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Rapid determination of creatine, phosphocreatine, purine bases and nucleotides (ATP, ADP, AMP, GTP, GDP) in heart biopsies by gradient ion-pair reversed-phase liquid chromatography

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ABSTRACT

A simple binary solvent method has been developed for the simultaneous determination of creatine (Cr), phosphocreatine (PCr), ATP, ADP, AMP, GTP, GDP, IMP, NAD, inosine, adenosine, hypoxanthine and xanthine. This allows separation of the most important nucleotides present in myocardial biopsies as, for example, in studies using ^{31}P NMR spectroscopy. In NMR spectra ATP and PCr are the only visible high-energy phosphates, therefore the status of other nucleotides and bases cannot be determined. The nucleotides, AMP degradation products, PCr and Cr in pig and rat heart muscle were resolved with 35 m*M* K_2HPO_4, 6 m*M* tetrabutylammonium hydrogensulfate buffer, pH 6.0, and a binary acetonitrile gradient on medium-bore, 250 mm or 125 mm × 3.9–4.6 mm I.D. steel octadecyl-bonded (C_{18}) columns at a flow-rate of 1.5 or 1.0 ml/min. This method, optimized for use with older high-performance liquid chromatography pumps (100 μl displacement heads), resolves the major porcine and rat myocardial nucleotides and degradation products within 22 min. The amounts found in normoxic porcine muscle are: Cr 9.21 ± 0.75; hypoxanthine 1.40 ± 0.14; PCr 7.20 ± 1.2; IMP 1.34 ± 0.13; βNAD 1.82 ± 0.23; AMP 0.10 ± 0.04; GDP 0.05 ± 0.02; ADP 1.23 ± 0.09; GTP 0.19 ± 0.01; ATP 4.45 ± 0.32 μmol/g wet weight. The method, incorporating adenosine tetraphosphate as an internal standard, allows the documentation of changes in both the high-energy phosphates and their degradation products in a single analysis of myocardial samples as small as 200 μg (wet weight).

INTRODUCTION

Biopsy of heart muscle has become an accepted investigative tool in both the experimental and clinical environment. The role of adenine nucleotides and pyridines, in the maintenance of energetic and redox states, is now well appreciated, and their levels are used as an indicator of cellular integrity [1–3]. High-performance liquid chromatographic (HPLC) analyses of cell extracts have been of tremendous utility in studying energetics [4–6] and separating mixtures of nucleotides, nucleosides and bases [4,7,8]. Although anion exchange (*e.g.*, Partisil-10 SAX) has proven useful, the analysis took about 1 h, at flow-rates of 1.5–5.0 ml/min, and required long equilibration times. With anion exchange the bases elute close to the solvent front and are poorly resolved, reducing data reliability [7]. Reversed-phase separation on C_{18} columns while superb in resolving the bases, is not as useful in separating the charged nucleotides, because they also elute close to the solvent front, and are poorly resolved [9–11] with ATP, ADP and AMP eluting close to guanosine, uridine and cytosine phosphates. This can be a problem when samples with low amounts of these adenosine nucleotides are analysed. Ion-pair chromatography of nucleotides

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allows the use of octadecyl-bonded columns, which are less susceptible to degradation than ion-exchange columns, resulting in superior retention, resolution and consistent determination of compounds of interest [12,13].

Studies of human and experimental heart disease indicated that failing heart muscle contained low concentrations of ATP [14,15]. Most studies using biochemical analyses of tissue samples do not report changes in phosphocreatine (PCr), since the methods used are not suitable for measuring the full range of high-energy phosphate compounds. One limitation is that the creatine compounds do not absorb light within the range most useful for determining adenosine and guanosine nucleotides. However, the creatine compounds absorb in the UV range 205–220 nm, and adenosine nucleotides can be determined within this range. In isocratic methods developed to analyze PCr and adenosine nucleotides the retention time of the latter is long and results in significant peak broadening and loss of sensitivity. This has traditionally been addressed by using three approaches, with detection of PCr at 210 nm and adenine nucleotides at 254 nm [13], by detection of both PCr and adenine nucleotides at 206 nm using isocratic elution [11] or by using gradient elution conditions for both PCr and adenosine nucleotides with detection at 210 nm [16]. Therefore an assay, which combines good resolution, selectivity, sensitivity, specificity and allows the simultaneous determination of PCr, the adenine and pyrimidine nucleotides, and their degradation products in *in vivo* biopsy samples, was developed. The method described here uses gradient ion-pair, reversed-phase HPLC, optimized for high resolution, and rapid quantification of these compounds.

EXPERIMENTAL

Chemicals

Nucleotides, adenosine diphosphate, diadenosine tetraphosphate (AP_4A), adenosine tetraphosphate (AP_4), βNAD, creatine, phosphocreatine and all other chemicals were obtained from Sigma (St. Louis, MO, USA) unless noted otherwise. HPLC-grade acetonitrile was from BDH (Toronto, Canada). HPLC-grade water was prepared on demand using a Milli-Q system (Millipore, Toronto, Canada).

Extraction of tissue samples

Pig and rat heart samples frozen in liquid nitrogen (−196°C) were extracted by homogenizing in ice cold 0.4 *M* perchloric acid ($HClO_4$) containing 0.5 m*M* ethylene glycol-bis(β-aminoethyl ether)tetraacetic acid (EGTA) in a ratio of 100 mg/ml (w/v). Needle biopsy samples were extracted using a ratio of 8 mg/ml (w/v). After 10 min on ice, the acid extract was centrifuged at 10 000 *g* for 2 min, and an aliquot of the supernatant was neutralized with 0.5 *M* K_2CO_3 to pH 6.8 (25:1, v/v). The neutralized extract was centrifuged to precipitate the insoluble potassium perchlorate ($KClO_4$), and the supernatant was frozen in a dry ice bath. Within 1 h of extraction the sample was thawed out and 25 μl of the sample were used for HPLC separation and determination.

There is no direct method for introducing a reference marker intracellularly for determining nucleotide and nucleoside losses during extraction. These losses were therefore determined indirectly by adding 0.25 g of frozen heart to either 0.25 ml of frozen solution containing added nucleotides or to 0.25 ml of frozen dilution buffer. The samples were extracted with 0.4 *M* $HClO_4$ and neutralized with 0.5 *M* K_2CO_3. The $KClO_4$ was precipitated by centrifugation and the supernatant analysed. After chromatographic separation the peak area of each nucleotide in the chromatogram of the sample without added reference standards was subtracted from the corresponding peak area in the chromatogram of the sample with added standard nucleotides. Under these conditions the recovery of reference nucleotides and bases ranged from 90 to 105% with an average of 95%, while the recoveries for βNAD and GTP were slightly lower (93 and 85%, respectively).

In an attempt to incorporate a reference standard into the extraction procedure AP_4A was evaluated. The intracellular concentration of

AP_4A is extremely low (0.1–1 m*M*) [3], making it a potentially good candidate. There were two problems: (i) AP_4A obtained from commercial sources was contaminated with low levels of other nucleotides and (ii) in ion-pair chromatography its retention time was considerably longer than that of any of the nucleotides of interest. This precluded its adoption for this purpose. AP_4 unlike AP_4A can be used as an internal standard for the adenosine nucleotides, since its retention time and recovery were acceptable.

Chromatographic procedure

Nucleotide analyses were carried out with a dual-pump (M6000) gradient HPLC system (Waters Chromatography, Toronto, Canada), equipped with a U6K injector, an M490 UV absorbance detector and an M480 controller and data station. Chromatography was carried out using either an LC18-T (5 μm particle size, 250 mm × 4.6 mm I.D., Supelco Canada, Oakville, Canada) or a Nova-Pak C_{18} (5 μm particle size, 150 mm × 3.9 mm I.D., Waters Chromatography, Toronto, Canada) column protected with a C_{18} guard column (25 mm × 4.6 mm I.D., Supelco Canada). The mobile phase consisted of buffer A (35 m*M* KH_2PO_4, 6 m*M* tetrabutylammonium hydrogensulfate, pH 6.0, 125 m*M* EDTA) and buffer B (a mixture of buffer A and HPLC-grade acetonitrile in a ratio of 1:1, v/v), all filtered

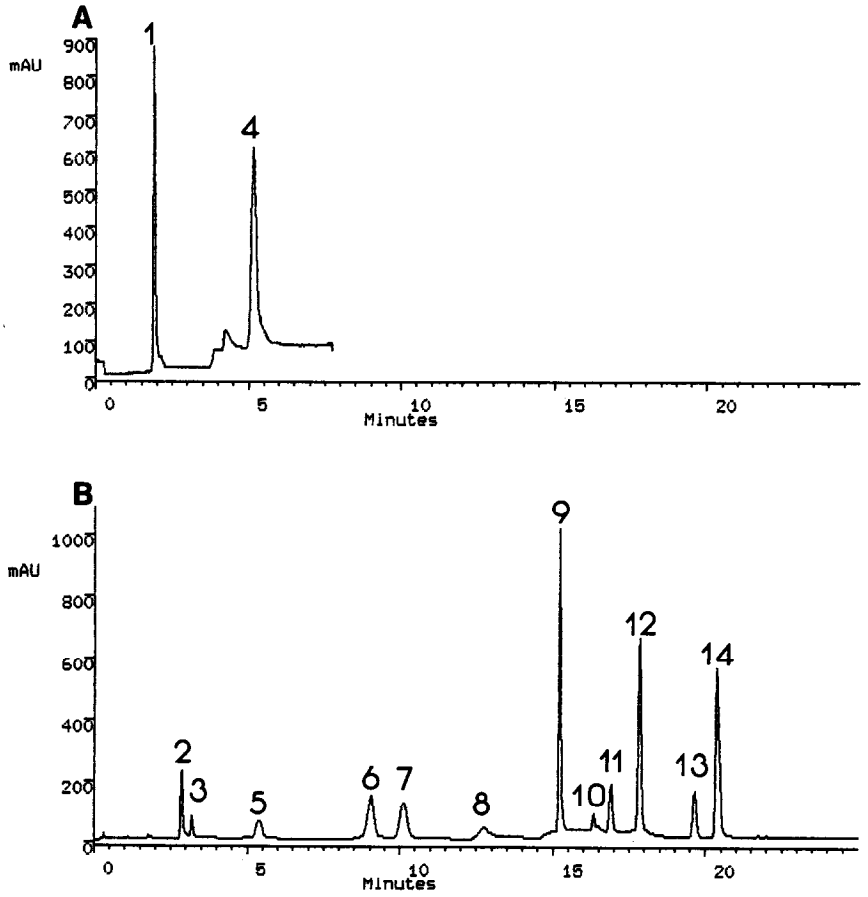

Fig. 1. Elution pattern of fourteen standards with dual-channel detection (LC18-T, 5 μm, 250 mm × 4.6 mm I.D. column). (A) 210 nm detection (acquisition turned off after 7 min). Peaks: 1 = creatine; 4 = phosphocreatine. (B) 260 nm detection (0–25 min). Peaks: 2 = hypoxanthine; 3 = xanthine; 5 = inosine; 6 = IMP; 7 = βNAD; 8 = adenosine; 9 = AMP; 10 = cAMP; 11 = GDP; 12 = ADP; 13 = GTP; 14 = ATP.

through a 0.2-μm membrane filter and helium degassed. The binary gradient program used the Waters curvilinear program No. 3, beginning after 10 min isocratic elution with 98% A–2% B. The gradient ended at 45% A–55% B after 10 min. This composition was maintained for an additional 5 min, at a flow-rate of 1.5 ml/min. After the analysis the column was reequilibrated with 98% buffer A–2% buffer B. The total time for an analysis was 22 min.

Calibration stock solutions were prepared in 0.1 *M* KH_2PO_4, pH 7.0 and stored at −70°C to minimize the degradation of PCr. The retention times of reference compounds, dissolved in the supernatant prepared from charcoal plus C_{18} stripped deproteinized and neutralized heart muscle extract, and processed in a manner similar to that of tissue samples, did not differ from those prepared by dilution in 0.1 *M* KH_2PO_4. The working standard mixture was therefore routinely prepared by dilution of these stock solutions in 0.1 *M* KH_2PO_4. A 25-μl aliqout of each extract or standard was injected on column. The standard curves (peak areas *versus* picomoles of compounds injected on column) were subjected to linear regression analysis, and calibration factors were determined. Since AP_4 elutes shortly after ATP its use as an internal standard is recommended only if ATP, ADP, AMP are of primary interest. The cellular energy status (adenylate energy charge) can then be readily calculated (ATP + ADP/2)/(ATP + ADP + AMP) [17].

RESULTS AND DISCUSSION

The method developed had two thrusts: (i) to establish a fast robust separation of cAMP, βNAD, GTP, GDP, IMP and the high-energy phosphorylated compounds found in the myocardium, and (ii) within the same analysis allow identification of degradation products produced during ischemia and hypoxia. With ion-pair chromatography the buffer can be maintained at pH 6, which is compatible with good retention and resolution of the nucleotides, while ensuring that the acid-labile PCr is not degraded during the analysis. To optimize PCr retention and resolution some analytical speed was sacrificed, still a full analysis can be completed in 22 min (see Fig. 2 and Table II). Any determination of NAD or NADP with this method using acid extracts represents the sum of NAD + NADH and NADP + NADPH since the acidity converts NADH and NADPH to their oxidized forms. This method will resolve NADH and NADPH from NAD and NADP if a suitable non-acidic extract, such as that described for red blood cells, is prepared [10]. To optimize detection 340 nm should be used for NADH and NADPH, and 276 nm for CTP and CDP.

The complete separation of a standard mixture containing PCr, Cr, purine bases, their monophosphates, diphosphates and triphosphates, is shown in Fig. 1. Note that two detection wavelengths (210 and 260 nm) were used, since PCr has a low molar absorptivity at 260 or 254 nm, while the absorptivities of the adenine nucleotides are excellent at either of the three wavelengths. It was found that the analysis of tissue extracts at 210 nm resulted in an unacceptable

TABLE I

RETENTION TIMES OF REFERENCE NUCLEOTIDES AND BASES PRESENT IN HEART EXTRACTS

Reference compounds were dissolved in the supernatant prepared from charcoal and C_{18} stripped heart muscle extract and processed in a manner similar to that of tissue samples. Results are mean ± S.D. of six determinations.

Compound	Retention time (min)	Standard deviation
1. Creatine	2.60	0.02
2. Hypoxanthine	2.84	0.02
3. Xanthine	3.16	0.02
4. Phosphocreatine	5.18	0.06
5. Inosine	5.48	0.10
6. IMP	9.02	0.05
7. βNAD	10.11	0.08
8. Adenosine	12.83	0.16
9. AMP	15.50	0.02
10. cAMP	16.33	0.00
11. GDP	16.88	0.00
12. ADP	17.82	0.00
13. GTP	19.62	0.02
14. ATP	20.42	0.03

TABLE II

LEVELS OF PCr, NUCLEOTIDES, NUCLEOSIDES AND BASES IN PORCINE HEART

Pig hearts were subjected to 20 min of regional ischemia *in vivo* by occluding the left anterior decending coronary artery, proximal to the second lateral branch, followed by 180 min of reflow. Myocardial biopsy samples were obtained from a reference area on the upper ventricle outside the area subjected to ischemia and from the reperfused muscle. Results are given as mean ± S.D., of duplicate determinations of four experiments in each group.

Compound	Concentration (μmol/g wet weight)	
	Normoxic heart muscle	Reperfused heart muscle
Creatine	9.21 ± 0.75	8.15 ± 2.3
Hypoxanthine	1.40 ± 0.14	1.81 ± 0.14
Xanthine	Not detected	Not detected
Phosphocreatine	7.20 ± 1.2	9.20 ± 1.5
Inosine	Not detected	Not detected
IMP	1.34 ± 0.13	1.22 ± 0.16
βNAD	1.82 ± 0.23	1.71 ± 0.15
Adenosine	Not detected	Not detected
AMP	0.10 ± 0.04	0.02 ± 0.01
cAMP	Not detected	Not detected
GDP	0.05 ± 0.02	0.01 ± 0.01
ADP	1.23 ± 0.09	1.15 ± 0.07
GTP	0.19 ± 0.01	0.22 ± 0.02
ATP	4.45 ± 0.32	3.81 ± 0.42
AEC[a]	0.876	0.880

[a] Adenylate energy charge.

baseline elevation during the acetonitrile gradient phase of the separation. This was not eliminated by using HPLC-grade potassium phosphate monobasic to make the buffer. In practice, of greater benefit in minimizing baseline drift during gradient elution was switching from 210 nm to either 260 or 254 nm at 7 min (after the elution of PCr but before the elution of βNAD).

The retention times and names of reference compounds are listed in Table I and the numerical identity is shown in Fig. 1. The amounts of reference compounds used are approximately 33% of those found in the pig myocardium (Table II), with the exception that cAMP, adenosine and inosine were at a concentration similar to that of AMP. The coefficients of variation (C.V.) of the quantification of the reference compounds ranged from 0.4% (βNAD) to a maximum of 2.6% (cAMP). The reproducibility of the retention times was very good (Table I, maximum C.V. of 1.7%). Over a period of months there was some variation resulting from column aging, change in ambient temperature and erosion of pump seals. This was readily corrected by the routine injection of calibration standards. In addition to retention times, verification and identification of the PCr peak and adenine nucleotides in the myocardial extracts were confirmed using the following criteria: coelution with standards, comparing peak-area ratio at two wavelengths, enzymatic degradation of neutralized extracts using creatine kinase and $MgCl_2$ to degrade PCr, glucose plus hexokinase to degrade ATP, glucose plus hexokinase and adenylate kinase to degrade both ATP and ADP, while mononucleotides were degraded with 5'-nucleotidase [18].

The analysis of a pig heart sample shown in Fig. 2A demonstrates the usefulness of single-channel monitoring with wavelength switching after 7 min to 260 nm, while in Fig. 2B the analysis monitored at 254 nm shows the loss of PCr and Cr visibility. The single-channel switching of wavelengths is preferable to using two separate assays or investing in multi-channel or array detectors, to get the information obtained here with a single short analysis. Note that ten nucleotides and bases were readily detected. To highlight the applicability of the method to small samples, the analysis of a needle biopsy sample of the porcine ventricle is shown in Fig. 3; the aliquot injected on column is equivalent to 200 μg tissue (wet weight), which is approximately 40 μg tissue dry weight. With a standard biopsy needle (1.2–2.0 mm I.D.) samples ranging from 2.8 to 6.9 mg dry weight, depending on the thickness of the ventricle, provide sufficient myocardial muscle for analysis. Note that Cr, PCr, βNAD, GTP, AMP, ADP and ATP are readily seen, but baseline drift is more prominent at the higher sensitivity, as compared to Fig. 2. The substitution of the less expensive tetrabutylammonium hydrogensulfate for tetrabutylammonium hydrogenphosphate reduces the cost of the assay without compromising

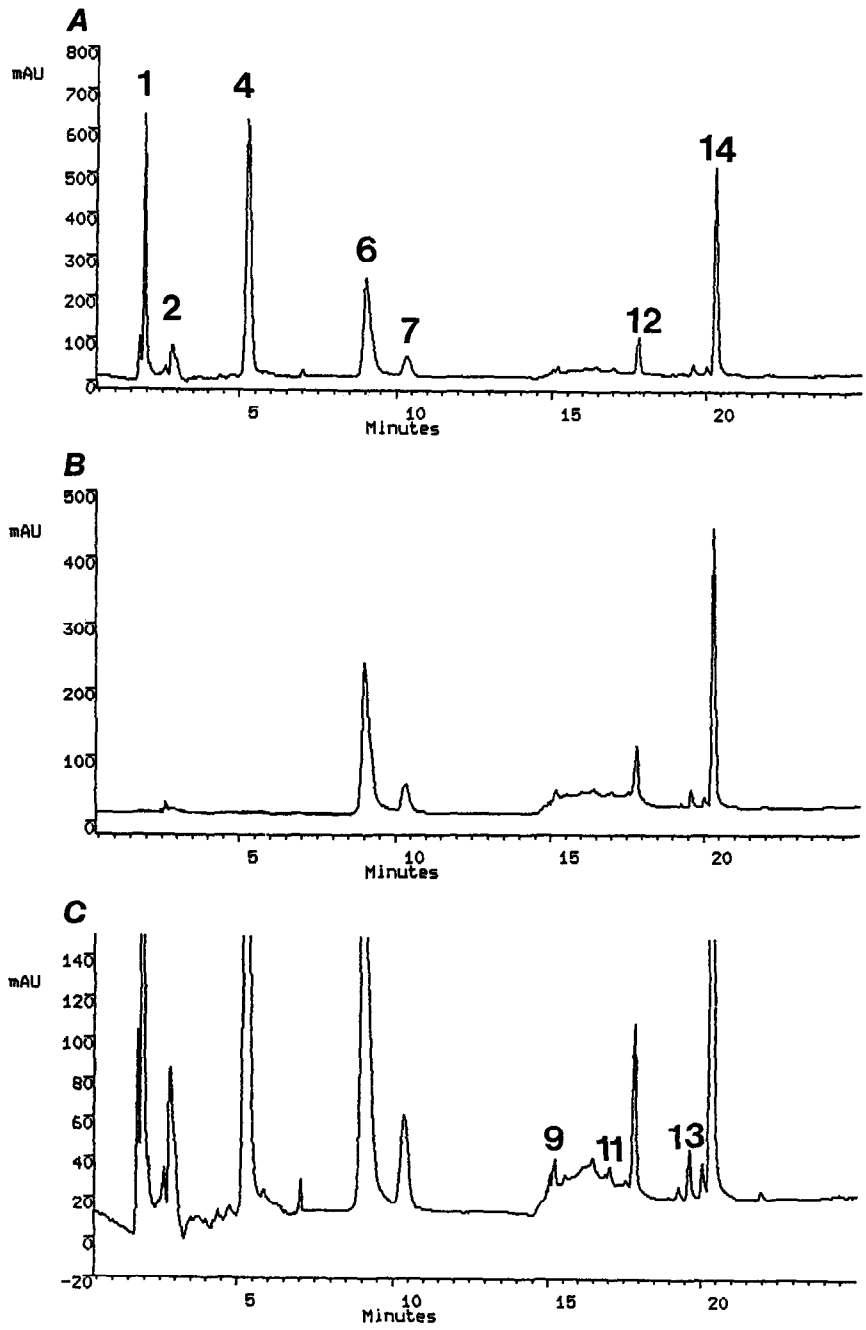

Fig. 2. Separation by ion-pair reversed phase HPLC of the adenine nucleotides, guanidine nucleotides, Cr, PCr and NAD present in porcine left ventricular muscle. Tissue (100 mg) was extracted with 1 ml of perchloric acid and 25 μl of the neutralized supernatant analysed (equivalent to 2.5 mg wet weight or 575 μg dry weight). Peak identification as in Fig. 1 and Table I. (A) 210 nm up to 7 min, then switching to 260 nm; (B) detection at 254 nm; (C) expanded scale showing small amounts of AMP, GDP and GTP.

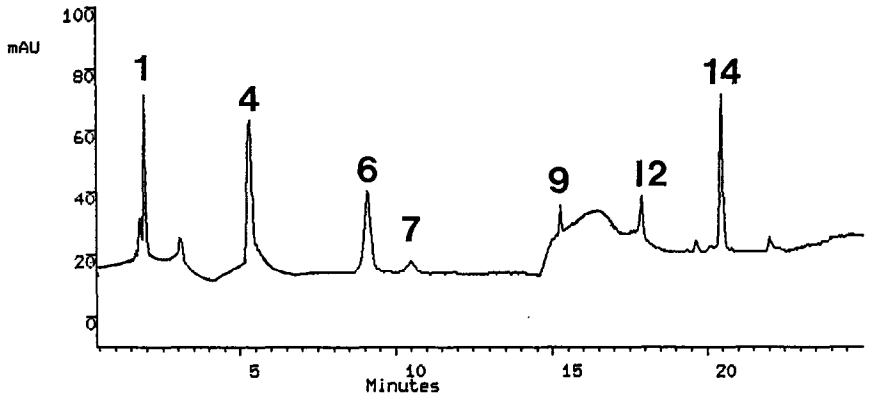

Fig. 3. Chromatographic profile of an extract of porcine ventricle obtained by needle biopsy (8 mg tissue wet weight/ml perchloric acid) eluting from a 250 mm × 4.6 mm I.D. C_{18} column. A 25-μl aliquot of the neutralized extract was injected on column (equivalent to 200 μg tissue wet weight or 40 μg dry weight). Note that Cr (1), hypoxanthine (2), PCr (4), βNAD (7), AMP (9), ADP (12), GTP (13) and ATP (14) are still readily detected.

resolution. To optimize separation while minimizing analysis time on a 250 mm × 4.6 mm I.D. column, it was necessary to use 6 m*M* tetrabutylammonium hydrogensulfate and an aggressive convex organic phase gradient (see Experimental).

The concentrations of compounds determined in biopsies of porcine myocardial muscle before and after a short ischemic insult *in vivo* are shown in Table II. Pig hearts *in situ* were subjected to 20 min of regional ischemia, followed by 180 min of reflow. Myocardial biopsy samples were obtained from normal and reperfused muscle. Note that xanthine was not detected in these samples. This is consistent with the occurrence of low levels of xanthine oxidase activity in normoxic and ischemic porcine heart [19], while cAMP levels were below the sensitivity of the method. The concentrations of PCr and adenosine nucleotides in pig heart (Table II) are similar to published values [2], with the exception that the reported PCr values (Table III) are higher, resulting in a PCr/ATP ratio close to 2. This is similar to the *in vivo* PCr/ATP ratio for pig heart determined by ^{31}P NMR spectroscopy [1]. In their report Cordis *et al.* [2] did not quantify creatine, hypoxanthine, inosine, βNAD, IMP, GDP or GTP, whereas these are reported here in Table II.

The adaptability of the method to other columns is illustrated in Fig. 4, which shows the separation of a normoxic rat heart extract spiked with the internal standard AP_4 on a Nova-Pak C_{18} column (150 mm × 3.9 mm I.D., Fig. 4A). Note that AP_4 is well resolved from ATP, allowing quantification and determination of the adenylate energy charge. Furthermore, on this shorter column it is still possible to separate the high-energy phosphates and their metabolites in is-

TABLE III

CHANGES IN HIGH-ENERGY PHOSPHATES DETERMINED USING THE INTERNAL STANDARD AP_4 IN ISOLATED PERFUSED RAT HEARTS

Values are mean ± S.D. of seven hearts perfused with oxygenated Krebs–Henseleit buffer (pH 7.4) for 60 min or subjected to 20 min of normothermic global ischemia after 40 min perfusion. Ischemic hearts were sampled without reflow or reoxygenation.

Compound	Concentration (μmol/g wet weight)	
	Control	Ischemia
ATP	5.08 ± 0.26	0.90 ± 0.17
ADP	1.32 ± 0.07	1.23 ± 0.11
AMP	0.19 ± 0.15	0.18 ± 0.02
PCr	7.92 ± 0.35	1.74 ± 0.18
AEC[a]	0.871	0.656

[a] Adenylate energy charge.

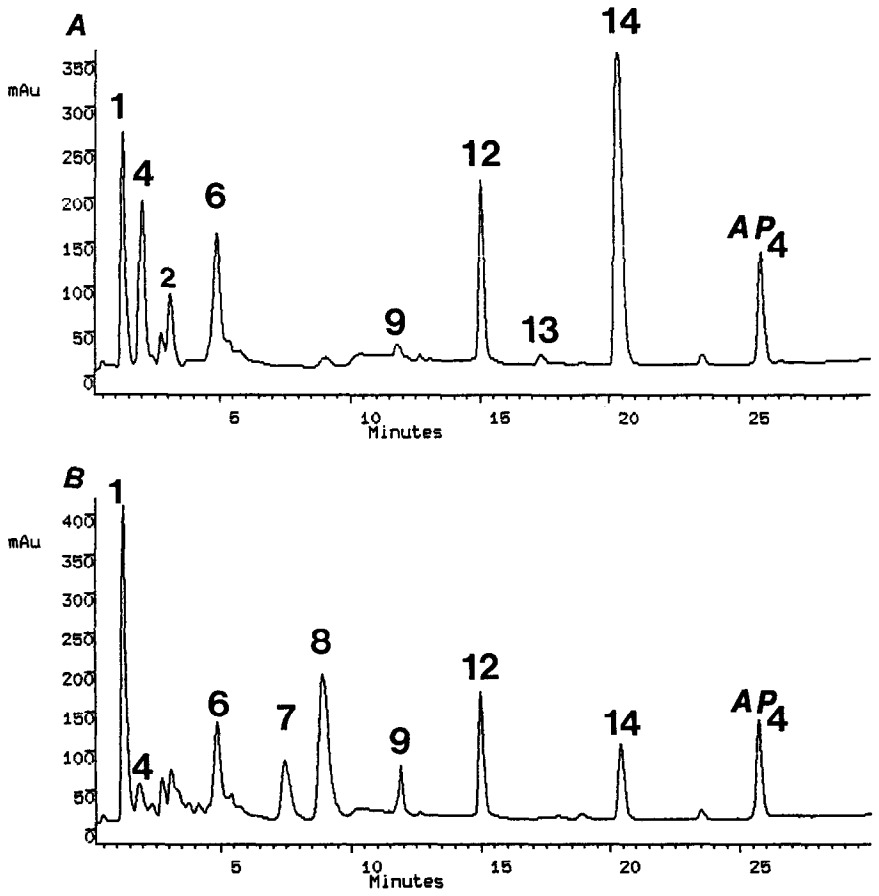

Fig. 4. Chromatographic profile of extracts of rat heart spiked with the internal standard ASP_4. Neutralized heart extracts (25 μl) were injected on a Nova-Pak C_{18} column (150 mm × 3.9 mm I.D.), at a buffer flow-rate of 1.0 ml/min. (A) Heart perfused for 1 h with Krebs–Henseleit buffer. (B) Heart subjected to 15 min global ischemia, showing the appearance of prominent IMP (7), adenosine (8) and AMP (9) peaks, and the disappearance of PCr (2). Note that the internal standard AP_4 is well resolved, eluting after ATP.

chemic tissue with resolution superior to that obtained with isocratic methods using either reversed-phase or ion-pair chromatography (Fig. 4B). In Table III, the concentrations of the high-energy phosphates found in rat hearts subjected to 20 min of normothermic global ischemia are contrasted with those obtained from normoxic hearts perfused for a similar duration. As expected, global ischemia resulted in a precipitous decrease in the concentrations of ATP, ADP, AMP and PCr, underscored as a reduction in the calculated adenylate energy charge (AEC) from 0.871 to 0.656. The longer column (250 mm × 4.6 mm I.D.) permits enhanced retention and resolution of PCr, and is therefore preferred for routine use.

CONCLUSION

In summary, an ion-pair HPLC method for simultaneously determining PCr, nucleotides, nucleosides and purine bases in tissue samples, with the option of incorporating an internal standard, is described. This method is adaptable to different columns, allowing the quantification of the more significant compounds in a single, short (22 min) analysis of myocardial extracts. This method, while intended primarily for analysing heart samples, can readily handle samples prepared from other tissues (brain, skeletal muscle, liver, red blood cells) since the peaks are well resolved from each other.

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