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## Note

# A sensitive and rapid method for separating adenine nucleotides and creatine phosphate by ion-pair reversed-phase high-performance liquid chromatography

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The use of conventional enzymatic techniques[1] for the estimation of tissue high energy phosphate compounds [ATP, ADP, AMP, CrP (creatine phosphate)] has been progressively replaced by high-performance liquid chromatography (HPLC), a powerful technique for the separation and quantitation of such components. The different HPLC approaches used have recently been reviewed by Simpson and Brown[2]. Separation of nucleotides (ATP, ADP, AMP) by HPLC on an ion-exchange column with detection at 254 nm has been described[3,4], and the determination of nucleotides on reversed-phase columns with or without ion-pairing[5–7] has also been reported. However, creatine phosphate, which is a sensitive indicator of ischaemic damage[8], cannot be determined simultaneously on the same sample, since it is not detectable at 254 nm. Juengling and Kammermeier[5] detected CrP at 210 nm, but separated the nucleotides and CrP in two separate experiments, using two completely different buffer systems. Two different procedures (ion-exchange and reversed-phase chromatography) were also used by other workers[9,10] to determine adenine nucleotides on the same sample.

The simultaneous analysis of nucleotides in one experiment has been accomplished: Heldt *et al.*[11] and Harmsen *et al.*[12] separated these compounds by using an ion-exchange column together with a gradient buffer system. This technique proved to be rather time-consuming; 30 min per experiment and a further equilibration period (usually of the order of 10–15 min) due to the gradient, so that only a limited number of samples can be analyzed per day.

The purpose of the present study was to establish more rapid and sensitive chromatographic conditions for the separation of adenine nucleotides and creatine phosphate from other nucleotides of biological interest, and thus to obtain a fast quantitative analytical procedure. A technique will be described by which the high energy phosphates (ATP, ADP, AMP, CrP) can be separated in 12 min with an isocratic buffer system, using ion-pair reversed-phase HPLC.

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## EXPERIMENTAL

### *Reagents*

ATP, ADP, AMP, creatine, AMP-deaminase (E.C. 3.5.4.6), hexokinase (E.C. 2.7.1.1) and tetrabutylammonium phosphate (TBAP) were purchased from Sigma. Creatine kinase (E.C. 2.7.3.2) and creatine phosphate were obtained from Boehringer (Mannheim, F.R.G.) potassium dihydrogen phosphate from Merck and methanol from Burdick and Jackson. Water was purified with a Milli-Q system obtained from Millipore.

### *Preparation of samples*

For identification of the unknown peaks, a standard mixture was made up in water consisting of 45.35 $\mu M$ ATP, 58.53 $\mu M$ ADP, 72 $\mu M$ AMP and 76.4 $\mu M$ CrP. Tissue samples from different organs were removed from anaesthetized rats and were immediately freeze-clamped and plunged into liquid nitrogen. The frozen tissue was pulverized in a stainless-steel mortar and kept in liquid nitrogen. A protein-free acid extract was prepared from approximately 200 mg pulverized tissue according to the method of Gutman and Wahlefeld[13] and neutralized with potassium hydroxide–Tris [40% potassium hydroxide in saturated potassium chloride (4 parts); 0.2 $M$ Tris–HCl, pH 7.5 (6 parts)]. The samples were kept on ice and the high energy phosphate contents of the extract were determined on the same day.

### *HPLC analysis*

A Spectra-Physics SP 8100 liquid chromatograph, equipped with a SP 8440 variable wavelength UV detector and a Model SP 4200 integrator was used. Aliquots (10 $\mu$l) of the extract and of the standard mixture were applied separately to the column. The eluate was monitored at 210 nm. The buffer contained 0.52 $\mu M$ potassium dihydrogen phosphate, 0.04% TBAP (w/v) and 1.25% (v/v) methanol. The pH was adjusted to 4.00 with concentrated phosphoric acid. Buffers were prepared on the day of use and filtered through 0.45-$\mu$m Millipore filters. Separations were carried out at 36°C with a Supelcosil LC-18, 5-$\mu$m (25 cm × 0.46 cm) reversed-phase column (Supelco, Crans, Switzerland), at a flow-rate of 2 ml/min.

### *Enzymatic analysis*

In addition to the use of standards for peak identification, the following enzymatic procedure was applied: peaks of the adenine nucleotides and creatine phosphate were shifted or disappeared completely by reaction with different enzymes as proposed by Brown[3]. For breakdown of ATP to ADP, an aliquot of the sample was incubated for 30 min at 37°C with hexokinase (50 mU) and D-glucose (0.67 $M$). CrP was broken down by addition of creatine kinase (70 U), hexokinase (50 mU), D-glucose (0.67 $M$) and magnesium chloride (13 m$M$): overall reaction, CrP + glucose → creatine + glucose-6-phosphate. In a similar manner, ADP was converted into ATP with creatine kinase (70 U). Finally the AMP peak was shifted with AMP-deaminase (1 U): AMP → IMP + $NH_3$.

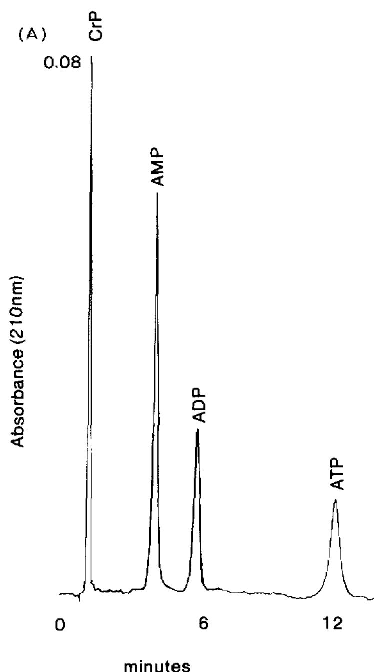


Fig. 1. Chromatogram of standards. Separation of standard containing 76.4 *μM* CrP, 72 *μM* AMP, 58.53 *μM* ADP and 45.35 *μM* ATP. Injection volume: 10 μl. Flow-rate: 2 ml/min. Buffer: 0.52 *μM* potassium dihydrogen phosphate, 0.04% TBAP and 1.25% methanol, pH 4.0. Column: Supelcosil LC-18, 5 μm (25 cm × 0.46 cm). Detection: UV 210 nm.

## RESULTS

A combination of the retention times, wavelength ratios and enzymatic peak shifts was used to identify the individual adenine nucleotides and creatine phosphate, as well as to confirm their homogeneity.

The retention times of the standards, Fig. 1, were used to identify the adenine nucleotides and creatine phosphate in a tissue extract (Fig. 2A). From these results it is evident that the complete separation was completed in 12 min, after which the column could be used for a second separation. By using the separation system described, no intermediate regeneration of the column between successive separations was necessary.

The peaks of the different adenine nucleotides and creatine phosphate can be shifted or abolished by using different enzymes as proposed by Brown[3]. Hexokinase was used for the breakdown of ATP to ADP (Fig. 2B). It is evident that the ATP peak disappeared, while the increase in the area of the ADP peak corresponded to the area of the original ATP peak. Subsequently CrP was broken down by the addition of creatine kinase and hexokinase (Fig. 2C). In a similar manner, when creatine kinase was used, ADP was converted into ATP (Fig. 2D). Finally, the AMP peak was shifted with AMP-deaminase (Fig. 2E).

From the data presented, it is clear that complete removal of ATP, ADP, AMP

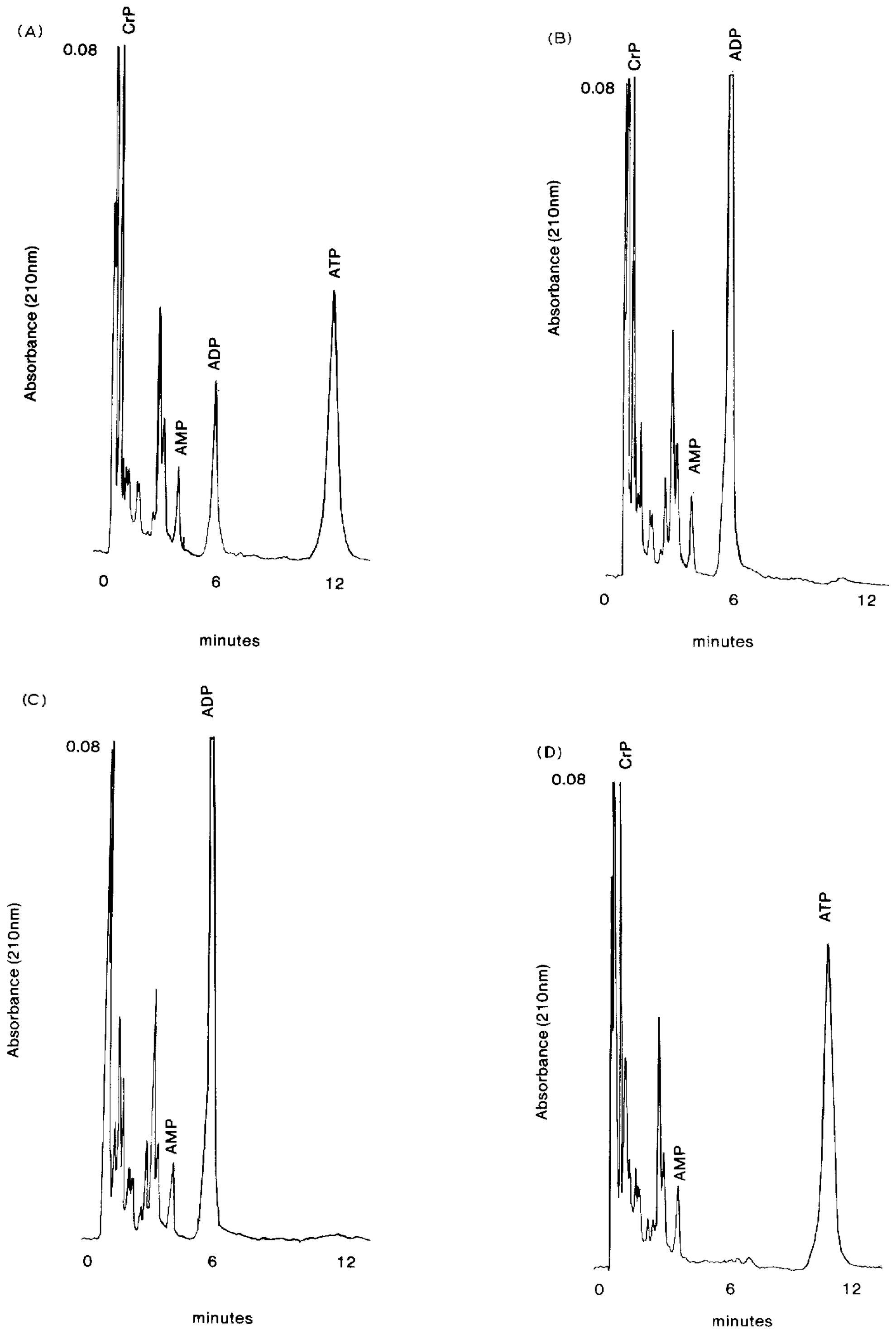


Fig. 2.

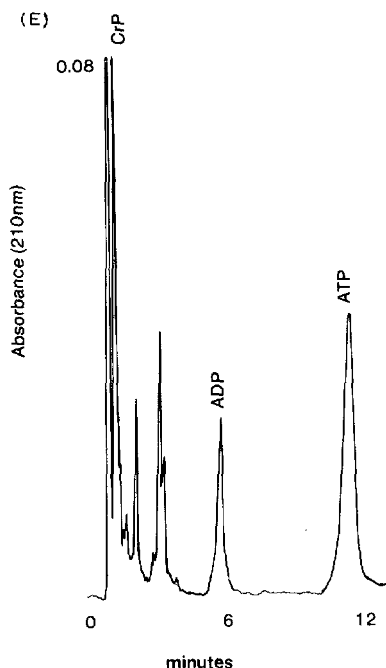


Fig. 2. (A) Chromatogram of a rat heart extract. A protein-free acid extract of freeze-clamped myocardial tissue was prepated as described in Experimental. Conditions as in Fig. 1. The retention times were similar to those obtained with a standard solution (Fig. 1). (B) Chromatogram of a rat heart extract with hexokinase. An aliquot of the tissue extract was incubated for 30 min at 37°C with hexokinase (50 mU) and D-glucose (0.67 *M*) and chromatographed (conditions as in Fig. 1). Disappearance of the ATP peak was associated with an increase in the area of the ADP peak which corresponded to the area of the original ATP peak. (C) Chromatogram of a rat heart extract with hexokinase and creatine kinase. CrP was broken down by addition of creatine kinase (70 U), hexokinase (50 mU), D-glucose (0.67 *M*) and magnesium chloride to an aliquot of the tissue extract. The mixture was incubated for 30 min at 37°C and then chromatographed (conditions as in Fig. 1). (D) Chromatogram of a rat heart extract with creatine kinase. ADP was converted into ATP by addition of creatine kinase (70 U) and D-glucose (0.67 *M*) to the tissue extract. The mixture was incubated for 30 min at 37°C and then chromatographed (conditions as in Fig. 1). (E) Chromatogram of a rat heart extract with AMP deaminase. The AMP peak in the tissue extract was shifted by addition of AMP-deaminase (1 U). Chromatographic conditions as in Fig. 1.

and CrP can be achieved by use of the different enzymes. Therefore, although the method described allows sufficient separation of adenine nucleotides and creatine phosphate, it is not sufficient for complete separation of the enzymatic conversion products. In addition, similar values of the peak area ratios at 210/254 nm and 210/220 nm for the adenine nucleotides and CrP respectively were obtained for the individual peaks of both the sample and standards, indicating pure resolved peaks (data not shown).

To assess the linearity of the described procedure, a 10-$\mu$l sample loop was used. Increasing concentrations of the individual standards were injected and the integral units of the peak areas recorded. A linear relationship up to 1 m*M* was obtained for all standards, the correlation coefficients for ATP, ADP, AMP and CrP being 0.983, 0.982, 0.982 and 0.991 respectively. The recovery determined by adding

known amounts of standards to the tissue extract was 101 ± 1, 97 ± 1, 99 ± 2 and 101 ± 2% for ATP, ADP, AMP and CrP, respectively.

## DISCUSSION

The method described is quick and accurate for the routine determination of adenine nucleotides and creatine phosphate in biological samples. The individual peaks are pure and well identified by using the standard chromatographic approach for peak identification (standards, retention times, enzymatic analysis, wavelength ratios). Furthermore, the concentrations of adenine nucleotides and creatine phosphate in tissue extracts obtained were similar to those obtained by using conventional enzymatic procedures[14]. Other identification procedures such as mass spectrometry were not used due to their cost and unavailability.

The procedure is extremely sensitive and linear over a wide concentration range and can be used on minute samples (10 mg) such as tissue biopsies. It is important to emphasize that an isocratic buffer system is used and that no column regeneration between samples is necessary as in the method of Harmsen *et al.*[12], where use was made of a gradient elution system. The isocratic elution also avoids excessive baseline absorbance changes, simplifying interpretation of the chromatogram. No carryover of samples occurred and up to five samples can be analysed per hour. We used the system without any pre-column, 500 samples being analyzed without any significant loss in column performance. However, it is important to filter the samples through a 0.45-$\mu M$ nitrocellulose filter, before loading on to the column.

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