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# Note

## Isocratic high-performance liquid chromatographic analysis of myocardial creatine phosphate and adenine nucleotides

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Assessment of myocardial ischemic damage requires the determination of adenosine nucleotides, particularly adenosine triphosphate (ATP) and creatine phosphate (CP). Rapid isocratic high-performance liquid chromatographic (HPLC) methods were devised for analysis of adenosine nucleotides [1,2]. Simultaneous isocratic analysis of CP and nucleotides has not been achieved. Using Nova-Pak $C_{18}$ columns we devised quantitative elution of adenine nucleotides in less than 7 min. In this procedure CP or creatine are among the first peaks to be eluted and the possibility arises that they are eluted in the dead volume along with other substances.

In the present report we describe an isocratic method for HPLC analysis of adenine nucleotides and we compare analysis of standard solutions and myocardial extracts by enzymatic and HPLC methods. A means of determining CP in conjunction with the isocratic method for adenine nucleotides is presented.

## EXPERIMENTAL

### *Methods*

Dog heart biopsies stored in liquid nitrogen were extracted for nucleotides using a modification of the procedure of Holland *et al.* [3]. In brief: 30–60 mg of tissue were crushed in a liquid nitrogen-cooled mortar and pestle together with

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400 μl of 0.6 *M* perchloric acid (PCA). The tissue was homogenized using a conical homogenizing tissue grinder (Kontes, Vineland, NJ, U.S.A.) at −20°C. The homogenized PCA-treated tissue was centrifuged (Sorvall Super Speed Model RC2-B, Ivan Sorvall, Norwalk, CT, U.S.A.) at 6000 *g* for 15 min at −5°C. A 300-μl aliquot of the supernatant was added to an Eppendorf tube and then frozen in liquid nitrogen. A 70-μl aliquot of neutralizing solution (4 g of potassium bicarbonate in 13 ml of 0.5 *M* Tris buffer at pH 7.5) was added on top of the frozen supernatant and the mixture was centrifuged (Model 59, Fisher Scientific, Itasca, IL, U.S.A.) at 7000 *g* for 1 min. The neutralized PCA extract was allowed to stand at 0–4°C for 1–2 h to precipitate potassium perchlorate. The pH of the extract was between 6.0 and 7.0. A 20-μl aliquot of the supernatant was analyzed for ATP and CP, using a coupled enzyme system (creatine phosphokinase, hexokinase and glucose 6-phosphate dehydrogenase) [3]. The remaining extract was stored in liquid nitrogen for HPLC analysis. Standards were prepared from weighed samples of CP or ATP (Sigma, St. Louis, MO, U.S.A.) diluted with neutralized PCA.

Standards and samples were analyzed in two steps. First, 20 μl of each standard or 20 μl of the neutralized extract were diluted in 240 μl of neutralized PCA, and 20 μl of the mixture were analyzed by HPLC. Another 20-μl aliquot of a standard or sample was incubated at room temperature for 30 min in 240 μl of a solution containing 50 m*M* imidazole · HCl at pH 7.0, 1 m*M* $MgCl_2$, 60 μ*M* ADP, 1 μ*M* diadenosine pentaphosphate (Ap5A) and 3 μg/μl creatine phosphokinase (EC 2.7.3.2). The reaction was stopped by heating in a microwave (Quasar MQ Model 6635yw) for 2 min. The mixture was then passed through a 4-μm non-sterile Milex filter (Waters Assoc., Millford, MA, U.S.A.) into a mini-glass vial, and a 20-μl sample was analyzed by HPLC. All chemicals were purchased from Sigma.

*Materials*

A Waters Assoc. 840 HPLC system was used to separate nucleotides. The system consisted of a Model 710B WISP automatic injector, Model 440 absorbance detector with fixed 254-nm and 214-nm wavelengths, a 150 mm × 3.9 mm I.D. Nova-Pak $C_{18}$, 4-μm column and Model 510 solvent delivery pumps. The pumps were used at a flow-rate of 1.0 ml/min. Ammonium phosphate (0.1 *M*) at pH 5.0 was used to isocratically separate nucleotides.

## RESULTS AND DISCUSSION

As shown in Table I authentic samples of ATP and CP gave similar values when analyzed by the enzymatic method or HPLC. A typical HPLC elution pattern is shown in Fig. 1. Mixtures of ATP, ADP and AMP also yielded close agreement when analyzed by enzymatic and HPLC procedures (data not shown). CP was detected at 214 nm and appears as the first eluted peak. Rapid isocratic

TABLE I

COMPARISON OF ENZYMATIC AND HPLC ANALYSES OF CREATINE PHOSPHATE AND ATP

Values are means ± S.D. ($n$ = 5) or ranges.

| Creatine phosphate | | ATP | |
|---|---|---|---|
| HPLC | Enzymatic | HPLC | Enzymatic |
| *Standards (μmol)* | | | |
| 1.00 ± 0.05 | 0.97 ± 0.10 | 1.06 ± 0.05 | 1.02 ± 0.15 |
| *Myocardial extracts (μmol/g wet weight)* | | | |
| 7.60–11.82 | 5.08–10.12 | 3.90–11.66 | 4.36–11.86 |
| 10.08 ± 1.61 | 7.95 ± 1.81[a] | 6.61 ± 3.20 | 6.70 ± 2.33 |

[a] $p < 0.05$, significance of paired sample difference.

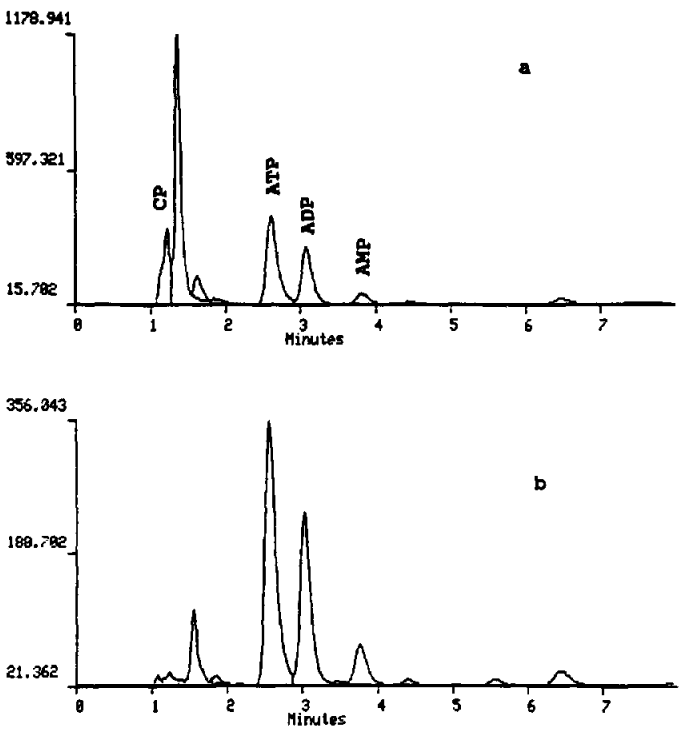


Fig. I. Effect of incubating myocardial extract with creatine phosphokinase. Myocardial extracts were treated with creatine phosphokinase and diadenosine pentaphosphate (a) prior to incubation and (b) after the incubation. The abcissa represents time in minutes and the ordinate indicates mV measured by the absorbance detector at 254 nm. For the chromatographic method, see Experimental.

separation of CP and adenine nucleotides has been reported by Sellevold *et al.* [4] and by Hull-Ryde *et al.* [5]. Both studies used reversed-phase techniques. In studies by Hull-Ryde *et al.* [5], CP was the first substance to be eluted. As shown in Table I, myocardial tissue samples analyzed by enzymatic and HPLC methods exhibited closely similar values for ATP (as well as ADP and AMP; data not shown). However, CP values obtained by the enzymatic method were always significantly less than those obtained by HPLC. Similar comparisons using HPLC and another analytical method for tissue CP have not been previously reported.

The higher CP values obtained by HPLC could be explained by co-elution of other tissue substances as a result of non-entry of CP and such other substances into the column. This would explain the close correspondence between the enzymatic and HPLC methods when authentic CP is used and the divergence when tissue samples are analyzed.

We reasoned that transfer of phosphate of CP to ADP using creatine phosphokinase would increase the ATP concentration by an amount equal to the CP concentration. However, the effect of the ubiquitous enzyme adenylate kinase would have to be inhibited to avoid further metabolism of ATP. This was done by

TABLE II

COMPARISON OF CREATINE PHOSPHATE LEVELS (μmol/g) IN MYOCARDIAL TISSUE AS DETERMINED BY ENZYMATIC ANALYSIS AND BY HPLC ANALYSIS OF ADDITIONAL ATP AFTER CREATINE PHOSPHOKINASE ACTION

| | Creatine phosphate | | ATP | |
|---|---|---|---|---|
| | HPLC | Enzymatic | HPLC | Enzymatic |
| | 4.23 | 4.21 | 3.97 | 3.95 |
| | 9.97 | 9.94 | 11.64 | 11.68 |
| | 4.45 | 4.42 | 4.74 | 4.72 |
| | 3.68 | 3.62 | 3.69 | 3.73 |
| | 4.74 | 4.72 | 2.33 | 2.36 |
| | 5.90 | 5.84 | 2.46 | 2.50 |
| | 5.82 | 5.76 | 3.70 | 3.73 |
| | 1.62 | 1.61 | 0.92 | 0.93 |
| | 3.27 | 3.26 | 1.79 | 1.80 |
| | 4.85 | 4.84 | 2.95 | 2.93 |
| | 2.85 | 2.84 | 1.69 | 1.68 |
| | 2.04 | 2.03 | 1.05 | 1.04 |
| | 1.72 | 1.71 | 1.35 | 1.34 |
| Mean | 4.24[a] | 4.21 | 3.21 | 3.22 |
| S.D. | 2.23 | 2.22 | 2.79 | 2.80 |

[a] $p > 0.05$, significance of difference between paired samples.

addition of Ap5A [6]. ATP predicted (mean ± S.D. 2.82 ± 0.89, range 1.27–3.39 $\mu M$) when authentic CP and ADP were incubated with creatine phosphokinase buffer in the presence of Ap5A, was not significantly different from the ATP found (mean ± S.D. 2.90 ± 0.91, range 1.36–3.27 $\mu M$), based on paired comparisons of HPLC results ($n = 5$). A similar analysis was carried out on fifteen samples of neutralized PCA extracts of mycoardial tissue. Again, ATP predicted (mean ± S.D. 3.22 ± 0.76, range 1.63–6.70 $\mu M$/g wet weight) was not significantly different from ATP found (mean ± S.D. 3.14 ± 0.33, range 1.62–6.70 $\mu M$), based on paired comparisons.

The creatine phosphokinase method for measuring CP concentration was then applied to myocardial tissue samples (Table II), and the result compared to enzymatic analysis. It is seen that mean CP, measured as the increase in ATP, closely agrees with CP found enzymatically although the HPLC-determined CP is slightly greater than the enzymatically determined CP on a paired sample basis. As before, HPLC-determined ATP was not significantly different from the enzymatically determined ATP.

Two separate runs were needed to obtain CP and adenine nucleotides from the same sample. However, the isocratic elution was rapid, reproducible, took less than 7 min each and no regeneration between samples was required.

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