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Simultaneous determination of myocardial nucleotides, nucleosides, purine bases and creatine phosphate by ion-pair high-performance liquid chromatography

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ABSTRACT

An ion-pair reversed-phase high-performance liquid chromatographic method is described for the separation and quantification of myocardial nucleotides, nucleosides, their metabolites and creatine phosphate-related compounds in a single run. Separation of a standard mixture containing 21 compounds was achieved on a 5-μm Hypersil ODS column with a 5-min isocratic elution (buffer: 0.1 *M* NaH_2PO_4, pH 5.5, containing 5.9 m*M* tetrabutylammonium hydrogensulphate) followed by a slow linear gradient to 17% acetonitrile. The method was applied to extracts of freeze-clamped rat heart tissue samples as well as to extracts of neonatal rat heart cardiomyocytes, and it provided good resolution of high-energy phosphates, including creatine phosphate, as well as of their degradation products.

INTRODUCTION

Adenine and pyridine nucleotides are involved in the energetic charge and redox state of cells and participate as cofactors in enzymic reactions. Creatine phosphate (CreP) serves as an energy shuttle between the mitochondrion and the myofibril [1]. Recent investigations have also shown the involvement of purine metabolites in lipid peroxidation in the case of ischemia and subsequent reoxygenation [2]. Superoxide radicals and hydrogen peroxide are generated during oxidation of the accumulated purine metabolites hypoxanthine (HX) and xanthine (X) to xanthine and uric acid (UA) via the xanthine–xanthine oxidase path. Therefore it is essential to provide a method for the determination of both the high-energy phosphates, which are directly related to the cell energy level, and the purine metabolites, which serve as substrates for the generation of oxygen-free radicals.

Various high-performance liquid chromatographic (HPLC) methods have been reported for the separation of nucleotides and nucleosides. Ion-exchange HPLC applied to nucleotide analysis yields good retardation of CreP but leads to peak broadening of the later-eluting phosphates ATP and GTP [3,4]. Furthermore, high buffer concentrations are not suitable for UV detection at 214 nm, which is necessary for the quantification of CreP. Additionally, reversed-phase columns chosen for the determination of nucleosides and nucleobases are unable to retain sufficiently creatinine or CreP [5,6].

Thus, ion-pair chromatography seems to be suitable for simultaneous determination of cre-

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atine compounds, nucleotides, nucleosides and related bases. Some papers have described this technique for nucleotide and nucleoside analysis [2,7], but CreP levels are commonly assayed in a separate chromatographic system [6,8]. It was the aim of this study to establish the conditions for quantification of all these compounds in a single run.

EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile, sodium dihydrogenphosphate, sodium hydroxide, hypoxanthine and xanthine were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogensulphate (TBA), uric acid, creatine, creatinine and AMP-deaminase (EC 3.5.4.6, 60 U/mg) were purchased from Sigma (St. Louis, MO, USA). Hexokinase (EC 2.7.1.1, 140 U/mg), creatine kinase (EC 2.7.3.2, 800 U/mg), adenylate kinase (EC 2.7.4.3, 360 U/mg), nucleotides and nucleosides were obtained from Boehringer (Mannheim, Germany).

Equipment

Separation was performed on a Hypersil ODS column (5 μm, 250 mm × 4 mm I.D.) using two Model 510 HPLC pumps, a WISP 710B autosampler and a Model 680 gradient controller (all Waters Assoc., Milford, MA, USA). A variable-wavelength UV detector (Model 481, Waters Assoc.) monitored the absorbance of the eluents at 214 nm. Detector signals were recorded and integrated by a CI-4100 integrator (Milton Roy, FL, USA).

Chromatography

Buffers were prepared with bidistilled water and degassed by sonication before use. Buffer A consisted of 0.1 *M* NaH_2PO_4 and 2 g/l TBA, adjusted to pH 5.5 with NaOH. Buffer B was a 75:25 (v/v) mixture of buffer A and acetonitrile.

The initial 5-min period of isocratic elution at 100% buffer A was followed by a 20-min linear gradient to 70% buffer B. After 3 min of continuous delivery of a 30:70 (v/v) mixture of buffer A and buffer B, the initial conditions were re-established by a 2-min gradient back to 100% A. Prior to the next injection, the column was flushed with buffer A for 20 min.

Sample preparation

Myocardial tissue samples were freeze-clamped from five Sprague–Dawley rats anesthetized with nembutal, and stored in liquid nitrogen until further treatment. The weighed tissue was homogenized with 500 μl of 0.4 *M* perchloric acid in a ball mill (Braun, Melsungen, Germany) precooled with liquid nitrogen. After thawing in ice–water and centrifugation at 12 000 *g*, 200 μl of the acid extract were neutralized with 25 μl of 2 *M* K_2CO_3. The $KClO_4$ was spun down and the supernatant was stored at −28°C.

Neonatal rat cardiomyocytes were extracted with 400 μl of precooled perchloric acid from each well of a multi-dish six-well plate (Nunclon Plastics, Nunclon Delta Si, Roskilde, Denmark). The whole cell extract was centrifuged at 9600 *g*, and a 300-μl aliquot of the supernatant was immediately neutralized with 2 *M* K_2CO_3. The neutralized supernatant was stored at −28°C until analysis.

The pellets were resuspended with 1 ml of 0.1 *M* NaOH for protein determination according to the method of Lowry *et al.* [9]. The cardiomyocytes were extracted after a culture period of 48 h. The preparation of spontaneously beating neonatal rat cardiomyocytes has been described in detail elsewhere [10].

RESULTS AND DISCUSSION

Fig. 1A shows the chromatogram from a mixture of 21 standard substances related to the purine and CreP catabolism. All of the relevant phosphates and their metabolites, especially HX, X and CreP, were well resolved and separated within 30 min.

The detection limit varied between 50 and 100 pmol. The linearity was tested for each compound up to 20 nmol without loss of resolution. The near-neutral pH of the eluents (pH 5.5) also allows quantification of the reduced pyridine

TABLE I

CONCENTRATIONS OF PURINE NUCLEOTIDES AND CREATINE PHOSPHATE IN RAT HEARTS

Compound	Concentration (mean ± S.D., n = 5) (μmol/g wet weight)
ATP	4.11 ± 0.09
ADP	0.70 ± 0.05
AMP	0.15 ± 0.02
CreP	6.98 ± 0.66
GTP	0.19 ± 0.02
HX	0.11 ± 0.01
X	0.04 ± 0.01

nucleotides (NADH and NADPH), if an alkaline extraction procedure is used. Compared with UV monitoring at 210 nm, which is generally used for the detection of CreP, detection at 214 nm was adequate and led to an improvement in the gradient baseline shifts. This allowed the simultaneous determination of the nucleotides and nucleosides. Furthermore, the described chromatographic conditions permitted the simultaneous determination of oxypurinol, the main me-

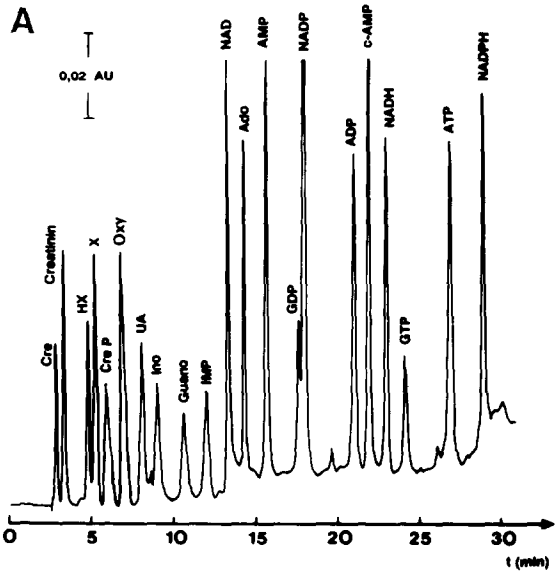

Fig. 1.

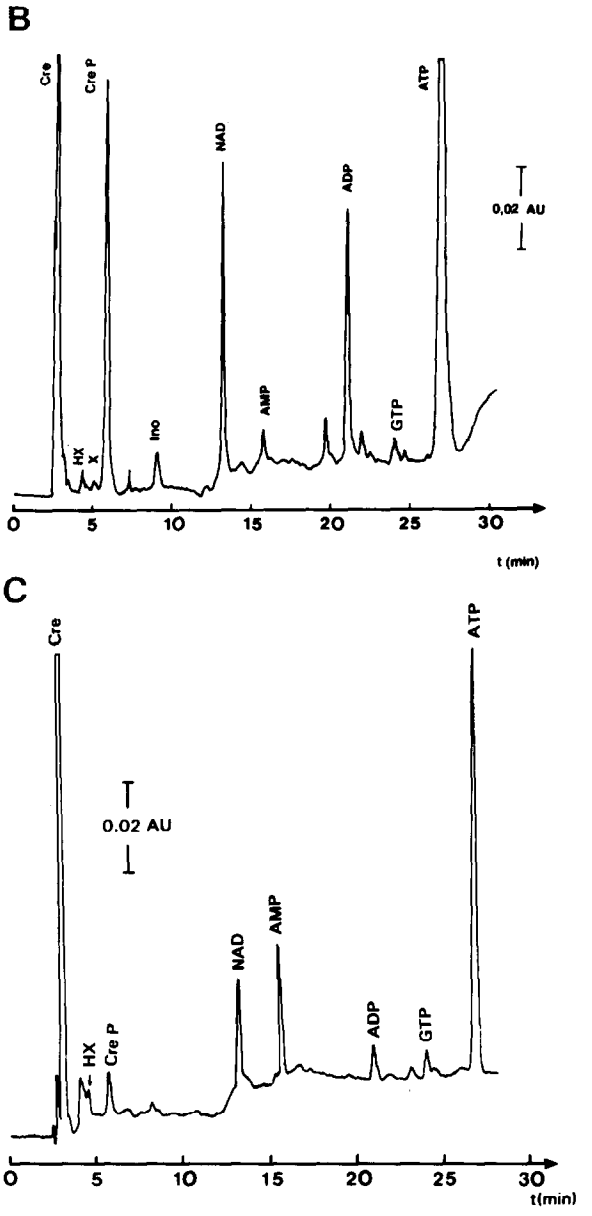

Fig. 1. (A) Separation of a standard mixture containing 1 nmol nucleotides and nucleosides, 1 nmol oxypurinol and 2 nmol creatine compounds. (B) and (C) Chromatograms of extracts from a rat heart biopsy (B) and from neonatal rat cardiomyocytes (C). Injection volumes, 10 and 40 μl (cardiomyocyte extract); column, Hypersil ODS (5 μm, 250 mm × 4 mm I.D.); flow-rate, 0.8 ml/min; eluents, A = 0.1 M NaH_2PO_4, 2 g/l TBA · HSO_4, adjusted to pH 5.5 with NaOH, B = buffer A–acetonitrile (75:25, v/v); 0–70% B linear gradient over 20 min started 5 min after injection; detection, UV 214 nm. Peaks: Cre = creatine; HX = hypoxanthine; X = xanthine; CreP = creatine phosphate; Oxy = oxypurinol; UA = uric acid; Ino = inosine; Guano = guanosine; Ado = adenosine.

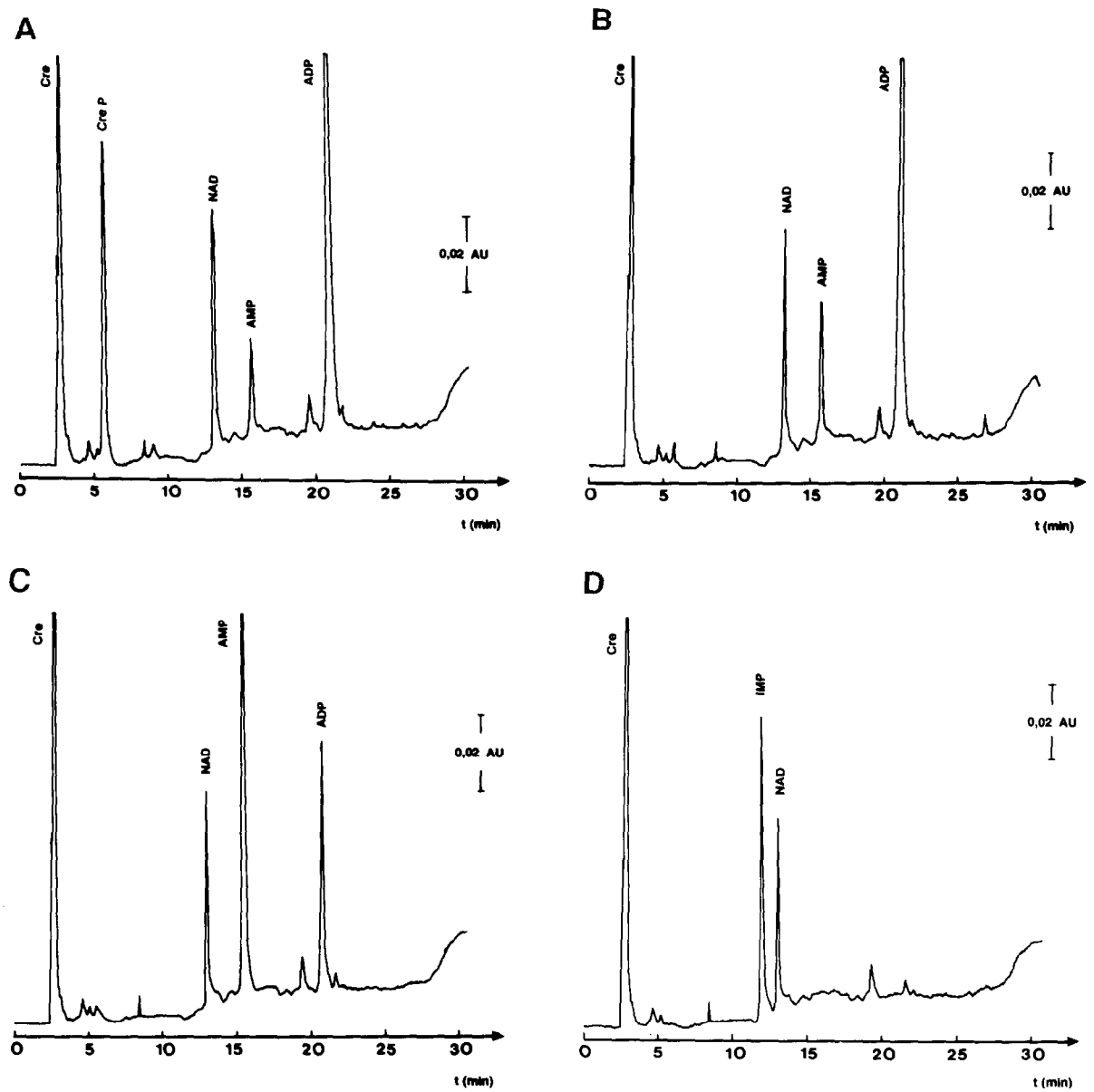

Fig. 2. Peak identification of high-energy phosphates by enzymic peak-shifts according to the method of Brown [11]. A neutralized aliquot of a heart extract was incubated with the following enzymes for 30 min at 37°C: hexokinase to convert ATP into ADP (A); creatine kinase for the breakdown of CreP (B); adenylate kinase (C); and finally AMP-deaminase (D) to shift ADP and AMP. Conditions and detection as in Fig. 1.

tabolite of allopurinol (a xanthine oxidase inhibitor often used in lipid peroxidation studies).

Recovery studies were carried out by adding a mixture of different standards to a frozen biopsy before processing the sample. The recovery varied between 95 and 102%, with a standard deviation lower than 5% ($n = 5$).

Fig. 1B is a representative chromatogram of a

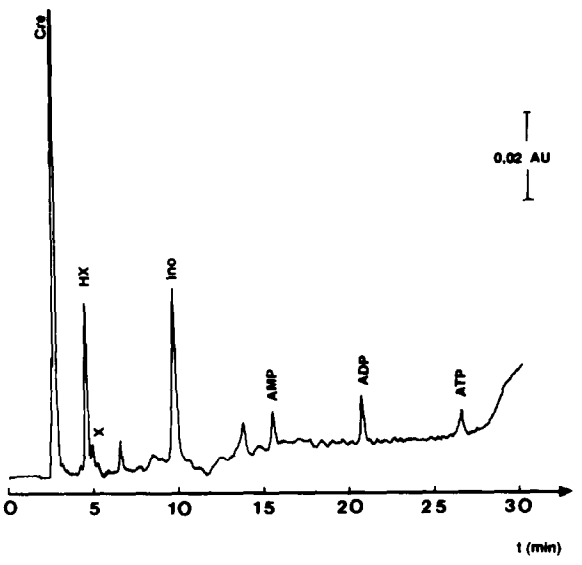

Fig. 3. Autolysis of rat myocardial tissue at 37°C for 30 min. Compared with controls (Fig. 1B) there is complete loss of CreP and a decline in ATP and ADP with a commensurate rise in hypoxanthine and inosine. Conditions and detection as in Fig. 1.

rat heart tissue extract obtained from a control animal. Besides the adenine nucleotides and CreP, HX, X, inosine and GTP can also be quantified. Data from myocardial biopsy extracts obtained from five rats are summarized in Table I.

To exclude the possibility that unidentified metabolites coelute with compounds of interest, enzymic peak-shifting was performed with neutralized rat heart extracts according to the method of Brown [11], as shown in Fig. 2A–D.

Fig. 3 represents the chromatogram of a rat heart biopsy, which was subjected to autolysis at 37°C for 30 min to mimic a severe ischemic event. Results of *in vitro* autolysis have previously been presented by Jennings *et al.* [12]. The graph illustrates the decline of ATP and ADP with a concomitant increase in HX and inosine due to autolysis.

A further application of this method, utilizing its low detection limit, was the analysis of neonatal rat heart myocytes containing not more than 500 μg of total protein. Fig. 4A shows the profile of nucleotides and CreP of a perchloric acid extract from cultured myocytes. Table II summarizes the data on purine nucleotide and CreP from five extracts of neonatal rat heart cells after a 48-h culture period.

TABLE II

CONCENTRATIONS OF PURINE NUCLEOTIDES AND CREATINE PHOSPHATE IN NEONATAL RAT HEART CELLS AFTER A 48-h CULTURE PERIOD

Compound	Concentration (mean ± S.D., n = 5) (μmol/mg of protein)
ATP	21.60 ± 0.96
ADP	2.05 ± 0.05
AMP	3.46 ± 0.60
CreP	12.70 ± 0.15
GTP	3.56 ± 0.20

CONCLUSION

An ion-pair reversed-phase HPLC method designed for measuring nucleotides and nucleosides as well as creatine phosphate and related compounds in small biological samples has been developed. The resolution, reproducibility and linearity have been proven. The run time of 50 min (including reconditioning) appears long, but the information provided by one sample in a single run is worth the effort. Therefore we are convinced that this method might be helpful in the study of energy metabolism in cardiac tissue.

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