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Note

High-performance liquid chromatographic method for the simultaneous determination of myocardial creatine phosphate and adenosine nucleotides

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The quantitation of energy stores [creatine phosphate (CP) and adenosine nucleotides, adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP)] is of paramount importance in the study of living-tissues metabolism. This is more so in the heart, which depends to a very great extent on an uninterrupted aerobic metabolism to maintain its normal function. Availability of data on myocardial energy stores is, therefore, indispensable for assessing the responses of the heart to drugs or stresses such as ischemia and hypoxemia.

Until recently this information was not easily obtainable due to a cumbersome and time-consuming methodology. At least two different assays were needed to determine the levels of CP with ATP and ADP with AMP, in relatively large amounts of tissue. Recently, the technique of high-performance liquid chromatography (HPLC) has been successfully applied to this problem [1—7]. Even this approach required the run of two separate assays, one for CP and another for adenosine nucleotides [5]. More recently, simultaneous determination of these compounds has been reported by Harmsen et al. [6, 7]. Their method, however, had to be adapted to our needs for the reasons discussed below. This report is a description of their modified methodology as used at present in our laboratory. It provides an easy, rapid and reliable way of determining energy stores in small quantities of myocardial tissue.

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EXPERIMENTAL

Equipment

The HPLC system was a Beckman 332 model consisting of two Model 110A pumps, a wavelength-selectable detector Model 160 set at 214 nm, a Series 210 universal injection valve and a Model 420 controller/programmer. The latter component allowed the programming of flow-rates and buffer ratios. The results were processed by a Hewlett-Packard integrator Model 3390A.

The column used (25 × 0.46 cm) contained Partisil-10-SAX (10-μm particles) and was provided with a guard column filled with Pellinox-Sax. Buffer A was 0.4 *M* potassium dihydrogen phosphate in 0.4 *M* potassium chloride (pH 4.2 adjusted with potassium hydroxide) and buffer B, 0.05 *M* potassium dihydrogen phosphate (pH 3.3 adjusted with orthophosphoric acid). The characteristics of a typical run are shown in Table I.

TABLE I

CHARACTERISTICS OF THE SAMPLE RUNS

Time (min)	Gradients		
	Flow-rate (ml/min)	Buffer A (%)	Buffer B (%)
0—13	0 5	0	100
13—25	1 5*	30**	70**
25—35	1.5	100*	0*

*Change over 1 5 min.
**Change over 0.5 min.

Reagents, standards and procedure

Either HPLC-grade or the purest available chemicals were used for all solutions. Water was purified in a Barnstead Nanopure II four-cartridge system before making the buffers and other solutions.

CP, ATP, ADP and AMP, obtained from BMC Diagnostics (Dorval, Canada), were used to prepare suitable standards in 0.9 *M* perchloric acid, which were subsequently neutralized with 6 *M* potassium hydroxide.

Tissue samples were obtained from hearts in situ or after perfusion and quickly frozen with a Wollenberger clamp at liquid-nitrogen temperature or immediately homogenized with a Polytron sonicator/homogenizer (Brinkman, Westbury, NY, U.S.A.) in 2.0 ml of 0.9 *M* perchloric acid. The pH was adjusted to 5 with 3.75 *M* potassium carbonate. A 100-μl aliquot of the extract was then applied to the HPLC system. Tissue aliquots and the volume of the homogenate and the extracts could be varied according to the anticipated tissue concentrations and the sensitivity of the detector. In our system the latter was usually set at 0.4 a.u.f.s. (the lowest sensitivity being 2.0 a.u.f.s.).

For enzyme peak shifts, myocardial tissue was homogenized in the presence of 4 mg of glucose per ml of extract and 0.5 *M* magnesium sulfate and the pH adjusted to neutrality with 6 *M* potassium hydroxide. Specific enzymes [1, 7]

were added stepwise followed by incubation at 37°C for 30 min. At the end of each incubation period, aliquots of the extract were then run in the HPLC system.

RESULTS

Fig. 1A shows a chromatogram of a standard solution containing creatine, CP, ATP, ADP and AMP, to illustrate the separation and sharpness of the peaks. The duration of the run was 28 min.

Fig. 1B illustrates a chromatogram of an extract of fresh non-perfused rabbit myocardium.

Peaks were identified by their retention times, by spiking (adding known amounts of the compounds of interest to an aliquot of the extract), and by enzymatic shifting of the peaks. The latter is the most reliable approach, and the results are shown in Fig. 1C—F. In step 1 (Fig. 1C) the ATP peak disappears after the addition of hexokinase, while the ADP peak increases markedly. In step 2 (Fig. 1D) creatine kinase reduces the CP peak while increasing the concentration of creatine. In step 3 (Fig. 1E) ADP is markedly reduced by myokinase with concomitant elevation of AMP. Finally, in step 4 (Fig. 1F) AMP

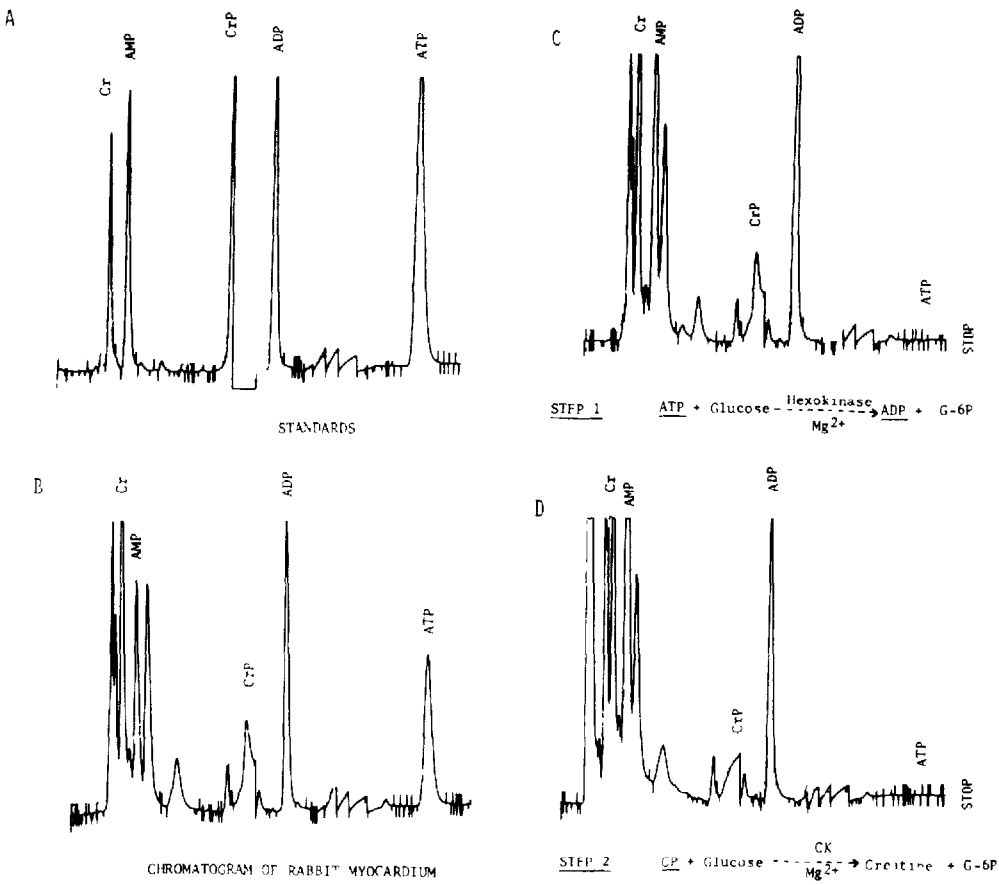

Fig. 1.

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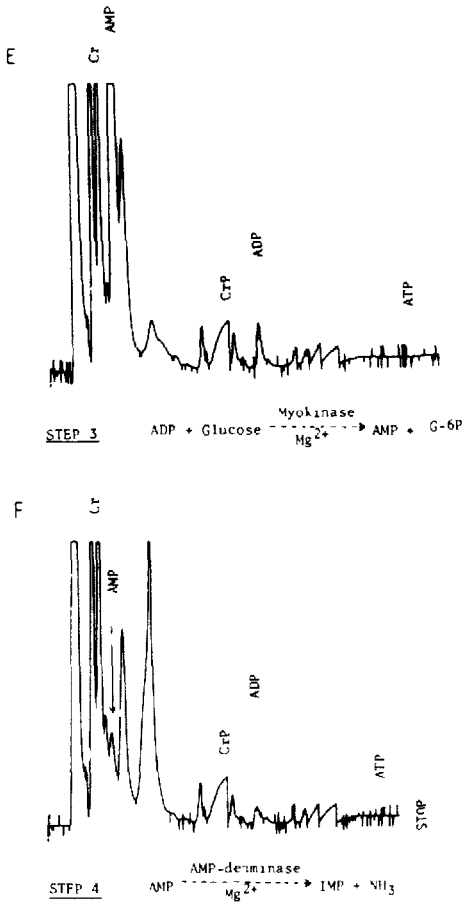

Fig. 1. (A) Run of standards. Cr = creatine; CP = creatine phosphate; ATP, ADP and AMP = adenosine tri-, di-, and monophosphate, respectively. (B) Chromatogram of an extract of non-perfused rabbit myocardium. The duration of the run is around 28 min (C) The addition of hexokinase in the presence of glucose and Mg^{2+} converts ATP to ADP with changes in the corresponding peaks. (D) After addition of creatine kinase (CK), CP disappears with increase in creatine. (E) The addition of adenylate kinase (myokinase) results in the conversion of ADP to AMP. The ADP peak is markedly decreased; its persistence may be related to the large amounts of ADP present or insufficient incubation time, since it is no longer present in the next step (see Fig. 1F). (F) The final step is the reduction of the AMP peak by AMP deaminase (5-adenylic acid deaminase) via its transformation to inosine monophosphate (IMP) (not identified)

concentration decreases after the addition of AMP deaminase, resulting in the production of inosine monophosphate (IMP) (not identified).

The present method allows determination of the energy stores in as little as 10—15 mg of mycardial tissue. The change in concentration of the energy stores in samples frozen up to four weeks at —30° C was less than 5%. After the addition of known amounts of the different compounds to a tissue extract, the recoveries were 101% for CP, 93% for ATP and 90% for ADP and AMP. The lower limits of detection were (in nmol) 2.27 for CP, 0.343 for ATP, 0.213 for ADP and 0.072 for AMP.

DISCUSSION

Our results show that CP and adenosine nucleotides stores in the myocardium can be determined simultaneously by HPLC in approximately 28 min. Further improvement of this method will shorten the duration of the run and increase its sensitivity. This can be achieved by reducing the volume of the extract, and/or increasing the volume of the injectate and the sensitivity of the detector. The latter, however, has limitations since it can markedly distort the baseline.

Harmsen et al. [7] were very kind to provide us with details of their methodology before publication. However, their method did not work in our system for several reasons. Firstly, the change in buffers (0.01 *M* orthophosphoric acid and 0.75 *M* potassium dihydrogen phosphate) during the run caused exaggerated baseline shifts in our system that could not be compensated; secondly, the use of a strong buffer B (0.75 *M* potassium dihydrogen phosphate) was hard on the pump because of salt precipitation; and thirdly, the high flow-rate (2.0 ml/min) did not allow us to achieve good peak separation.

By changing buffer A from 0.01 *M* orthophosphoric acid to 0.4 *M* potassium dihydrogen phosphate, buffer B from 0.75 *M* potassium dihydrogen phosphate to 0.05 *M* potassium dihydrogen phosphate, and reducing the flow-rate from 2.0 to 1.5 ml/min, we were successful in obtaining similar results.

In our hands, the described method is easy, rapid and accurate. It has the important advantage of permitting the simultaneous determination of CP and adenosine nucleotides in extracts of several tissue samples in the same day. Easier isocratic methods have been described for the separation only of nucleotides, but, as far as we are aware, always excluding creatine phosphate. The advantage of our method is that both CP and nucleotides can be now quantitated in an aliquot of tissue extract processed in a single HPLC run. This method could be easily adapted to other HPLC systems.

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