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DETERMINATION OF ADENINE NUCLEOTIDES AND INOSINE IN HUMAN MYOCARD BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic high-performance liquid chromatographic system for the quantitation of AMP, ADP and ATP is presented. The separations were achieved at room temperature by reversed-phase chromatography (Supelcosil LC-18). The standard solvent was 220 m*M* potassium phosphate, pH 6.9, 1% (v/v) methanol and 0.3 m*M* tetrabutylammonium hydrogen sulphate. A selective retention of the adenine nucleotides as a group relative to the mono-, di- and triphosphates of guanosine, uridine and cytidine was observed under these experimental conditions. The adopted procedure was applied to the separation of adenine nucleotides in biological extracts, *i.e.*, human myocard. The adenine nucleotides in an extract of myocard were quantitated in less than 20 min. Only 5–10 mg (wet weight) of myocard were needed in order to determine the energy charge of a myocardial sample. Also inosine was easily quantitated in this liquid chromatographic system.

INTRODUCTION

Recently, the morphological and biochemical changes of the myocard during ischemia and heart surgery have received considerable attention. Changes in the concentrations of the adenine nucleotides and creatine phosphate provide valuable information, reflecting the metabolic changes in the myocard under these conditions[1–4]. The concentration of ATP in heart muscle probably declines earlier than detectable morphological changes, and ATP depletion in a specific cell compartment may be the primary trigger for the contractile failure[1].

Both ADP and ATP, as well as AMP, modulate the activities of many regulatory enzymes[5]. Atkinson[5] suggested the term energy charge in order to describe cellular energy metabolism as reflected by the concentration of these nucleotides in cells:

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$$\text{energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

A biological system is fully charged when all adenylate is present as ATP, and the energy charge is close to 1. A system is fully discharged when only AMP is present. Different approaches have been used in order to estimate the energy charge in biological extracts by high-performance liquid chromatography (HPLC). Ion-exchange columns were employed in early work. This approach requires a gradient elution or two to three isocratic systems in order to quantitate AMP, ADP and ATP[6–8]. Recently, reversed-phase systems have been reported[9,10], also with pairing ions in the solvent[11–14]. The reversed-phase systems for the quantitation of adenine nucleotides do not show a selectivity towards the adenine nucleotides as a group. Thus, ATP has a retention time close to GTP, UTP and CTP, while ADP is eluted from the column close to GDP, UDP and CDP. The same pattern is also reported for AMP[11,13,14].

The purpose of the present work was to establish the chromatographic conditions for the elution of ATP, ADP and AMP as a group from a reversed-phase column, well separated from the other nucleotides of biological interest, and thus facilitate the quantitation of the adenine nucleotides in biological specimens. The purity and identity of the adenine nucleotide peaks in the adopted chromatographic procedure were verified by incubation of myocardial extracts with appropriate enzymes.

The AMP in myocard is degraded during ischemia[2]. The key degradation metabolite inosine is also separated in the present chromatographic system, and data on the retention of this compound under different chromatographic conditions are included.

EXPERIMENTAL

Materials

ATP, ADP, AMP, GTP, GDP, GMP, UTP, UDP, UMP, CTP, CDP, CMP, inosine, 5′-adenylic acid deaminase (E.C. 3.5.4.6), hexokinase (E.C. 2.7.1.1), myokinase (E.C. 2.7.4.3), nucleoside phosphorylase (E.C. 2.4.2.1) and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium hydrogen sulphate was obtained from Aldrich Europe (Beerse, Belgium).

Preparation of standards of nucleotides and myocard for HPLC analysis

The standards were dissolved in water. The application of the chromatographic system for the quantitation of adenine nucleotides in biological materials was tested with extracts of human myocard. A papillary muscle specimen was obtained from a patient undergoing mitral valve replacement owing to mitral stenosis. The patient was cooled to 25°C on a cardiopulmonary bypass. The aorta was clamped and cardioplegic solution (St. Thomas[15]) was infused into the aortic root. The valve was excised 15 min after the aortic clamping, and the papillary muscle specimen was immediately frozen in liquid nitrogen and stored at −80°C until analysis by HPLC.

About 30 mg of myocard (wet weight) from papillary muscle were suspended in 1000 μl 0.4 *M* perchloric acid and homogenized by hand for 3 min at 0°C in a small glass homogenizer fitted with a glass pestle. The suspension was then centrifuged in an Eppendorf Model 3200 centrifuge for 4 min. The perchlorate anion was precipitated by addition of 250 μl 1 *M* K_2HPO_4 to 500 μl of the supernatant. The precipitate was then removed by centrifugation as above, and the supernatant was used for nucleotide analysis.

HPLC analysis of nucleotides

The chromatograph was composed of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), and an Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.). The eluate was monitored at 254 nm with a Model UV III Monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The separation was carried out at room temperature with a column (25 × 0.46 cm) packed with Supelcosil LC-18 (Supelco, Crans, Switzerland). This material has a particle diameter of about 5 μm. The system was provided with a guard column (7.5 × 0.2 cm), packed with pellicular silica, *i.e.*, HC Pellosil ODS (Whatman, Clifton, NJ, U.S.A.). This material has a particle diameter of about 25–37 μm. The standard chromatographic solvent was 220 m*M* potassium phosphate (pH 6.9), 1% (v/v) methanol and 0.3 m*M* tetrabutylammonium hydrogen sulphate. The flow-rate was 1.3 ml/min at 1800 p.s.i. The potassium phosphate buffer with tetrabutylammonium hydrogen sulphate was filtered through a 0.45-μm Millipore filter Type HA and degassed for 10 min before addition of degassed methanol. The detector response was recorded either on a Model 1107 recorder (W + W Electronic, Basle, Switzerland), or a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The nucleotides were quantitated from the peak height in the chromatogram. A linear correlation between the peak height and the injected amount was observed for all the nucleotides.

RESULTS AND DISCUSSION

Chromatography of standards

Fig. 1 shows the chromatograms of the standards of the mono-, di- and triphosphates of cytidine (A), uridine (B), guanosine (C) and adenosine (D). The mononucleotides were eluted first, followed by the respective di- and trinucleotides. Injection of a mixture of the twelve nucleotides did not influence the selective retention of adenine nucleotides relative to the other nucleotides in the chromatographic system (Fig. 1E). Note that AMP was eluted after CTP, UTP and GTP. Apparently, the interactions of the pairing ion tetrabutylammonium with the adenine nucleotides are stronger under the adopted chromatographic conditions than with the other nucleotides (see below).

Fig. 2A–D shows the dependence of the capacity factor (k') for AMP, ADP, ATP and inosine upon changes in the chromatographic solvent. Since inosine is generated in myocard during hypoxia, and this compound is separated in the chromatographic system, we included this compound in the systematic study. Moreover, inosine is less polar than AMP, and thus provides a control of the solvent effects on k' for the nucleotides. If the pairing ion tetrabutylammonium was absent from the chromatographic system, we found that ATP was eluted first, followed by ADP and

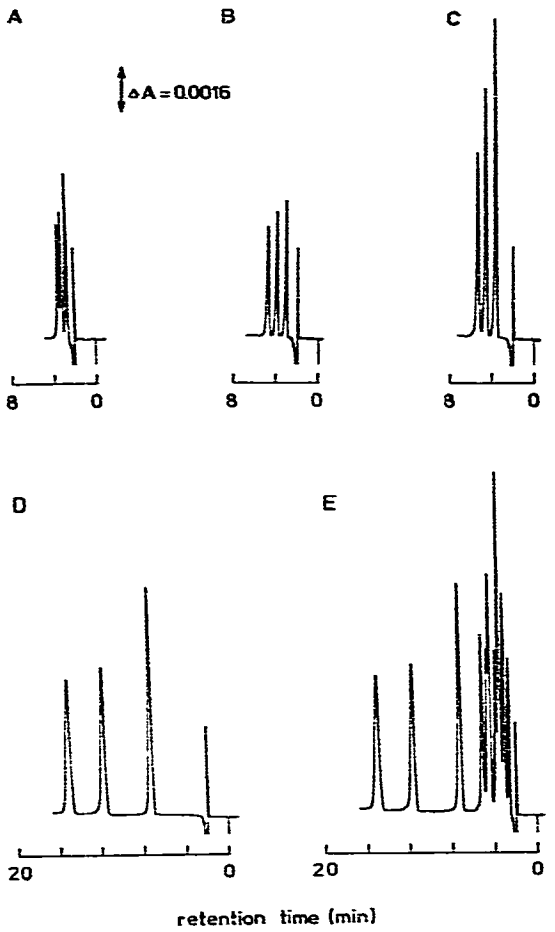

Fig. 1. HPLC elution pattern of standards. The chromatographic conditions are given in the text. Full scale deflection was 0.016 absorbance units, and the chart speed was 0.5 cm/min. The concentration of each of the nucleotides in the samples was 0.01 m*M*. Mixtures: A = CMP (retention time, t_R = 2.5 min). CDP (t_R = 3.1 min) and CTP (t_R = 3.4 min); B = UMP (t_R = 3.0 min), UDP (t_R = 3.9 min) and UTP (t_R = 4.7 min); C = GMP (t_R = 3.5 min), GDP (t_R = 4.4 min) and GTP (t_R = 5.2 min); D = AMP (t_R = 7.2 min), ADP (t_R = 11.7 min) and ATP (t_R = 15.0 min); E = all twelve nucleotides.

AMP (Fig. 2A). This is expected from current theories of reversed-phase chromatography[16,17]. The complex formation between the pairing ion and the adenine nucleotides influenced the retention times of the various nucleotides on the column, and the interaction increased with the number of negatively charged phosphate groups in the nucleotide molecule. As expected, the retention times of the adenine nucleotides were increased by higher concentrations of tetrabutylammonium hydrogen sulphate in the solvent. On the other hand, the number of negatively charged groups in the nucleotide did not alone determine the retention in the chromatographic system (see Fig. 1). The k' for inosine varied in a different manner. Increasing concentrations of tetrabutylammonium hydrogen sulphate in the solvent resulted in a marked decrease in k' for inosine. This may reflect the increased polarity of the packing material with

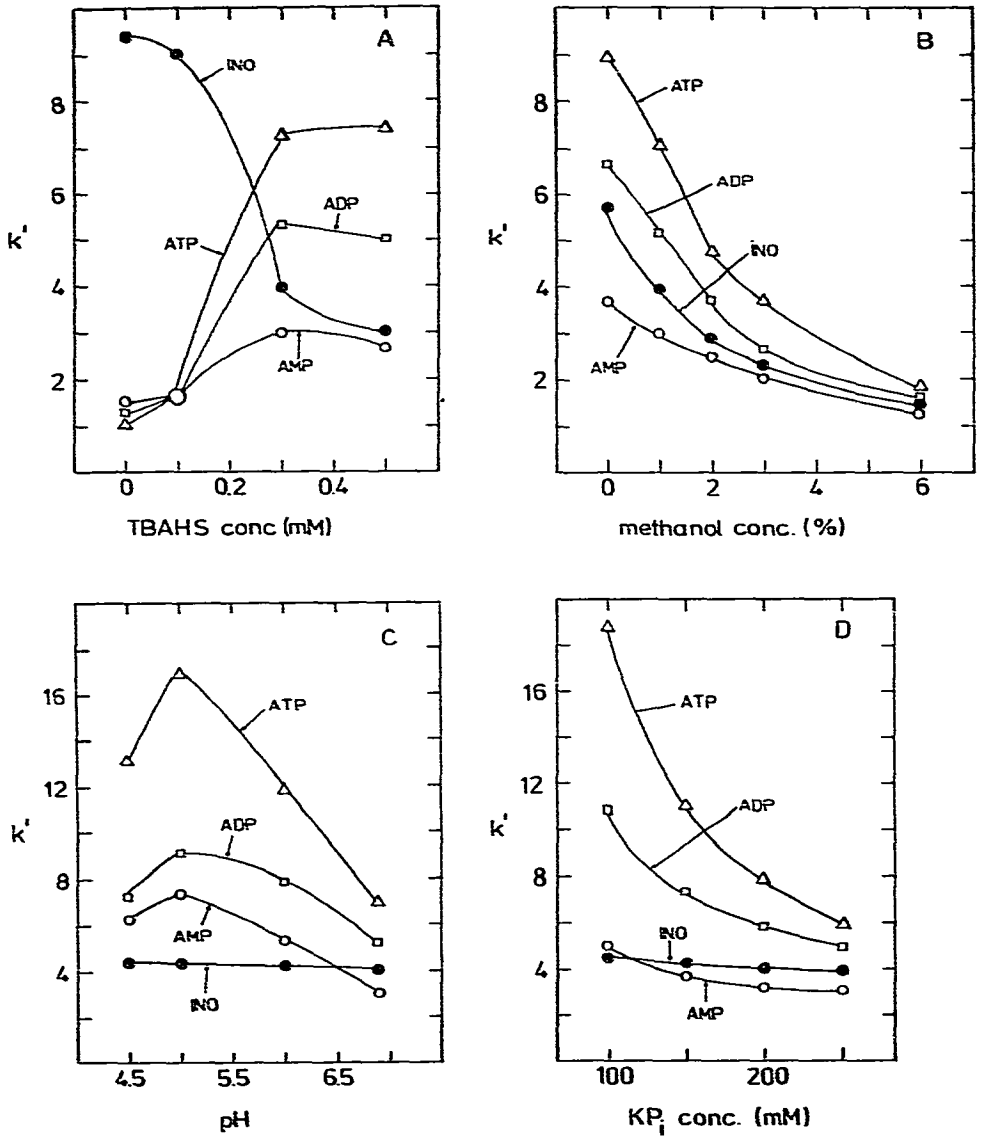

Fig. 2. Dependence of k' values on the composition of the chromatographic solvent. The adopted standard solvent (220 m*M* potassium phosphate pH 6.9, 1 % methanol and 0.3 m*M* tetrabutylammonium hydrogen sulphate) was used, and one parameter was varied in each experiment. The column was equilibrated for 90 min (*i.e.*, 117 ml) with each solvent, before injection of standards of adenine nucleotides and inosine. A, influence of tetrabutylammonium hydrogen sulphate concentration; B, influence of methanol concentration (%, v/v); C, influence of pH; D, influence of salt concentration (potassium phosphate).

the tetrabutylammonium ion bound to the hydrophobic surface (C_{18}) of the reversed phase[18].

Under otherwise identical conditions, the retention times of the nucleotides and inosine were determined by the concentration of methanol in the solvent, *i.e.*, higher methanol concentrations resulted in shorter retention times (Fig. 2B). The

relative position in the chromatogram of the nucleotides and inosine did not change significantly as a function of methanol concentration.

Fig. 2C shows the effects of changes in the pH of the chromatographic solvent. The retention time of inosine was constant in the pH range 4.5–6.9. On the other hand, the retention times for the nucleotides increased dramatically as the pH of the solvent decreased from 6.9 to 5.0. This observation can be explained by increasing adsorption of the tetrabutylammonium ion to the reversed-phase packing material at lower pH[18]. The decreased retention time of the nucleotides at pH 4.5 may be an effect of their decreased ionization at this pH.

Fig. 2D shows the effect of the salt concentration of the solvent on the retention times of the adenine nucleotides and inosine. Again, the retention time of inosine was almost unaffected by the salt concentration. The retention times of the nucleotides increased at lower salt concentrations. We previously reported that the apparent efficiency of a reversed-phase column is influenced by the salt concentration of the solvent in analyses of highly polar compounds by reversed-phase chromatography[19]. The increased retention times of the nucleotides at lower salt concentrations, and thus broader peaks, is probably the main factor in the decreased efficiency at lower salt concentrations in this chromatographic system.

Application of the method to biological extracts

Fig. 3A shows the chromatogram of an extract of human myocard. The mono-, di- and triphosphates of adenosine are easily quantitated. The energy charge of the extract shown in Fig. 3A is 0.86. Under optimal conditions, an energy charge of about 0.9 is found in most cells[5]. Inosine was also observed in this myocardial extract (retention time 9.6 min).

The purity and identity of the AMP, ADP and ATP peaks in the chromatogram of the biological extract were confirmed by different methods. First, the retention times of the assigned ATP, ADP and AMP peaks were the same as those obtained for the respective standards of the nucleotides (see Figs. 1 and 3). Moreover, the peaks responded in a typical way following the incubation of the myocardial extract with specific enzymes. Thus, the ATP peak was quantitatively removed after incubation with appropriate amounts of hexokinase (E.C. 2.7.1.1) and glucose (Fig. 3A and B). The ADP peak was then quantitatively removed in a subsequent incubation with myokinase (E.C. 2.7.4.3) (Fig. 3C). Finally, more than 90% of the AMP peak were removed by addition of 5′-adenylic acid deaminase (E.C. 3.5.4.6) (Fig. 3D). Inosine 5′-monophosphate is produced in this reaction (retention time 3.7 min). In control experiments, deaminase was also added directly to the myocardial extract (Fig. 3A). Also in this case, with deaminase as the only enzyme in the incubation mixture, the AMP peak was decreased by over 90% with the same amount of enzyme and the same incubation time as used in Fig. 3C (data not shown).

The identity and purity of the assigned inosine peak were established in the same manner. First, inosine standard had the same retention time. Moreover, the peak with the retention time of 9.6 min was quantitatively removed following the incubation of the myocardial extract with nucleoside phosphorylase (E.C. 2.4.2.1) (0.025 units per 100-μl extract in the presence of 0.1 *M* sodium phosphate pH 7.4). Concomitantly, the appearance of a peak with a retention time identical to that of hypoxanthine (3.7 min) was observed in this case (data not shown).

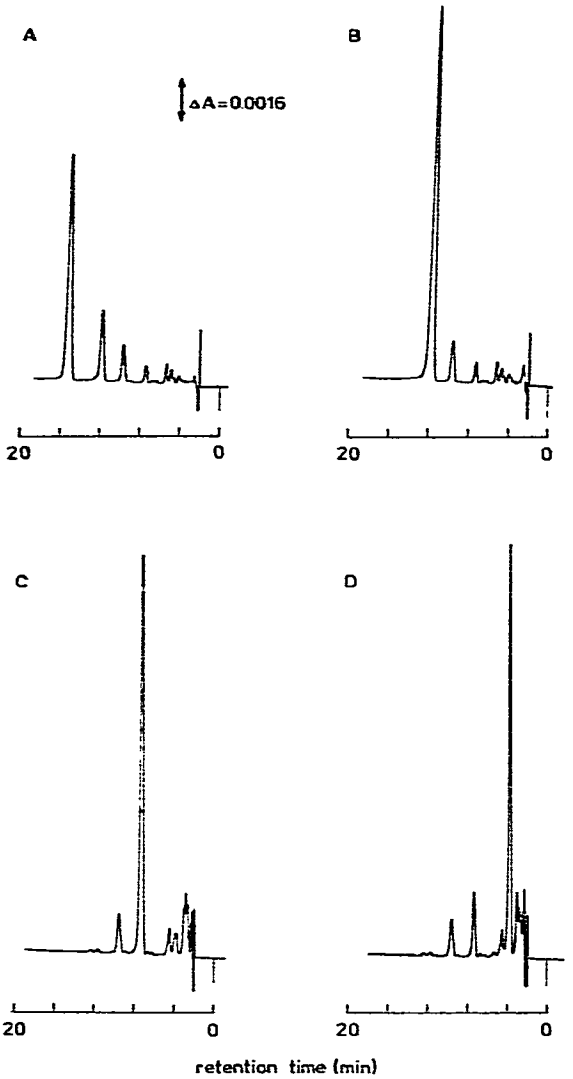

Fig. 3. HPLC elution pattern of an extract of human myocard. Full scale deflection was 0.016 absorbance units. A, 50 μl of the myocardial extract (see Experimental) were mixed with 150 μl of 0.075 *M* Tris pH 8.5, containing 5 m*M* glucose, and a 20-μl sample was injected into the liquid chromatograph. The same retention times for the adenine nucleotides were obtained as in Fig. 1. Inosine has a retention time of 9.6 min, *i.e.*, between those of AMP and ADP. B, The mixture from A above was incubated with 23 units of hexokinase at 25°C for 15 min. 20 μl were injected into the liquid chromatograph. C, The mixture from B above was then incubated with 8 m*M* $MgCl_2$ and 20 units of myokinase at 37°C for 15 min at pH 7.6. 20 μl were injected into the liquid chromatograph. D, The mixture from C above was then adjusted to pH 6.5 with HCl, and incubated with deaminase (0.03 units) for 20 min. 20 μl were injected into the liquid chromatograph. Negligible volume changes occur during the overall procedure, as shown by the constant peak height of inosine in the chromatograms (t_R = 9.6 min).

Only 5–10 mg (wet weight) of myocard are needed in order to determine the energy charge of the myocard by this method. This means that biopsies obtained with a Tru-cut Travenol needle (1.2 mm I.D.) provide ample myocard for analysis. This is of practical clinical significance in the current search for optimal myocardial protection during heart surgery.

The liquid chromatographic procedure compares favourably in sensitivity and convenience with alternative methods for the quantitation of adenine nucleotides, *e.g.*, isotachophoresis[20,21] and the luciferase assay[22]. Isotachophoresis is more time-consuming, and the luciferase assay may be sensitive to other constituents in the assay mixture[23,24].

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