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Note

# Analysis of adenosine, inosine and hypoxanthine in suspensions of cardiac myocytes by high-performance liquid chromatography

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Adenosine has been implicated as a physiological regulator in many biological systems, as amply reviewed by Arch and Newsholme [1]. In the heart, adenosine is a potent coronary vasodilator [2] and much evidence suggests that it is a mediator substance in the autoregulation of coronary blood flow [3—6]. In order to study adenosine release by heart cells, we sought a method of quantification for adenosine and its catabolites, inosine and hypoxanthine. It was necessary to obtain a method which would allow us to quantify the desired nucleosides in heart tissue where adenine nucleotide levels far exceed nucleoside levels. Although there are reported methods for the analysis of one or more nucleosides [6—10], each had limitations or presented special problems. The purpose of this note is to describe our high-performance liquid chromatographic (HPLC) method for the quantification of adenosine, inosine and hypoxanthine in nucleotide-rich tissue extracts.

## EXPERIMENTAL

### *High-performance liquid chromatography*

Our system consists of a Milton Roy mini-pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) 7120 sample injection valve and a Perkin-Elmer (Norwalk, CT, U.S.A.) LC-55 variable-wavelength detector set at 260 nm. Flow-rate under all conditions was constant at 1.12 ml/min. The analysis scheme used has two stages. In the first stage (clean-up stage), nucleosides and bases were isolated as a group completely free of nucleotides using a Whatman (Clifton, NJ, U.S.A.) Partisil SAX anion-exchange column (25 cm × 4.6 mm; 10-$\mu$m particle size). In the second stage, the separation of nucleosides and bases from one another was

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accomplished using a Whatman Partisil ODS-1 reversed-phase $C_{18}$ column (25 cm × 4.6 mm; 10-$\mu$m particle size).

In the clean-up stage (Fig. 1) the group separation of nucleosides and bases, with complete removal of contaminating nucleotides, was accomplished by injecting 500 $\mu$l of tissue extract onto the Partisil SAX anion-exchange column equilibrated with 0.005 *M* ammonium phosphate buffer, pH 2.8. The peak containing all of the nucleosides and bases (hereafter referred to as the nucleoside peak) was eluted isocratically and collected in a measured time interval, 55 sec (volume, 1.03 ml), while the bulk of the nucleotides remained bound to the column. Four individual 500-$\mu$l samples of tissue extract could be processed in this manner before it was necessary to purge the column of accumulated nucleotides. Purging was accomplished with a step gradient of ammonium phosphate buffers as follows: 0.20 *M*, pH 3.7, 2 min; 0.75 *M*, pH 3.7, 15 min; 0.20 *M*, pH 3.7, 2 min; and re-equilibration with 0.005 *M*, pH 2.8, 15 min.

Samples of collected nucleoside peak material were stored at —20°C until analyzed by reversed-phase chromatography. Nucleoside and base analysis was carried out with isocratic elution, using the Whatman ODS-1 reversed-phase column equilibrated with a solution of 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v). Sample injection volume on the ODS-1 column was usually 200 $\mu$l.

Concentrations of adenosine, inosine and hypoxanthine in tissue samples were calculated by comparing peak areas from chromatograms of tissue extract with peak areas from chromatograms of standard solutions of authentic compounds (Fig. 2). Peak area was determined as peak height × peak width at half peak height. Standard solutions of authentic adenosine, inosine and hypoxanthine were prepared to have a concentration of approximately $3 \cdot 10^{-5}$ *M*. The exact concentrations of these compounds were then determined spectrophotometrically using millimolar extinction coefficients, $ml \cdot \mu mole^{-1} \cdot cm^{-1}$, of 15.4 at 259 nm [11], 12.2 at 248.5 nm [12], and 10.6 at 250 nm [12] for adenosine, inosine and hypoxanthine, respectively. Standards, prepared in a buffer of 0.05 *M* 3-(N-morpholino)propanesulfonic acid with 0.13 *M* sodium chloride, pH 5.2, were subjected to exactly the same chromatographic scheme as the tissue extracts.

Proof of the identity of the chromatographic peaks of interest (inosine, hypoxanthine and adenosine) was obtained by three independent means: (1) retention time as compared with pure known substance, (2) comparison of UV spectra (220—300 nm) of peak material in question with that of a known substance and (3) enzymic peak shift [13], using adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase.

*Preparation of tissue*

Protein-free tissue extracts were prepared from cell suspensions obtained from adult rat hearts dissociated with crude collagenase [14]. Ice-cold trichloroacetic acid (TCA), 1 g/ml, was added to the cell suspensions to give a concentration of 10% TCA and the sample was homogenized immediately. After centrifugation to remove solids, the TCA was removed with five extractions of equal volumes of cold, water-saturated diethyl ether. The resulting

aqueous extract was bubbled with water-saturated air for 5 min to remove the dissolved ether. Finally, the pH was adjusted to 4.5—5.0 with potassium hydroxide, and the sample was frozen at —20°C until HPLC analysis.

The efficiency of recovery of nucleosides from tissue during tissue extract preparation was determined by comparing the amount of added nucleoside that can be recovered from a TCA tissue extract versus a non-extracted standard solution. Recovery was 111.3 ± 0.6% and 105.7 ± 4.0% (mean ± S.D.) for adenosine and inosine, respectively.

*Reagents and chemicals*

Ammonium hydroxide and phosphoric acid were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Ammonium dihydrogen phosphate was from Baker (Phillipsburg, NJ, U.S.A.) and methanol (acetone-free) was obtained from Fisher (Fair Lawn, NJ, U.S.A.). Nucleosides, related bases and enzymes were obtained from Sigma (St. Louis, MO, U.S.A.). Collagenase was obtained from Worthington (Freehold, NJ, U.S.A.).

## RESULTS

A chromatogram representative of the first stage (clean-up stage) of analysis is shown in Fig. 1. The compounds eluting in the broad peak following each nucleoside peak are nucleotides which were not specifically identified. The bulk of the nucleotides in each sample remain bound to the column. As shown in Fig. 1, for each sample injected, the nucleoside peak (numbered peaks) was reasonably narrow allowing this material to be collected in a rela-

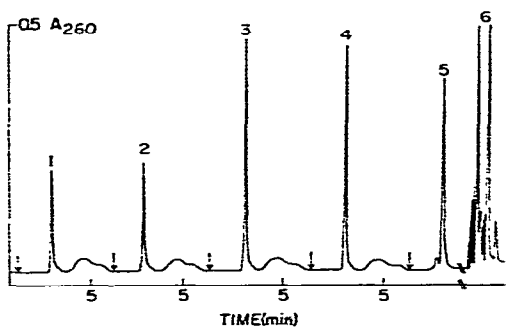


Fig. 1. The first stage of nucleoside analysis: removal of nucleotides. Representative chromatogram of multiple injections (indicated by arrows) of cardiac myocyte extracts on anion-exchange column chromatography. Peaks 1, 2, 3, and 4, containing the nucleosides and bases (free of nucleotides) were each collected in a volume of 1.03 ml (collection time, 55 sec) and saved for reversed-phase chromatography (Fig. 2). Upon the fifth sample injection, the usual result was a split peak (labeled 5) which, if collected would be undesirably diluted. Purging the column of accumulated nucleotides (peak 6, shown on a compressed time scale) usually after the fourth sample injection, allowed a new cycle of four sample injections to be made. Sample injection volume, 500 $\mu$l; column, Whatman SAX at ambient (ca. 22°C) temperature; detector at 260 nm; flow-rate, 1.12 ml/min. Isocratic elution, 0.005 *M* ammonium phosphate (pH 2.8); nucleotide purging (6) accomplished by changing eluent from 0.005 *M* to 0.750 *M* ammonium phosphate (pH 3.7) for 15 min.

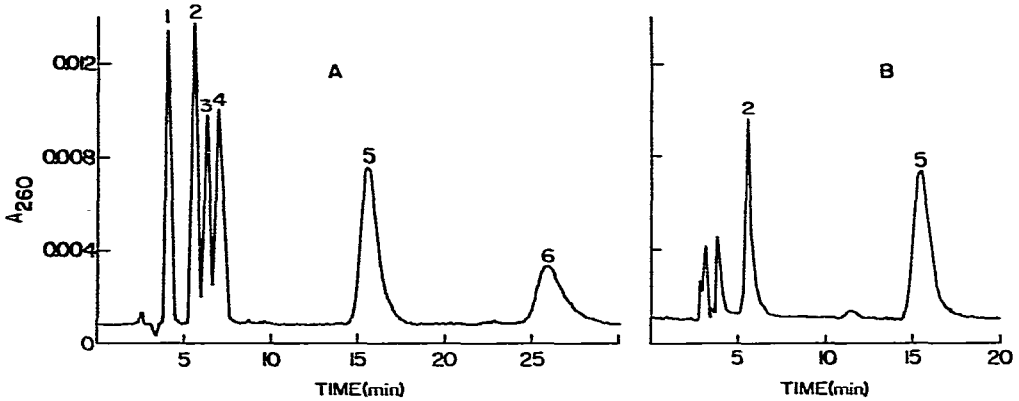


Fig. 2. (A) Chromatogram of authentic compounds, uridine (1), inosine (2), hypoxanthine (3), thymidine (4), adenosine (5) and adenine (6); quantities injected were 0.5, 1.0, 0.6, 0.7, 0.9, and 0.6 nmole, respectively. (B) Chromatogram of peak material collected from the anion-exchange column (Fig. 1), showing the presence of inosine (2) and adenosine (5), but an absence of hypoxanthine in cardiac myocyte suspensions. Sample injection volume, 200 μl; column, Whatman ODS-1 $C_{18}$ at ambient (ca. 22°C) temperature; detector at 260 nm; flow-rate, 1.12 ml/min. Isocratic elution, 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v). Retention times vary with individual columns.

tively small volume (1.03 ml). However, peak splitting usually occurred upon the fifth in a series of injections (Fig. 1, peak 5). Thus, we usually inject and collect four samples sequentially and then purge before making further injections. Purging the column of bound nucleotides restores the unity of the nucleoside peak, allowing another series of four injections and peak collections to be made.

Each collected nucleoside peak from the first stage was subsequently analyzed by reversed-phase column chromatography. The results, shown in Fig. 2, were obtained using an elution scheme optimized for the separation of inosine, hypoxanthine and adenosine. Specifically, we found that isocratic elution with 0.010 *M* potassium phosphate (pH 5.5)—methanol (90:10, v/v) allowed inosine and hypoxanthine to be almost baseline resolved, while adenosine was completely separated from all compounds present. Varying the phosphate concentration from 0.005 *M* to 0.020 *M* and the pH from 5.5 to 6.0 did not alter the results. Retention time decreased with increasing methanol concentration. Better resolution was not obtained using 5% or 20% methanol buffer or a linear gradient elution to 30% methanol.

In the clean-up stage of analysis we routinely injected 500 μl of tissue extract onto the anion-exchange column and collected the nucleoside peak in 1.03 ml (Fig. 1). However, we determined that we could inject up to 1.0 ml of tissue extract onto this column and still collect the resulting nucleoside peak in a volume of 1.03 ml. Also routinely, we injected only 200 μl (of the 1.03 ml collected peak) onto the reversed-phase column to separate and quantify inosine, hypoxanthine and adenosine (Fig. 2). We found, however, that virtually all (1.00 ml out of 1.03 ml) of the collected nucleoside peak from the anion-exchange column could be injected onto the ODS-1 column with no ill effects on peak separation or linearity of peak area response. Thus,

if 1.0 ml of tissue extract is loaded onto the anion-exchange column, almost all of it (ca. 97%) can be collected and subsequently loaded onto the reversed-phase column for quantitation of inosine, hypoxanthine and adenosine. This capability allowed us to maintain the maximum sensitivity possible in the HPLC analysis of purines, when using a spectrophotometric detector ($\lambda$ = 260 nm), about 50 pmole [10].

The observed retention times (Fig. 2) for inosine, hypoxanthine and adenosine were 5.44 ± 0.04, 6.19 ± 0.04 and 15.56 ± 0.07 min, respectively (mean ± S.D., $n$ = 10). An ODS-1 column used in earlier work exhibited shorter retention times during its useful life (e.g. 4.73, 5.36 and 12.0 min for inosine, hypoxanthine and adenosine, respectively).

Comparison of Fig. 2A and B indicates that cardiac myocyte suspensions possess adenosine (1.14 ± 0.25 nmole per mg cell protein) and inosine (0.73 ± 0.32 nmole per mg cell protein), but usually no measurable hypoxanthine. Occasionally, a cardiac myocyte sample exhibited a small hypoxanthine peak (ca. 5% of the inosine peak). Fig. 2A shows that uridine, thymidine and adenine although not measurable in heart cell extracts, can be separated under the isocratic elution conditions utilized.

## DISCUSSION

Several analytical methods for one or more nucleosides have been reported [6—10] and each has its advantages and disadvantages. We sought a method which would allow us to quantify not only adenosine but also its catabolites, inosine and hypoxanthine, in suspensions of cardiac myocytes. Certain methods were excluded because they were applicable only to adenosine [8, 9]. Others required equipment which was not available to us (a dual-wavelength spectrophotometer) [6, 7] or extensive sample clean-up [7, 8]. We examined one potential HPLC method [10] in which the tissue extract was injected directly onto a $C_{18}$ reversed-phase column and the nucleosides and bases eluted with a methanol gradient. Two problems were encountered. Firstly, the method required gradient elution, an operation which we wished to avoid. Secondly, we found that with heart tissue, which is extremely rich in nucleotides, the inosine and hypoxanthine chromatographic peaks eluted high along the trailing edge of the large early-eluting nucleotide region. Eluting in this manner with the nucleotides, the inosine and hypoxanthine peaks varied erratically in size (area) upon repetitive injection of the same sample, making quantification impossible.

In the present study using an isocratic HPLC method, we show that inosine, hypoxanthine and adenosine are easily separated and quantified in heart cell suspensions where potentially interfering nucleotides are present in amounts considerably in excess of nucleosides. Because of the high nucleotide levels in myocardial tissue extracts, the initial anion-exchange HPLC clean-up (nucleotide removal) was necessary in order to allow quantitation of inosine and hypoxanthine using reversed-phase HPLC. Although sample clean-up can be accomplished with thin-layer chromatography or with small open columns [7, 8] utilization of anion-exchange HPLC for this process allowed us to monitor (Fig. 1) the isolation and collection of the nucleosides and bases in

a sample, with no risk of loss of peak material or unnecessary dilution thereof and with a minimum of sample manipulation. The sample injection—collection procedure is rapid and allows four samples to be processed before the accumulated nucleotides must be purged from the column. If greater dilution of the peak material can be tolerated, the peak-splitting (Fig. 1, peak 5) can be ignored and a greater number of samples can be processed before column purging is necessary.

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