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Extraction and Assay of Creatine Phosphate, Purine, and Pyridine Nucleotides in Cardiac Tissue by Reversed-Phase High-Performance Liquid Chromatography

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The levels of creatine phosphate, purine, and pyridine nucleotides in tissues provide important information on energetic and oxidative cellular states. Nevertheless, technical, theoretical, and methodological difficulties in extraction and quantification procedures have so far limited our understanding of the exact role that these substances play in metabolic processes which take place in cells. The objective of our study was to find an easy and rapid method for extracting, separating, and quantifying creatine phosphate, purine, and pyridine nucleotides in solid tissues. We adapted the classic acid-extraction procedure with $HClO_4$ for purine and oxidized pyridine nucleotides and then developed a new alkaline extraction with phenol in a phosphate buffer solution (pH 7.8) for reduced pyridine nucleotides. Biopsies of myocardial tissue were frozen and ground at −180°C using the appropriate extraction procedure. The separation and quantification of the metabolites were performed using a reversed-phase 3-μm Supelchem C18 column, with the addition of tetrabutylammonium as an ion-pair agent to the buffer solution, by ultraviolet detection. The recovery of the external and internal standards always exceeded 90%. The autooxidation or interconversion processes were almost insignificant for each reduced form. This technique allowed us to avoid complex enzymatic procedures and difficulties in the selective assay of pyridine nucleotides with chemiluminescence and surface spectroscopy.
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High-energy phosphates and pyridine nucleotides play a central role in cellular oxidative metabolism. Therefore, knowledge of the cellular dynamics of adenine nucleotides, creatine phosphate, and NADH/NAD and NADPH/NADP redox couples provides fundamental insights into the pathophysiology of the different diseases affecting cardiac tissue. However, on one hand, technical difficulties have so far not allowed investigators to quantify the complete pattern of adenine and pyridine nucleotides in tissue. Problems arise due to the difference in chemical stability of oxidized and reduced forms of pyridine nucleotides in acid and alkali. Consequently, double-step extraction procedures are generally used. Acidic extraction is performed for adenine and oxidized pyridine nucleotides, and alkaline extraction for NADH and NADPH (1,2). Furthermore, alkaline extraction with KOH is long and tedious requiring considerable analytical work while recoveries can be less than 70% (3).

On the other hand, available techniques present theoretical and methodological difficulties. Given the relatively low specificity of luciferin/luciferase for distinguishing between NADH and NADPH (4–6), for instance, chemiluminescence analysis becomes less than ideal. Therefore, surface spectroscopy has been applied in research with solid tissue. Again, given the spectral similarities, NADH and NADPH cannot be adequately identified (7–9).

Concerning quantitative analysis, high-performance liquid chromatography (HPLC) (10–14) is a more powerful tool than are spectrophotometric, fluorimetric, or enzymatic cycling methods (8,15–17). Initially chromatographic methods were based on ionic exchange separation (10,11,18,19). In 1980, Jeungling and Kammermeier (20) described an isocratic HPLC protocol with reversed-phase, ion-pair separation of purines applicable to cardiac tissue. Recently, Stocchi *et al.* showed advantages, in terms of simplicity and versatility, of reversed-phase over ion-

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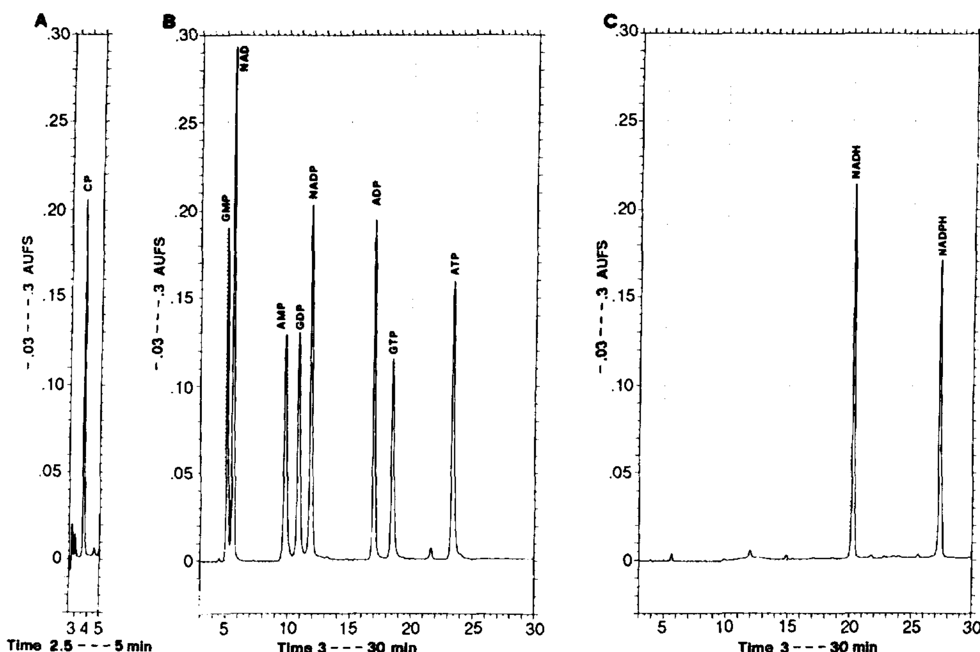

FIG. 1. Separation of standard mixtures by ion-pair reversed-phase HPLC: creatine phosphate (CP) (A; wavelength 205 nm), purine and oxidized pyridine nucleotides (B; wavelength 260 nm), and reduced pyridine nucleotides (C; wavelength 260 nm). NADPH and NADH standard chromatograms are usually obtained in our laboratory in a separate run to abolish the interconversion cycles with the oxidized forms, although these are insignificant. Adsorbance unit is reported in full scale (AUFS).

exchange HPLC for separation and simultaneous extraction and assay of adenine and pyridine nucleotides in red blood cells (3,21). Unfortunately, we found this procedure, which employs a single-step alkaline extraction and a membrane ultrafiltration, unsuitable for muscle tissues. This is probably due to the low stability of purines in the extraction medium that persists during the long tissue preparation procedures.

Having taken into account all of the disadvantages mentioned, we attempted to overcome them by developing a rapid and easy procedure for extracting, separating, and quantifying purine and pyridine nucleotides in cardiac muscle tissues and solid tissues in general. Here we describe: (a) our adaptation of the classical extraction method with perchloric acid for creatine phosphate, purine, and oxidized pyridine nucleotides; (b) a newly developed extraction procedure with alkaline phenol for reduced pyridine nucleotides; and (c) reversed-phase, ion-pair liquid chromatography with ultraviolet detection.

TABLE 1

Recovery of the Extracted External Standard of Creatine Phosphate (CP) and Most Relevant Purine and Pyridine Nucleotides

Compound	Recovery %
CP	100 ± 3.0
ATP	94 ± 4.0
ADP	90 ± 6.0
AMP	94 ± 5.0
GTP	102 ± 2.0
NAD	101 ± 2.9
NADP	99 ± 4.3
NADH	100 ± 3.2
NADPH	98 ± 3.0

Note. The data are expressed as the means ± SE of four experiments.

TABLE 2

Recovery of Creatine Phosphate (CP) and Purine and Oxidized Pyridine Nucleotides from Acid-Extracted Cardiac Tissue

Compound	Recovery %
CP	91 ± 5.0
ATP	90 ± 3.3
ADP	100 ± 7.0
AMP	94 ± 5.0
GTP	97 ± 2.0
NAD	98 ± 0.9
NADP	100 ± 0.6

Note. The data are expressed as the means ± SE of four experiments.

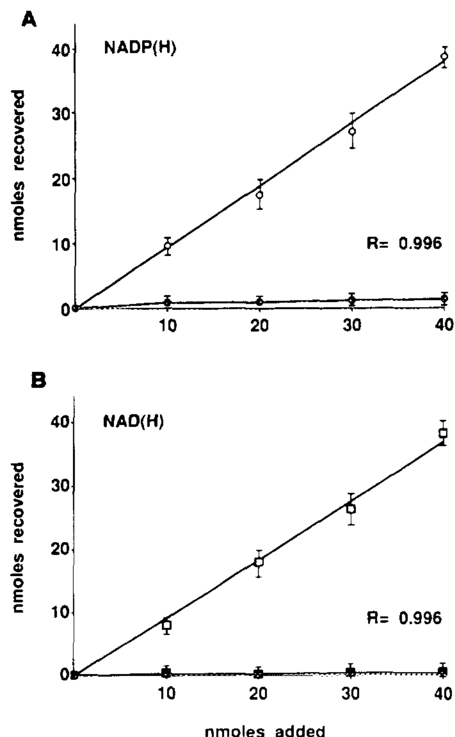

FIG. 2. Recovery of added pyridine nucleotides from an aerobic control of cardiac tissue. A, recovery and autooxidation of NADPH; B, recovery and autooxidation of NADH. The abscissas show the concentration after (open symbols) the addition of NADPH or NADH and the ordinates show the amount recovered after correction for the incomplete recovery of the internal standard. Data (closed symbols) for NADP (A) and NAD (B) are also shown, resulting from the interconversion of the reduced forms and represent the undesired interconversion during the procedures. Data are expressed as the means ± SE of five individual experiments.

MATERIALS AND METHODS

Chemicals. Nucleotide and coenzyme standards of the highest available grade were purchased from Sigma (St. Louis, MO). Analytical grade reagents and HPLC-grade acetonitril were obtained from Merck (Darmstadt, Germany), tetrabutylammonium hydrogen sulfate was from Serva (Heidelberg, Germany), while double-distilled water was prepared in our laboratory.

Tissue storage. Immediate arrest of metabolism is essential for obtaining correct data on high-energy phosphates and coenzymes (1). Tissues were freeze–clamped with liquid nitrogen precooled Wollemberger clamps (22). The whole heart was stored in liquid nitrogen and aliquots of the left ventricular apex of about 500 mg were used for further procedures.

Acid extraction of purine nucleotides and oxidized coenzymes. For the extraction of creatine phosphate, purine nucleotides, NAD, and NADP, we adapted existing procedures (1). In a mortar precooled in liquid nitrogen, preweighed portions of frozen tissue were ground in 0.4 N $HClO_4$ (3 ml/500 mg) until complete homogeneity was obtained. The mixture was then transferred from the mortar to a tube and allowed to thaw while being stirred on a Vortex mixer. It was further homogenized four times with an ultraturrax for 5 s, interspaced with 30-s cooling periods, and then centrifuged at 4000*g* for 10 min at 4°C. Supernatant pH was adjusted to 6–6.5 with 6 N KOH. Following the removal of $KClO_4$, the extract was used for chromatography. All procedures were performed at 4°C. A separate aliquot of tissue was dried at 100°C to measure the dry weight.

Alkaline extraction of NADH and NADPH. We developed a method of deproteinization and stabilization of the sample that is considerably simpler and faster than traditional extraction with KOH (2). Preweighed portions of frozen tissue (about 500 mg) were ground in phenol buffer (500 mg/3 ml of 0.64 M phenol, 0.07 M phosphate buffer solution, pH 7.8). The mixture was transferred from the mortar to a tube to which 3 ml of chloroform was added and allowed to thaw in the dark at 4°C. After centrifugation (4000*g* × 10 min at 4°C), the aqueous phase was washed five times with 5 ml of diethyl ether to remove the residual traces of phenol and organic material. The extract was used for chromatography.

Chromatographic apparatus and conditions. The HPLC system was obtained from Waters (Milford, MA). It consisted of a Model 600 E multisolvent delivery system and a Model 990 photodiode array detector. The injection volume was 20 μl (7161 rheodyne sampling valve). Separations were done on a Supelchem C18 3-μm reversed-phase column (0.46 × 15 cm).

Optimal conditions, in terms of ion-pair and organic modifier concentrations, were reached experimentally. In pilot experiments, at low concentration of acetonitril (2.5%), the concentration of tetrabutylammonium hydrogen sulfate was increased to obtain good separation of first eluted analytes (CP, GMP, and NAD) from unretained solute.

The mobile phase consisting of a gradient of buffer A (0.1 M KH_2PO_4, 5 mM tetrabutylammonium hydrogen sulfate, 2.5% (v/v) acetonitril, pH 6.0) and buffer B (0.1 M KH_2PO_4, 5 mM tetrabutylammonium hydrogen sulfate, 25% acetonitril, pH 5.5). The column was eluted at 0.8 ml/min for 3 min with buffer A, 2 min with buffer A plus buffer B, increasing to 11%, and then for 25 min with a further gradual increase (slope 6 bulletin software) of buffer B to 100%. Finally, the column was reequilibrated for 20 min with 100% buffer A. Chromatography separations were performed at room temperature.

Detection was done at 205 nm for creatine phosphate and 260 nm for nucleotides. The identities of the peaks were confirmed by coelution with standards, enzymatic peak shift, and spectral analysis. Quantitative measure-

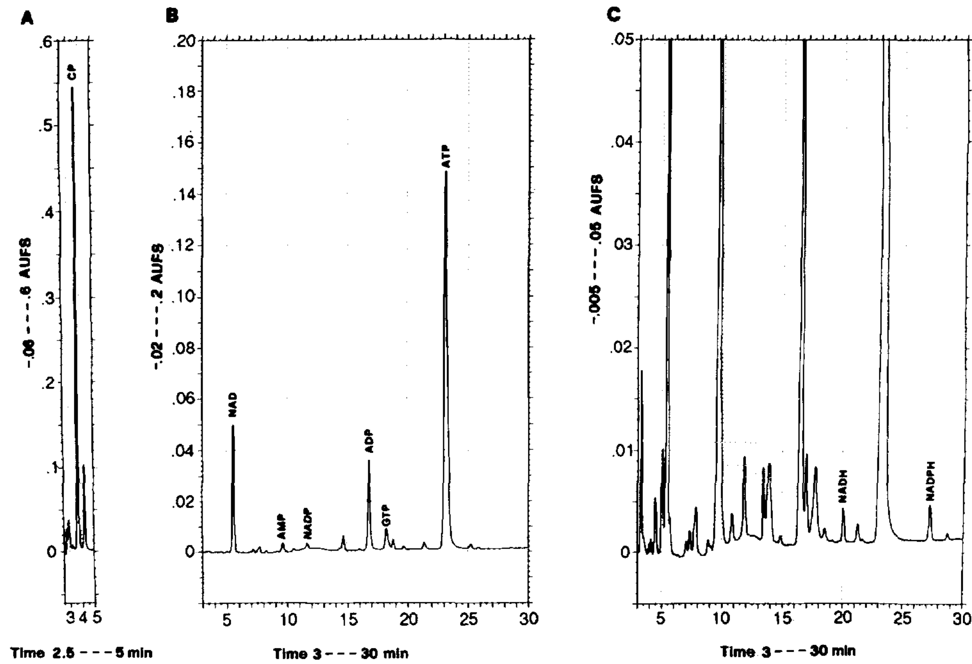

FIG. 3. Separation by ion-pair reversed-phase HPLC of a typical aerobic myocardial tissue sample: creatine phosphate (CP) (A; wavelength 205 nm), purine and oxidized pyridine nucleotides (B; wavelength 260 nm), and reduced pyridine nucleotides (C; wavelength 260 nm). The heart was perfused *in vitro* under aerobic conditions for 30 min, in a Langendorff-perfused preparation of rabbit heart (24). Adsorbance unit is reported in full scale (AUFS).

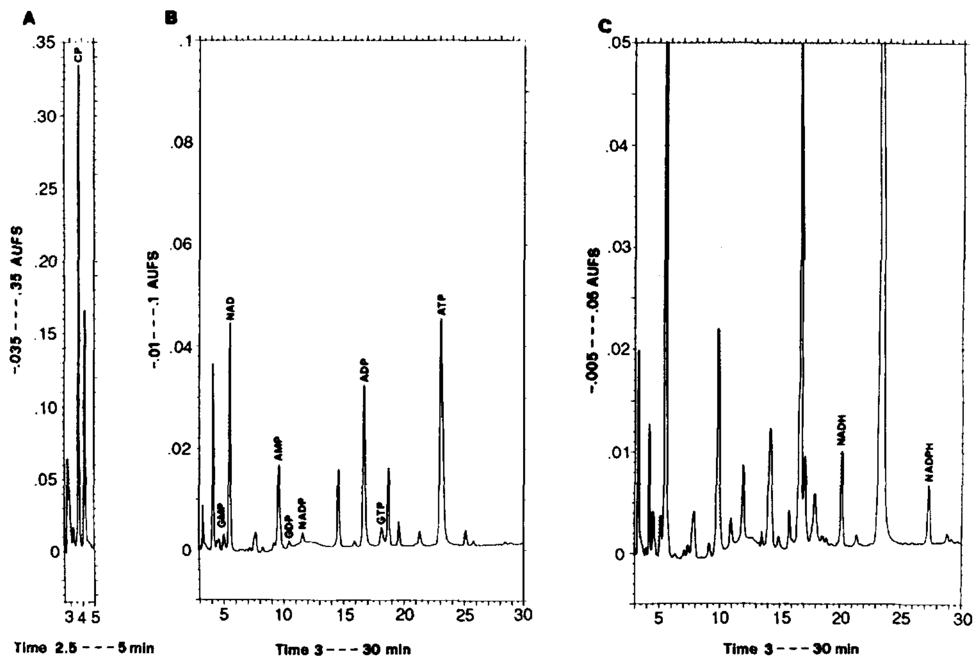

FIG. 4. Separation by ion-pair reversed-phase HPLC of a typical sample of myocardial tissue obtained after ischemia: creatine phosphate (CP) (A; wavelength 205 nm), purine and oxidized pyridine nucleotides (B; wavelength 260 nm), and reduced pyridine nucleotides (C; wavelength 260 nm). A short period of ischemia (30 min) was induced *in vitro* in a Langendorff-perfused preparation of rabbit heart (24). Adsorbance unit is reported in full scale (AUFS).

ments were made on the basis of the injection of standard solutions in known concentrations.

RESULTS AND DISCUSSION

The objective of our work was to develop a rapid HPLC method that allowed a complete separation of the most significant purine and pyridine nucleotides and relevant metabolic by-products. Typical chromatograms for creatine phosphate and the most significant nucleotide standards are reported in Fig. 1. We used acetonitril as an organic modifier of the mobile phase. This allowed for a low concentration of counter-ion and for analysis times as short as 25–28 min (Fig. 1).

Recovery rates for extracted external and internal standards were calculated. As we expected from our study of the relevant literature (1), acid extraction of an external standard gave an almost complete recovery of nucleotides (Table 1).

The recovery percentages of creatine phosphate, purine, and oxidized pyridine nucleotide internal standards, after the acid extraction, are reported in Table 2; physiological concentrations of the compounds are used. Specifically, each known standard solution was added singularly to the same homogenate of aerobic cardiac tissue. The average recovery was greater than 94%. These results confirm previous data reported in the literature (13,23) for the acid extraction applied to different tissue samples.

The check for an optimal recovery is more essential and necessary for the reduced form of pyridine nucleotides owing to the originality of our extraction procedure performed and the chemical instability of the compounds involved. For this reason, the recovery of NADH and NADPH internal standards was carried out over different concentration points. The results, in terms of the expected values, over a wide dynamic range of concentration and in terms of undesired interconversion into the related oxidized form, are reported in Fig. 2. The recovery of NADPH over a range of biological relevance is reported in Fig. 2A: the linearity is excellent (R = 0.996) and the recovery is almost complete.

In the same figure, the data of the codetermined oxidized form (NADP) are reported for each separate point. As no NADP was added, this represents undesired interconversion, or autooxidation, of NADPH. The percentage of NADP produced was always lower than 3% with respect to the NADPH value and therefore was low. The results for NADH are given in Fig. 2B: the dynamic linearity is excellent ($R = 0.996$) and recovery exceeds 90%, and again autooxidation and interconversion are negligible.

The typical chromatographic separation of compounds extracted from a myocardial aerobic tissue is shown in Fig. 3.

Stress of the myocardium caused by a restriction of oxygen, albeit for a very short period (30 min), is well represented in a different typical chromatogram obtained after ischemia (Fig. 4): the marked decrease in the peaks of creatine phosphate and ATP and the increase in AMP and reduced pyridine nucleotides (NADH) are evident.

We believe that the new extraction procedure of pyridine nucleotides and the chromatographic conditions described here represent an improvement over the available techniques. The procedure is fast enough and suitable to be applied to solid tissues such as the cardiac muscle. Furthermore, technical difficulties with the complex enzymatic procedures and in the selective assay of pyridine nucleotides with chemiluminescence and surface spectroscopy have been overcome.

The high specificity and reproducibility of our procedure, alongside the uncomplicated and easily adaptable manual technique, add a strong applicable impact.

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