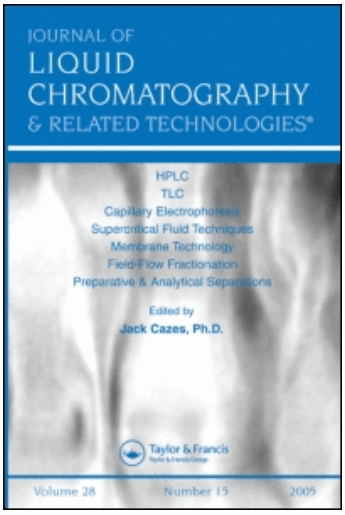

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MEASUREMENT OF FEMTOMOLAR CONCENTRATIONS OF ADENOSINE

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ABSTRACT

A method is described for the measurement of femtomole quantities of adenosine. Standards of adenosine or samples of biological fluids or tissues (after deproteinization) are subjected to standard, gradient, reversed phase HPLC. The adenosine fraction is collected, enzymatically converted to uric acid and the uric acid quantified by electrochemical detection. Peak height in millimeters or nanoamperes is linearly related to adenosine levels over a range of 25-2000 fmoles. As little as 5 fmoles of adenosine can be detected and 25 fmoles can be accurately measured. The adenosine concentration of Krebs-Henseleit solution in contact with the epicardial surface of the dog heart reaches apparent equilibrium with the interstitial fluid concentration of the myocardium within 2-4 min at a level of 0.15 μM (range 0.08-0.24 μM).

INTRODUCTION

There has been an increasing interest in the role of adenosine in several physiological phenomena such as local regulation of blood flow, chronotropic, dromotropic and inotropic effects on the heart, inhibition of neural activity, and lipolysis. Hence, an accurate and very sensitive method for the determination of adenosine concentrations in small amounts of fluid or tissue is necessary. Enzymatic conversion of adenosine and its degradative products, inosine and hypoxanthine to uric acid, and subsequent spectrophotometric

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determination of uric acid has been a useful but relatively insensitive method (1). With the advent of high performance liquid chromatography (HPLC), the measurement of adenosine became more sensitive, particularly with the fluorescence assay (2, 3). For determination of picogram samples of uric acid, electrochemical detection (ECD) has been successfully used (4). Combination of enzymatic degradation of adenosine, HPLC, and ECD now provides an even higher degree of sensitivity for the quantification of adenosine than has previously been achieved even with radioimmunoassay (5), and constitutes the basis of this report.

MATERIALS AND METHODS

Instrumentation

The mobile phase for ECD was delivered via a Beckman Model 112 Solvent Delivery Pump to which was attached a flow-through pulse damper. A Rheodyne 7125 Sample Injector with a 20 µl loop was used to introduce the samples onto a Biophase 5 µm ODS 4.6 x 250 mm reversed phase column (Bioanalytical Systems (BAS), West Lafayette, Indiana) which was preceded by a Biophase 10 µm ODS guard column. Oxidation of the samples took place using a single electrode glassy carbon cell (BAS) with a Ag/AgCl reference electrode enclosed in a Faraday cage. The oxidation potential was applied to the working electrode via a Model LC-4B amperometric detector (BAS) which also measured the resultant oxidative current, converted it to a voltage and delivered it to a single pen 10 inch strip chart recorder.

All biological samples were first fractionated by an HPLC gradient method before introduction into the ECD system, using two LKB 2150 pumps, an LKB 2152 controller, a Waters 710B automatic injector, an Altex 5 µm ODS ultrasphere 4.6 x 250 mm column, and a Kratos 773 variable wavelength detector.

Operating Parameters

The ECD mobile phase consisting of 0.05 M monochloroacetic acid, 0.07 mM di-sodium ethylenediamine-tetraacetic acid, and 0.11 M sodium octyl sulfate, adjusted to pH 3.0 with 10 N NaOH was filtered (0.2 µm Millipore GS filter) and degassed before use. The column and glassy carbon cell were kept at room temperature (approximately 22°C) and protected from air currents by an enclosure, since the system is temperature sensitive. The flow-rate was set at 1 ml/min. The tube carrying the effluent from the carbon cell was kept at a constant height and not immersed in the collecting flask because the system is very sensitive to flow. A slight change in the hydrostatic pressure alters

back pressure and hence flow. Sample volumes of 20 µl were injected and exposed to an oxidation potential of +0.8 V. All samples were measured at a sensitivity range setting of either 0.5 nAmp (0-1250 fmoles uric acid) or 1.0 nAmp (1250-2000 fmoles uric acid) full scale. The output from the amperometric detector in the form of a voltage was recorded at a range of 1 V full scale on 10 inch strip chart paper at a speed of 0.5 cm/min. The samples were quantified by measuring the peak heights in either millimeters or nanoamperes.

Sample Preparations

Standards. Adenosine and uric acid standards were prepared by dissolving the compounds in a 50 mM phosphate buffer at pH 7.4 (KH_2PO_4 - 9.5 mM/ Na_2HPO_4 - 40.5 mM) and then serially diluted to the desired concentration. Aliquots (1.0 ml) of the adenosine standards were enzymatically degraded to uric acid by incubation at 37°C for 2 hrs with a mixture of 1 µl each of adenosine deaminase and nucleoside phosphorylase and 2 µl of xanthine oxidase (all undiluted). The reaction was stopped by the addition of 20 µl of concentrated perchloric acid (final concentration = 0.2 M) and the precipitate removed by centrifugation for 10 min at 15,000 x g. The supernatant fractions with a final pH of 1.2 were stored overnight at 4°C, centrifuged for 5 min at 15,000 x g and the uric acid content determined.

Controls and uric acid standards were prepared as described above except that perchloric acid was added first to inactivate the enzymes. In addition, uric acid standards were analyzed in the absence of enzymes and perchloric acid to check for any possible variation in retention time and contaminants due to additives.

Biological Samples

After subjecting the biological samples to gradient HPLC (flow-rate 1.1 ml/min), the entire adenosine fraction (2.0 ml) was collected, evaporated to dryness, and dissolved in 0.5 ml phosphate buffer (50 mM, pH 7.4). The samples were then processed as described for adenosine standards except that the aliquot volumes were either 0.1 or 0.2 ml with the volume of enzymes and perchloric acid added in the same ratio.

Reagents

Adenosine, adenosine deaminase (EC 3.5.4.4), nucleoside phosphorylase (EC 2.4.2.1) xanthine oxidase (EC 1.2.3.2), and uricase (EC 1.7.3.3) were

obtained from Boehringer Mannheim (Indianapolis). Uric acid (Ultrex), monochloracetic acid, dibasic sodium phosphate, and monobasic potassium phosphate were all reagent grade purchased from J.T. Baker (Phillipsburg, N.J.). Octyl sodium sulfate (Eastman Kodak), EDTA - disodium salt (Sigma) and perchloric acid (MCB, Gibbstown, N.J.) were also used in solution preparation.

RESULTS AND DISCUSSION

Adenosine can be accurately measured by enzymatic degradation to uric acid and quantification of the uric acid by ECD. Adenosine is not oxidized at a potential of +0.8 V and hence gives no deflection on the ECD record. The relationship between the amount of adenosine in the sample and the height of the uric acid peak is linear over the range studied (25 to 2000 fmoles) as shown in figure 1. The presence of the enzymes produces a small peak (5 mm at 0.5 nAmp full scale) in the uric acid region of the record. Hence, this deflection must be determined by adding the enzymes after the addition of perchloric acid and subtracting this small peak from the uric acid peak. Without this correction the curve shown in figure 1 would be displaced slightly upward.

Verification of the uric acid was done by the addition of uricase to an aliquot of the mixture of adenosine and enzymes at the start of incubation, and as depicted in figure 2, the prominent uric acid peak vanishes. It is also evident that several deflections appear between zero time and approximately 7 min (Fig. 2). These deflections, which vary somewhat from sample to sample, are associated with the salts in the mobile phase and sample, but their identity has not been determined. However, they do not pose a problem in the measurement of adenosine since they occur prior to the appearance of the uric acid peak (9-9.5 min). Similar unidentified peaks are seen in figure 3 in which the sensitivity of the method is depicted.

The uric acid peak shown in figure 3 represents 25 fmoles of adenosine which is about the maximum accurate sensitivity we have been able to obtain with currently available equipment. One can detect as little adenosine as 5 fmoles but this cannot be precisely quantified.

One of the most important uses of the HPLC-ECD method is for the determination of the interstitial fluid content of adenosine in different tissues. Such measurements have been of particular interest in the heart since adenosine has been intensively studied as a mediator of coronary blood flow regulation.

To collect samples of cardiac interstitial fluid, a small chamber (exposed surface area 2 cm) is placed on the epicardial surface in a region of the left ventricle of the dog heart that is free of large blood vessels.

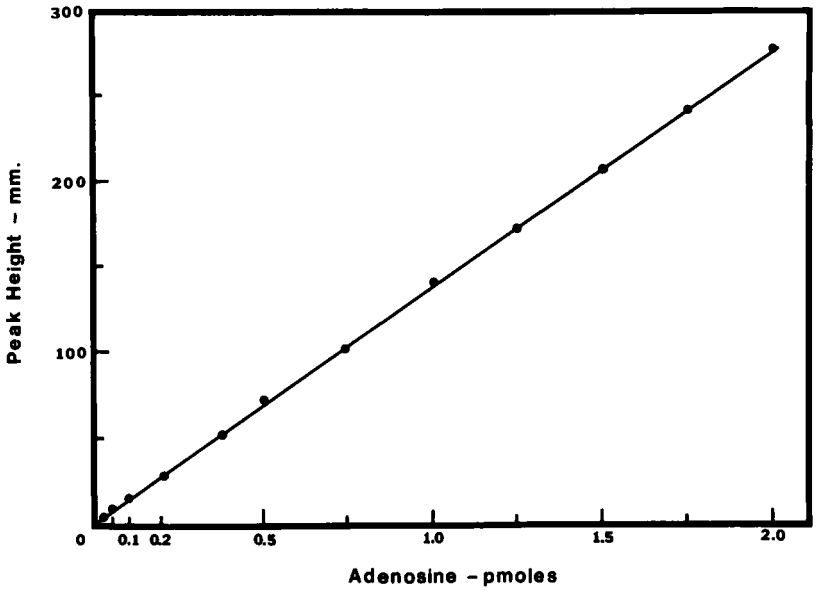

FIGURE 1. Relationship between adenosine added and peak height of uric acid determined by ECD after the adenosine standards were enzymatically converted to uric acid.

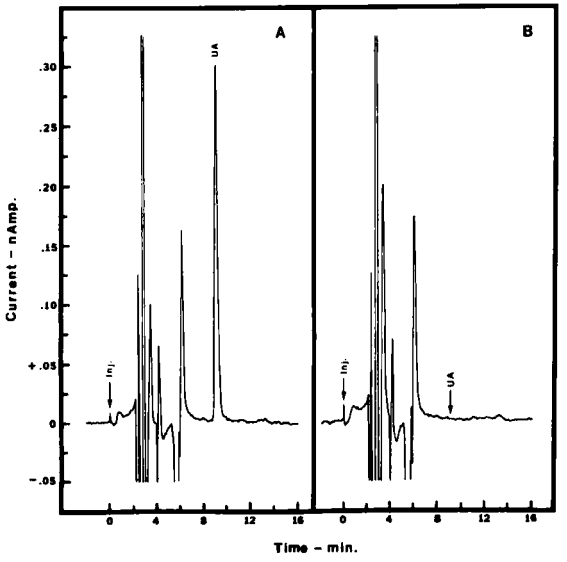

FIGURE 2. A. Uric acid peak by ECD (UA) = 1 pmole. B. Disappearance of the uric acid peak after incubation of the sample with uricase. Inj = injection artifact.

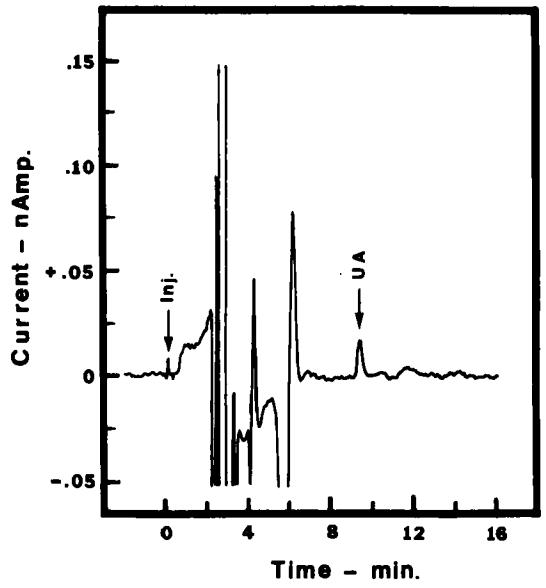

FIGURE 3. The uric acid peak (UA) represents 25 fmoles of adenosine. The detector was set at 0.5 nAmp full scale.

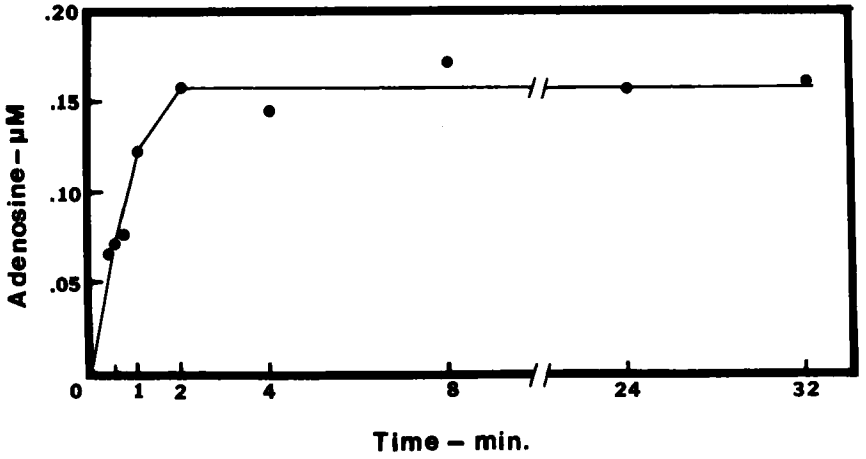

FIGURE 4. The change with time of the adenosine concentration in Krebs-Henseleit solution in a chamber placed on the surface of the left ventricle of the dog.

The chamber is held in place with rubber bands sutured to remote areas of the epicardium and a thin layer of silicone grease is used as a seal between the bottom of the chamber and the epicardial surface. To the chamber are added 200 µl aliquots of Krebs-Henseleit (K-H) solution which results in a layer of fluid 1 mm deep. The aliquots of K-H solution are removed after different time periods of contact with the epicardium. The chamber is washed once with 200 µl of K-H solution which is added to the sample and the mixture is placed in boiling water for 10 min to destroy any enzyme activity. The sample is evaporated to dryness, taken up in water to one-third of its original volume and the adenosine fraction separated by HPLC (2). This fraction is then analyzed in the manner described above.

The adenosine concentration in the cardiac chamber reached a plateau in 2-4 min and remained at an average level of 0.15 µM (range 0.08 - 0.24 µM) for as long as measured. A composite record of four experiments is shown in figure 4 for 30 sec to 32 min of contact with the epicardium. This concentration is in agreement with that reported for dog plasma (5).

This method can facilitate adenosine studies, since it will permit measurement of adenosine levels below basal levels (e.g. conditions in which cardiac metabolic activity is reduced) as well as concentrations in milligram samples of tissues such as the atrioventricular node of the heart, cardiac trabeculae and brain.

ACKNOWLEDGEMENTS

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REFERENCES

1. Dobson, J. G., Jr., Rubio, R., and Berne, R. M. Role of Adenine Nucleotides, Adenosine and Inorganic Phosphate in the Regulation of Skeletal Muscle Blood Flow, Circulation Res. 29, 375-384, 1971.

2. Hartwick, R. A., Assenza, S. P., and Brown, P. R. Identification and Quantitation of Nucleosides, Bases and Other UV-Absorbing Compounds in Serum, Using Reversed-Phase High Performance Liquid Chromatography, I. Chromatographic Methodology, J. Chromatog. 186, 647-658, 1979.

3. Preston, M. R. Determination of Adenine, Adenosine and Related Nucleotides at the Low Picomole Level by Reversed-Phase High-Performance Liquid Chromatography with Fluorescence Detection, J. Chromatog. 275, 178-182, 1983.

4. Pachla, L. A., and Kissinger, P. T. Estimation of Serum Uric Acid by High Performance Liquid Chromatography with Electrochemical Detection, Clin. Chim. Acta 59, 309-312, 1975.

5. Sato, T., Kuninaka, A., Yoshino, H., and Ui, M. A Sensitive Radioimmunoassay for Adenosine in Biological Samples, Analyt. Biochem. 121, 409-420, 1982.