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# NOVEL DUAL-WAVELENGTH MONITORING APPROACH FOR THE IMPROVED RAPID SEPARATION AND ESTIMATION OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A rapid and improved method for the simultaneous quantification of adenine nucleotides, including adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, as well as creatine phosphate by high-performance liquid chromatography is described. A programmable multi-wavelength UV detector was used to monitor the effluent initially at 210 nm for 4 min to measure creative phosphate, and then at 259 nm for 6 min to quantitate adenosine mono-, di- and triphosphate. The method is, thus, not only rapid, but also extremely sensitive because of the utilization of corresponding absorption maxima for creatine phosphate and adenine nucleotides, rather than measuring all of them at a single wavelength. In addition, computer-generated standard curves were used to estimate these compounds, thereby improving the accuracy of the measurements.

Since myocardial high-energy phosphate levels are widely measured because they are reliable parameters for myocardial preservation during ischemia, these compounds were assayed in pig heart prior to and following ischemia. The results were compared with those obtained by conventional high-performance liquid chromatographic assay methods in order to examine the validity of this method. Our results indicate that this is indeed a novel method, which is more rapid as well as sensitive and accurate compared with other methods.

## INTRODUCTION

Despite a number of methods now available for the separation and quantitative estimation of high energy phosphate compounds in biological tissues [1–6], a rapid and accurate method for the measurement of extremely low levels of these compounds is

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still lacking. In most of these methods, adenine nucleotides are measured at 254 nm, whereas creatine phospate (CP) is measured at 210 nm, in which the respective absorption maxima are observed [1]. These methods, thus, require relatively more time because of the necessity of the separate injections of the samples onto the high-performance liquid chromatographic (HPLC) column, with a subsequent change in wavelength and mobile phase in order to measure CP and to achieve complete separation of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). Ion-exchange HPLC methods[2] allow simultaneous determination, but all the compounds are measured at 210 nm. The method is lengthy, the baseline drifts due to the gradient, and baseline separation of AMP is not achieved. Reversed-phase methods[3] also allow simultaneous analysis; however, CP and the adenine nucleotides are measured at 214 nm, and baseline separation of ADP and ATP in myocardial extracts is not achieved. Recently we[4] and others[5,6] have developed methods which allow simultaneous measurement of ATP, ADP, AMP, and CP. However, these methods utilize a wavelength of 210 nm, where only CP shows absorption maxima; hence, extremely low levels of adenine nucleotides are not adequately resolved. The same investigators [5,6] used rapid isocratic methods for their simultaneous analysis of adenine nucleotide and CP. However, baseline separation of CP from various bases and nucleosides in a myocardial extract and subsequent accurate quantitation can only be achieved by the absence of an organic solvent in the initial mobile phase. We, therefore, used the gradient system in order to subsequently elute the adenine nucleotides in our separation.

Since the preservation of high-energy phosphate compounds, including ATP and CP, during ischemic insult such as myocardial infarction or open-heart surgery, is considered to be one of the most important determinants for the functional recovery of heart[7], rapid and accurate measurements of extremely low levels of these compounds are necessary to predict the appropriate therapeutic interventions[8,9]. In this report, we describe a rapid HPLC technique aided by computer to use 210 nm and 259 nm wavelengths for estimating pmole amounts of high-energy phosphate compounds in myocardial tissue with rapidity and high precision.

## EXPERIMENTAL

### *Materials*

Adenine nucleotides and creatine phosphate used as chromatographic standards were purchased from Sigma (St. Louis, MO, U.S.A.). 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 obtained from a Millipore water system (Millipore, Bedford, MA, U.S.A.). Centrifugal 0.45-$\mu$m nylon microfilters were from Schleicher & Schuell (Keene, NH, U.S.A.). Solvent 0.45-$\mu$m Nylon-66 filters were purchased from Rainin Instrument (Woborn, MA, U.S.A.).

### *Equipment*

Two Waters Assoc. HPLC systems were used. The first system consisted of a WISP Model 710B injector, Model 720 system controller, Model 730 data module, Model 490 programmable multi-wavelength UV detector, Model 6000A pump, Mod-

el 45 pump, μBondapak $C_{18}$ Guard-Pak precolumn, Z-Module, and a Radial-Pak Nova-Pak $C_{18}$ (10 cm × 5 mm I.D., 4 μm particle size) radial compression cartridge. The second HPLC system consisted of Model 820 full control MAXIMA computer system, Satellite WISP Model 700 injector, Model 490 programmable multi-wavelength UV Detector, two Model 510 pumps, μBondapak $C_{18}$ Guard-Pak precolumn, Z-Module, and a Radial-Pak Nova-Pak $C_{18}$ (10 cm × 5 mm I.D., 4 μm particle size) radial compression cartridge.

*Methods*

Myocardial left ventricular 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[4].

A volume of 25 μl of a filtered, neutralized, perchloric acid extract was injected onto a Nova-Pak $C_{18}$ column with a degassed initial mobile phase of 48 m*M* monobasic potassium phosphate, 1 m*M* tetrabutylammonium phosphate (pH 5.8, with potassium hydroxide) at a flow-rate of 2 ml/min. Using a Waters Assoc. Model 490 programmable multi-wavelength UV detector, the effluent was monitored at 210 nm for 4 min to measure CP, and then monitored at 259 nm for 6 min to measure the adenine nucleotides. The initial mobile phase was used for 4 min, followed by a step gradient to 20% acetonitrile in the degassed initial buffer. After the 10 min run, the column was equilibrated with the initial mobile phase for 25 min prior to the next injection. The UV detector was auto-zeroed upon injection and during the change in wavelength. A blank was initially run and used for baseline substraction.

A calibration curve was produced using MAXIMA 820 software (Waters) system for each of the high-energy phosphate compounds. Using external standards, six different concentrations ranging from 10 μ*M* to 1 m*M* were used. A response factor was then calculated from the slope of each curve.

The MAXIMA 820 software and the I-200 system interface module controlled the two HPLC pumps and the auto-injector. The computer system also acquired and stored data from the UV detector. Data processing allowed editing of the integration and production of calibration curves. The M-490 UV detector was used for changing the wavelength, auto-zeroing, and for baseline subtraction.

## RESULTS

*Separation of ATP, ADP, AMP, and CP using two different wavelengths*

High-energy phosphate compounds were separated according to previously described methods[4]. These methods utilize a single wavelength of 210 nm to monitor compounds eluted. The retention times of CP, AMP, ADP, and ATP were 5.7 min, 18.5 min, 22.6 min, and 28.1 min, respectively, making a total run time of *ca.* 35 min (Fig. 1A).

CP and the adenine nucleotides were then separated utilizing the two-wavelength approach. As described in *Methods,* the effluent was initially monitored at 210

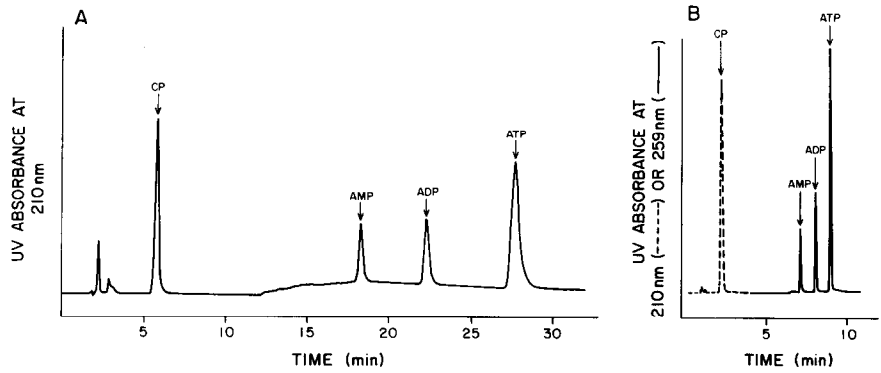


Fig. 1. Separation of CP, AMP, ADP, and ATP standards by ion-pair reversed-phase HPLC. A volume of 25 $\mu$l of standard solution of 1 m*M* CP, 0.1 m*M* AMP, 0.15 m*M* ADP, and 0.5 m*M* ATP was injected onto a $C_{18}$ column equipped with a programmable multi-wavelength detector. (A) Conventional method using a 10-$\mu$m particle size $C_{18}$ column and a linear gradient as described elsewhere[4]. (B) Modified method using a 4-$\mu$m particle size $C_{18}$ column, step gradient, baseline subtraction, and wavelength shift as described in Experimental (- - - -) 210 nm; (——) 259 nm.

nm for 4 min when CP was detected with a retention time of 2.2 min (Fig. 1B). The UV detector was pre-programmed, such that after 4 min the wavelength was changed to 259 nm when AMP, ADP, and ATP showed up with retention times of 7.1 min, 8.1 min, and 9.0 min, respectively. The peaks were identified by comparison with the authentic standards.

*Construction of calibration curve*

Using the second HPLC system described in Experimental, and employing the MAXIMA 820 software, a calibration curve and response factor were produced for each of the CP and adenine nucleotide standards. Six different concentrations in a ten-fold range for each standard were injected and chromatographed as described in Experimental. In Fig. 2, the concentrations of each standard, (A) CP, (B) AMP, (C) ADP, and (D) ATP, were plotted against the peak area obtained. A response factor was obtained from the slope of each curve. The calculated response factors for CP, AMP, ADP, and ATP were $2.234\,481 \cdot 10^7$, $1.167\,666 \cdot 10^7$, $1.270\,137 \cdot 10^7$, and $1.392\,313 \cdot 10^7$, respectively. The differences between the respective response factors can be explained because of the changes in peak shape as the component is retained on the column. In each case, however, the calibration curve was linear, with all points having a small standard deviation and falling on the curve. The *r* value was very close to 1 in each case.

*Quantitative estimation of high-energy phosphate compounds in pig heart*

Since the high-energy phosphate compounds are extremely susceptible to ischemic insult, we assayed ATP, ADP, AMP, and CP from pig heart prior to and after 60 min of normothermic ischemia, as well as after 60 min of ischemia followed by 60 min of revascularization. The quick-frozen myocardial biopsies were processed as described in *Methods*, and loaded onto the HPLC column equipped with programmable multi-wavelength UV detector. The results from control pre-ischemic heart

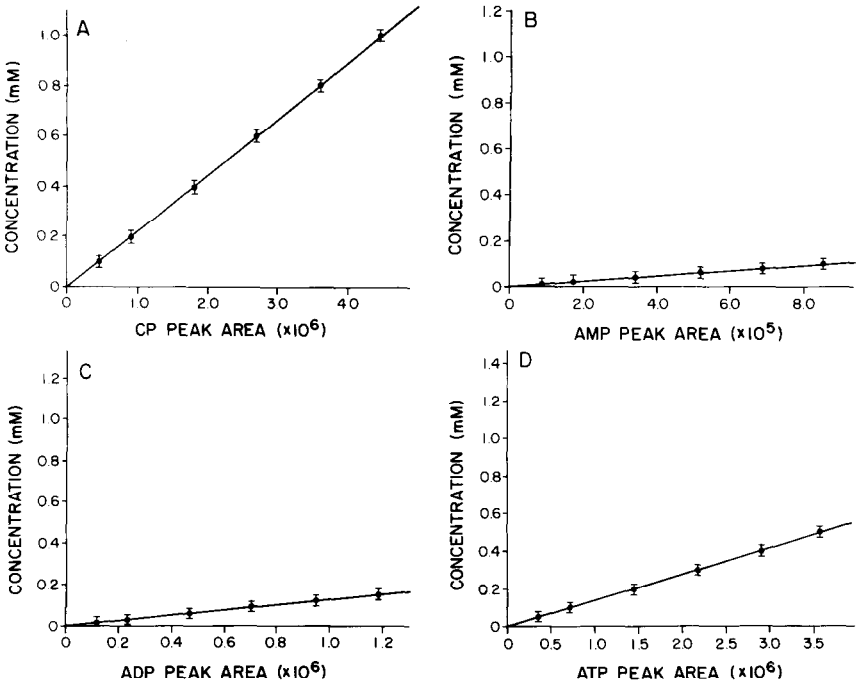


Fig. 2. Calibration curves for CP, AMP, ADP, and ATP standards. Six different concentrations of each standard, as indicated on the graph, were injected onto a $C_{18}$ column as described in Experimental, and the different concentrations were plotted against the peak area. Each value represents mean ± S.E. of at least three different experimental results. The *r* values are: CP = 0.999 859 58; AMP = 0.999 860 05; ADP = 0.999 948 50; ATP = 0.999 768 51 . (A) CP; (B) AMP; (C) ADP; (D) ATP.

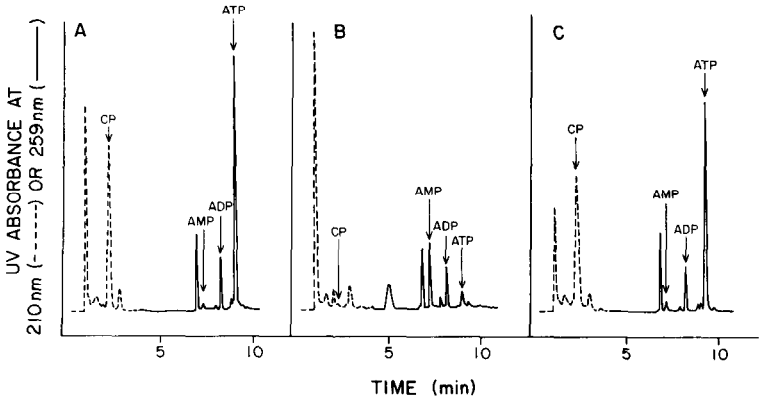


Fig. 3. Separation of CP, AMP, ADP, and ATP in porcine myocardial perchloric acid extracts, filtered and neutralized, as described in Experimental. Each injection consisted of 25 μl of neutralized perchloric acid extract containing 1 mg of tissue biopsy. The UV detector was set at 0.8 a.u.f.s. (A) Control heart; (B) ischemic heart; (C) reperfused heart.

TABLE I

LIMIT OF DETECTION FOR ATP, ADP, AMP AND CP BY HPLC

Standards of each component were injected onto a $C_{18}$ column as described in Experimental using the UV detector set at 0.001 a.u.f.s.

| | *Lowest limit of detection (pmol)* | *S/N ratio* |
|---|---|---|
| CP | 10.0 | 4.0 |
| AMP | 1.5 | 4.0 |
| ADP | 2.0 | 4.0 |
| ATP | 1.0 | 4.0 |

biopsies are shown in Fig. 3A, whereas the results from ischemic heart biopsies are demonstrated in Fig. 3B. As shown in the figure, even though the amounts of CP and ATP were extremely low, they were still detected with reasonable peak heights, enough to obtain accurate estimates. Peak-shift studies were done as described previously[4]. A porcine myocardial extract was acidified with hydrochloric acid and then heated to 50°C. This resulted in complete decrease in the CP peak and subsequent increase in the creatine peak. Addition of 5'-adenylic deaminase to the extract caused a disappearance of the AMP peak, with an increase in the inosine monophosphate (IMP) peak. Myokinase converted ADP to AMP and ATP, and adenosine 5'-triphosphatase caused the decrease of ATP and an increase in ADP. Close examination of Fig. 3 indicates the presence of another peak in close proximity to AMP. This peak, with a retention time of 6.8 min, indicates the presence of NADH, which is known to decrease during ischemia and increase to the preischemic value after reperfusion. Fig. 3 also shows the appearance of a peak only in the ischemic heart, which disappears after reperfusion. This peak, with a retention time of 5.0 min, was identified as IMP. Reperfusion following ischemia is generally associated with the rebouncing of the values towards the pre-ischemic levels[7–9]. As expected, the values of these compounds were much higher in the reprefused hearts then ischemic hearts, as evidenced by the relatively higher peaks shown in Fig. 3C. Accuracy of the method was determined by standard addition technique. Additions of even 10 pmol of ATP, 20 pmol of ADP and AMP, and 50 pmol of CP were accurately reflected in the peak heights. The lowest limit of detection for the adenine nucleotides and CP and corresponding signal-to-noise (S/N) ratios are given in Table I. Within-run and inter-animal variations were 1% and 5%, respectively.

## DISCUSSION

High-energy phosphate compounds, including ATP and CP, are considered as one of the most important biochemical gauges for proper functioning of mammalian heart. Myocardial infarction and open-heart surgery are usually associated with prolonged episodes of ischemic insult. During ischemia, depletion of the ATP level by adenylate kinase occurs, resulting in an extremely low level of ATP with a corresponding rise in tissue ADP and AMP[7]. CP also suffers appreciable loss, often becoming almost negligible after severe ischemic insult. Since CP serves as an "energy

buffer" and plays a significant role in the energy transport process between various cellular compartments, it is important to know the exact amounts of CP during the ischemic episode. At the onset of reperfusion, redistribution of adenine nucleotides usually takes place, which is reflected in the value of adenylate energy charge. Accordingly, ATP and CP, particularly the latter, often rise to the pre-ischemic levels[7].

It is, therefore, conceivable that extremely low levels, of ATP, ADP, AMP, and CP must be measured in the heart during ischemia with the utmost accuracy in order to be able to predict the functional recovery after the revascularization. In addition, issue size is also an important factor to be considered, because often only an extremely small amount of biopsy (less than 50 mg) may be obtained to measure the high-energy phosphate compounds.

In the present method, with less than a 50-mg biopsy we were able to accurately measure the levels of adenine nucleotides and CP during ischemia. We used standard addition technique to examine the accuracy of the method. The success of the method primarily depends on the fact that we measured CP and adenine nucleotides at two different wavelengths, corresponding to respective absorption maxima, instead of measuring at a single wavelength.

Another important feature of this method is the rapidity of the separation. The complete separation of CP, ATP, ADP, and AMP was achieved within 10 min, instead of *ca.* 35 min separation time for another previously described method[4]. The 25-min period of equilibration, however, remained unchanged. Such rapid determination is often very useful, because the results of the tissue levels of the high-energy phosphate compounds may be obtained prior to the initiation of reperfusion.

This computer-assisted method not only detected very low levels of high-energy phosphate compounds, but it also estimated them with a high degree of precision (within-run and inter-animal variations were 1% and 5%, respectively). Using a Waters Model 820 full-control MAXIMA computer, calibration curves were constructed for each compound. When various concentrations were plotted against the peak area, straight lines were obtained which, when extrapolated, passed through zero. Response factors for each compound were derived from these standard curves and used to calculate the unknown concentration of each compound in the pig heart extracts. This method thus increases the accuracy of the result significantly, as observed by the need for low amounts of standards in the standard addition technique (data not shown).

As mentioned earlier, the exact determination of high-energy phosphate compounds is of the utmost importance in evaluating proper myocardial performance during ischemia and reperfusion. Because of the great sensitivity, precision, and accuracy, as well as the rapidity, this computer-aided dual-wavelength method will be very useful in analyzing the tissue levels of ATP, ADP, AMP, and CP in ischemic and reperfused hearts. The method we report here should be easily adaptable for use with other tissues.

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