Journal of Chromatography, 230 (1982) 131—136
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1229

Note

Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography

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(First received October 7th, 1981; revised manuscript received January 18th, 1982)

ATP is one of the most important substances for the proper functioning of the heart [1]. Creatine phosphate (CrP) serves as an "energy buffer", and could also play a role in the rapid transport of energy between cell compartments [2]. Separations by high-performance liquid chromatography (HPLC) of nucleotides on an ion-exchange column with detection at 254 nm have been carried out [3, 4]. Recently, determinations of nucleotides on reversed-phase columns [5], with or without ion-pairing [6, 7], have also been made. CrP, however, cannot be detected at 254 nm. Juengling and Kammermeier [6] detected CrP at 210 nm, but their HPLC system could not separate adenine nucleotides. Heldt et al. [8] used a phosphate analyzer as a detector and were able to detect CrP in one run with nucleotide quantitation. We modified the separation and detection conditions of Edelson et al. [9]. Analysis time was decreased by a factor of two and detection at 210 nm was introduced. We obtained sufficient resolution to quantitate CrP and adenine nucleotides within 30 min.

EXPERIMENTAL

Reagents

Hexokinase (EC 2.7.1.1, 140 U/ml), creatine kinase (EC 2.7.3.2, 25 U/ml), and adenylate kinase (EC 2.7.4.3, 360 U/ml) were purchased from Boehringer, Mannheim, G.F.R.; AMP-deaminase (EC 3.5.4.6, 30—60 U/mg) was obtained from Sigma (St. Louis, MO, U.S.A.). KH_2PO_4, H_3PO_4, KOH and $HClO_4$ were from Merck (Darmstadt, G.F.R.), the nucleotides and CrP were from Boehringer and creatine was from Technicon (Tarrytown, NY, U.S.A.). Water

0378-4347/82/0000—0000/$02.75

was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA, U.S.A.).

High-performance liquid chromatography

A Varian 8520 HPLC system (Varian, Palo Alto, CA, U.S.A.) was used, which consisted of two positive displacement pumps, a variable-wavelength UV detector (Varichrom) set at 210 nm, a pneumatic sampling device, and a chromatographic data system (CDS-111) connected with a chart recorder. Buffers were prepared on the day of use and filtered through a 0.45-μm filter (Millipore). Buffer A consisted of 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH; buffer B consisted of 0.75 *M* KH_2PO_4, pH 4.40. The column (Partisil-10-SAX, 0.4 × 25 cm, particle size 10 μm; Whatman, Maidstone, Great Britain) was eluted with buffer A at a flow-rate of 2.0 ml/min. Five minutes after injection, a gradient started with an increase of 4% B per minute until 100% B.

Rat heart perfusions and sample treatment

Isolated hearts from male rats (Wistar strain, 300—400 g) were perfused retrogradely for 30 min and quickly frozen as described before [10]. After weighing, cardiac tissue was ground in a mortar, precooled with liquid nitrogen. One half of the sample (about 0.5 g) was freeze-dried for determination of percentage dry weight; the other half was extracted with 3.0 ml of 0.8 *M* $HClO_4$. After thawing and centrifugation, 2.0 ml of the supernatant fluid were neutralized at 0°C with about 200 μl of 6 *N* KOH and the $KClO_4$ was spun down at 4°C. A 20-μl volume of this supernatant was applied to the HPLC column.

RESULTS AND DISCUSSION

Chromatograms

Fig. 1 shows the separation of a standard mixture of thirteen nucleotides and creatine compounds. Fig. 2A gives the separation of the adenine nucleotides and CrP in an extract of an oxygenated rat heart.

Linearity, recovery and sensitivity

The lower detection limit with a 20-μl sample loop varies between 20 pmol for AMP and 100 pmol for CrP, and the determination is linear up to 400 nmol for all compounds indicated. Recoveries, determined in a model system (high-energy phosphates added to a 1 g/ml albumin solution), after deproteinization and neutralization, exceeded 95%, with a standard deviation of $<3\%$ for the adenine nucleotides and $<6\%$ for CrP ($n = 5$).

Peak identification

Fig. 2A gives the chromatogram of a rat heart extract. The main peaks in the extract are creatine, NAD, CrP, ADP and ATP. Peak identification was made by comparing retention times with standards and by enzymic peak shifts [3]. Fig. 2B—E shows chromatograms after subsequent incubation with hexokinase (B), creatine kinase (C), adenylate kinase (D) and AMP-deaminase (E). Complete removal of ATP, CrP and AMP was observed, with a concomitant increase of the conversion products ADP, creatine and IMP. ADP in the chromatogram

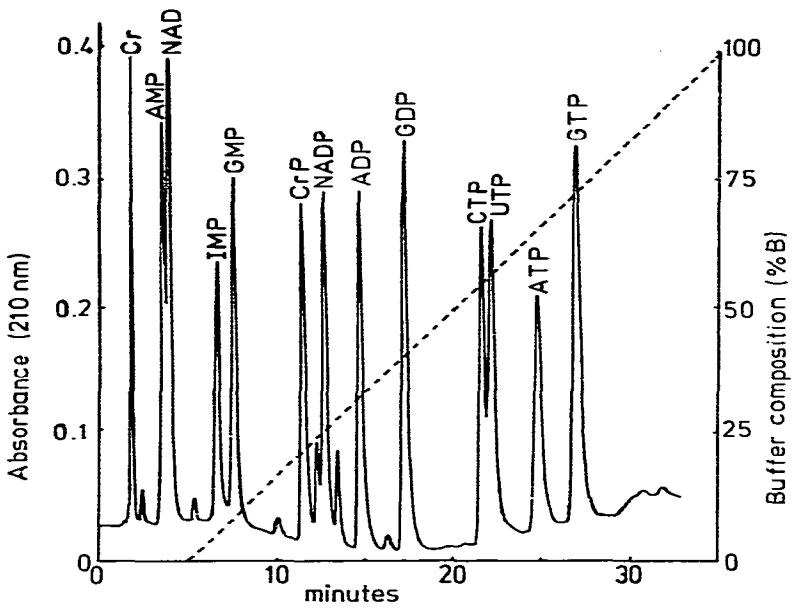

Fig. 1. Separation of standard nucleotides and creatine (Cr) compounds (0.2—0.5 mmol/l). Injection volume, 20 μl; flow-rate, 2.0 ml/min; eluents, A = 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH, B = 0.75 *M* KH_2PO_4 (pH 4.40); column, Partisil-10-SAX.

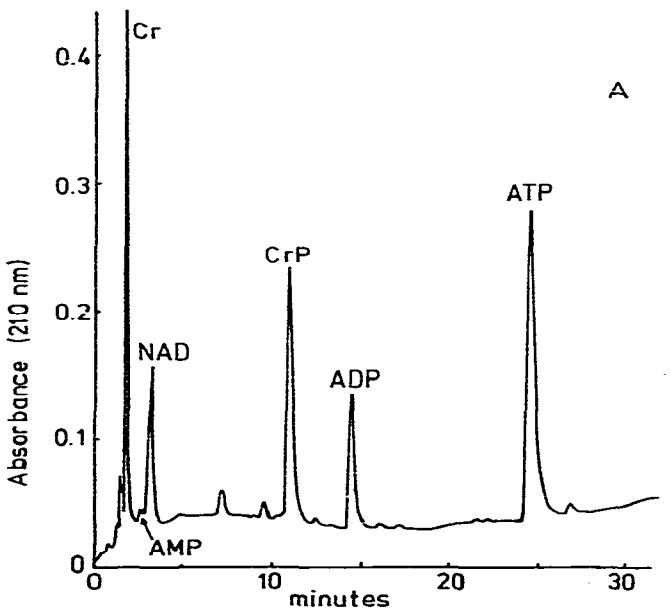

Fig. 2.

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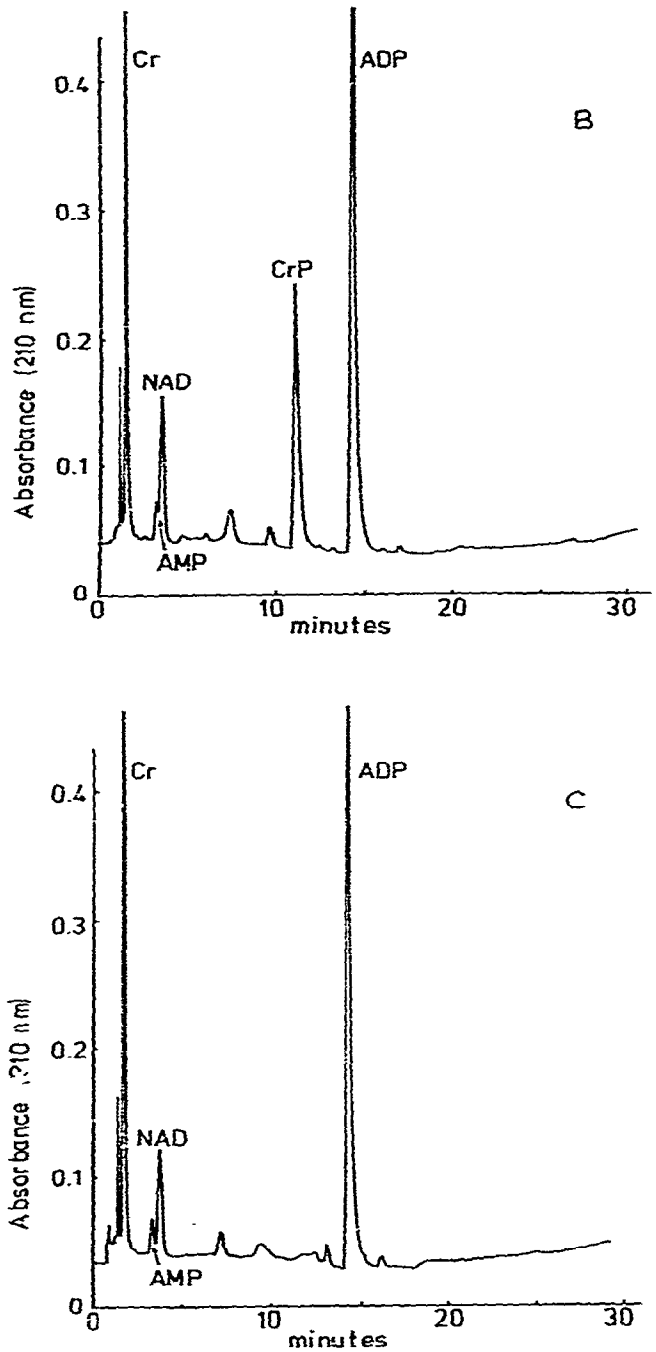
Cr
ADP
B
CrP
NAD
AMP
Absorbance (210 nm)
0.4
0.3
0.2
0.1
0
0
10
20
30
minutes
Cr
ADP
C
NAD
AMP
Absorbance (210 nm)
0.4
0.3
0.2
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minutes

Fig. 2.

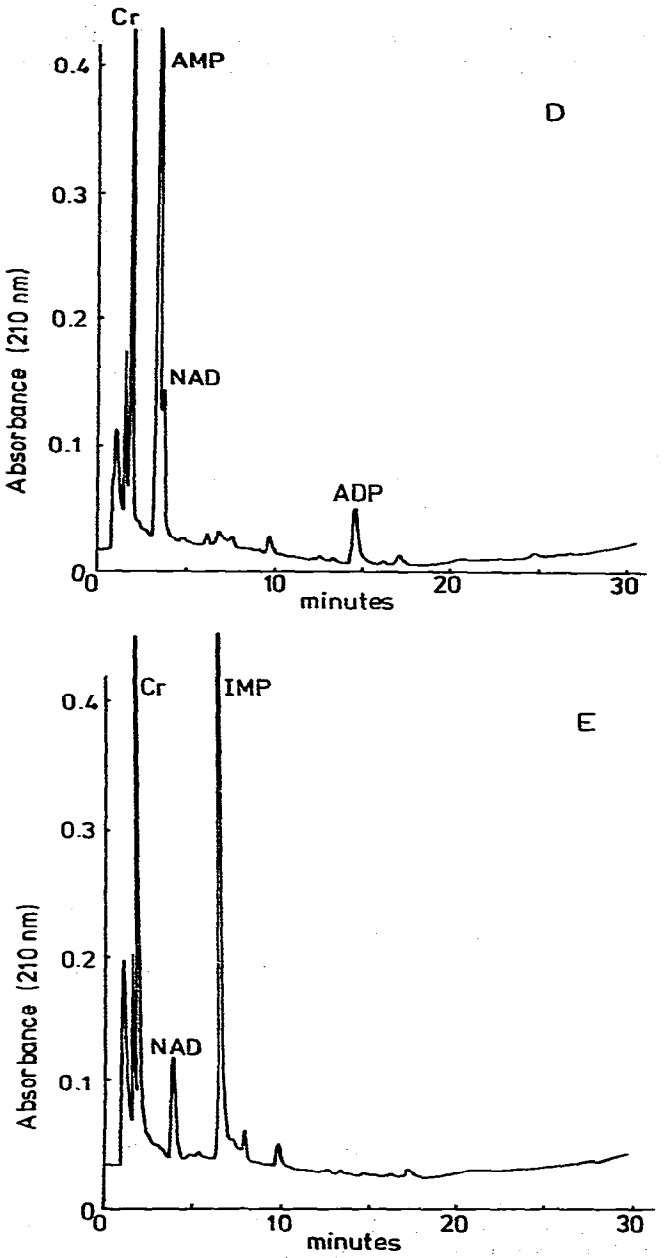

Fig. 2. Chromatogram of a rat heart extract and peak identification by enzymic conversions. HPLC was carried out with a 20-µl extract, prepared as described in the text. For HPLC conditions, see Fig. 1. Panel A gives the chromatogram of untreated extract. Enzymic peak shifts were carried out according to the method of Brown [3]. An aliquot of the extract was incubated for 30 min at 37°C with 0.7 mU hexokinase and D-glucose to convert ATP: ATP + glucose → ADP + glucose-6-phosphate (panel B). Subsequently CrP was broken down by addition to the extract of 0.1 mU of creatine kinase and $MgCl_2$. Under the influence of creatine kinase and hexokinase, the overall reaction is: CrP + glucose → creatine + glucose-6-phosphate (panel C). In a similar way ADP was converted with 1.8 mU of adenylate kinase: 2ADP + glucose → 2AMP + glucose-6-phosphate (panel D). Finally, the AMP peak was shifted with 0.4 mU of AMP-deaminase: AMP → IMP + NH_3 (panel E).

shown in Fig. 2D is not completely removed, presumably because of an unfavorable equilibrium of the adenylate kinase reaction. After addition of AMP-deaminase, which removes AMP, all ADP is converted (Fig. 2E).

Rat heart concentrations

In Table I concentrations are given for adenine nucleotides and CrP, measured in normal and anoxic rat hearts. These values are in the same range as found in the literature [1, 11, 12].

TABLE I

CONCENTRATION OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN RAT HEART

Hearts were perfused retrogradely (Langendorff perfusion) for 30 min with a modified Tyrode solution, equilibrated with 95% O_2—5% CO_2 (normoxia), or for 15 min perfused with 95% O_2—5% CO_2 and 15 min with 95% N_2—5% CO_2 (anoxia). Heart extracts were prepared as described in the text. For HPLC conditions and peak identification, see Figs. 1 and 2.

Condition	μmol/g dry weight (± S.E.M.)			
	ATP	ADP	AMP	CrP
Normoxia	20.9 ± 0.5	7.3 ± 0.4	0.2 ± 0.1	29.3 ± 2.8
Anoxia	9.9 ± 1.9*	9.5 ± 2.2	5.1 ± 1.6*	7.4 ± 1.8*

*$p<0.005$ vs. normoxia, $n = 5$—7.

Conclusion

We conclude that the method presented here is a quick and accurate way to determine myocardial high-energy phosphates. Within 30 min the concentration, energy charge [13] and ATP/CrP ratio can be read from the chromatogram.

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