 0.11	3.87 ± 0.12
ADP	254/280	3.98 ± 0.08	4.08 ± 0.08
ATP	254/280	4.04 ± 0.01	3.93 ± 0.01
CP	210/220	3.52 ± 0.01	3.52 ± 0.07

homogeneity of the desired peaks. The absorbance ratios for each compound are shown in Table I.

A peak shift study was performed, as described previously[1], by acidifying the porcine myocardial extract with hydrochloric acid, followed by heating, which resulted in a decrease in the CP peak and increase in the creatine peak.

Enzymatic degradation of the adenine nucleotide peaks was also investigated[4]. Addition of 5'-adenylic acid deaminase to the extract caused a disappearance of the AMP peak and an increase in the IMP peak. Myokinase converted ADP to AMP and ATP, while adenosine 5'-triphosphatase gave a decrease in ATP and a resulting increase in ADP.

Quantitative estimation

Quantification of adenine nucleotides and CP by peak area determinations was performed for seven samples in each group. Isolated *in situ*, a pig heart was subjected to 60 min of regional ischemia by occluding the left anterior descending coronary

TABLE II

ATP, ADP, AMP, CP, AND ADENYLATE ENERGY CHARGE IN PIG HEART AS A FUNCTION OF ISCHEMIA AND REPERFUSION

Isolated pig heart was subjected to 60 min of regional ischemia by the occlusion of left anterior descending coronary artery, followed by 60 min of global hypothermic, cardioplegic arrest. Hearts were then reperfused for a further period of 60 min. Heart biopsy specimens were withdrawn and assayed as described in Experimental. Results (μmol/gm wet weight) are expressed as mean ± S.D. of seven experiments in each group.

	ATP	*ADP*	*AMP*	*Energy charge*	*CP*
Control (pre-occlusion)	4.37 ± 0.26	1.75 ± 0.14	0.15 ± 0.06	0.83 ± 0.02	6.92 ± 1.05
Occlusion (60 min)	0.75 ± 0.20	0.68 ± 0.18	1.29 ± 0.10	0.40 ± 0.03	1.98 ± 0.97
Arrest (60 min)	0.58 ± 0.14	0.61 ± 0.15	0.88 ± 0.14	0.42 ± 0.05	1.07 ± 0.52
Reperfusion (15 min)	0.65 ± 0.14	0.63 ± 0.10	0.84 ± 0.07	0.46 ± 0.04	1.85 ± 0.54
Reperfusion (60 min)	1.03 ± 0.19	0.59 ± 0.15	0.75 ± 0.12	0.56 ± 0.04	4.84 ± 0.61

artery, followed by 60 min of a global, hypothermic, cardioplegic arrest[7]. Hearts were then reperfused for a further period of 60 min. Myocardial biopsy specimens were withdrawn prior to ischemia after occlusion, arrest, 15 min reperfusion, and 60 min reperfusion. The tissues were processed as described previously in *Methods*. The results are shown in Table II. Concentrations of ATP, ADP, AMP, and CP found were in the same range as that found in the literature[8,9], indicating the reliability and accuracy of this method.

DISCUSSION

ATP and CP levels are considered as the most important compounds for the proper functioning of the heart. During ischemia, depletion of ATP level by adenylate kinase occurs, resulting in a rise in cell ADP content, accompanied by a proportionally large increase in AMP content[7]. At the onset on reperfusion, redistribution of adenine nucleotides takes place, which is reflected in the value of adenylate energy charge, defined by (ATP + ½ADP)/(ATP + ADP + AMP). This energy charge is generally considered a gauge for the degree of rephosphorylation of the adenine nucleotides[10]. In addition, it is equally important to monitor the levels of CP in the ischemic, reperfused myocardium, because CP is known to serve as an "energy buffer" and to play a significant role in the energy transport between various cellular compartments[11]. Therefore, we have focused our attention on developing a rapid, accurate, and reproducible technique of analyzing ATP, ADP, AMP, and CP in the ischemic, reperfused myocardium. Tissue size is also an important parameter to be considered, because in many cases several biopsy specimens must be taken from a single heart during an experiment. In our method, we are able to use tissue size as small as 50 mg.

We have used an absorbance wavelength of 210 nm, since creatine phosphate has a low extinction coefficient at 254 nm, while the absorbance at 210 nm due to adenine nucleotides does not differ significantly from that at 254 nm. A low pH is used for optimal interaction of compounds with a C_{18} column[12], and pH 5.8 gives the best separation of the desired compounds. The paired-ion, tetrabutyl ammonium phosphate, ensures complete separation of ADP from ATP. The concentration of 2.9 m*M* maximizes the separation of CP from the void volume. The absence of acetonitrile for 7 min allows nucleobases and CP to be eluted separately, while the acetonitrile gradient elutes the adenine nucleotides. Higher concentrations of acetonitrile, while shortening the separation, did not result in homogeneous separation of the adenine nucleotides, especially AMP. Changing the concentration of monobasic potassium phosphate or tetrabutylammonium phosphate during the gradient also did not improve the separation of the adenine nucleotides. A 30-min equilibration of the column with the initial mobile phase washes off the acetonitrile.

The homogeneity of the CP and adenine nucleotide peaks in the porcine myocardial extracts was confirmed by the retention times of standards, chromatography of spiked samples, acid degradation of CP, enzymatic degradation of peaks, and the absorbance ratios at various wavelengths. Our method achieved a stable baseline with baseline separation of CP and the adenine nucleotides.

The method described in this report is rapid; the extraction of ATP, ADP, AMP, and CP and their complete separation are achieved in less than 45 min. A

tissue biopsy as small as 50 mg can be processed with a high degree of accuracy and reproducibility. These extraction and chromatographic procedures have been used to successfully quantify ATP, ADP, AMP, CP and adenylate energy charge defined by (ATP + ½ADP)/(ATP + ADP + AMP) values from ischemic, reperfused pig myocardium. The data are in general agreement with those reported by other investigators[8,9], who also found significant decrease in ATP, CP, and energy charge values during ischemia. After 60 min of normothermic ischemia, ATP declined by about 40%, energy charge by 50%, and CP by 70% compared to the preischemic control values. A period of 60 min of reperfusion following 60 min of ischemia increased CP and energy charge close to their normal values, whereas ATP values increased only slightly. In this study, we also found similar changes in ATP, energy charge, and CP values, demonstrating the reliability of this method. Since the values of these high-energy phosphate compounds are of utmost importance in evaluating proper myocardial functions during ischemia and reperfusion,the assays of these compounds are routinely performed by most investigators as the most reliable and important criterion of myocardial recovery after reperfusion of ischemic myocardium. Because of the great sensitivity and rapidity, a large number of samples can be processed within a minimum time period. The method we report here should be easily adaptable for use with other tissues.

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