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Quantitation of adenosine, inosine and hypoxanthine in biological samples by microbore-column isocratic high-performance liquid chromatography

R. H. GAYDEN

Division of Cardiology, Department of Internal Medicine, University of Texas, Health Science Center at Houston, Houston, TX 77030 (U.S.A.)

B. A. WATTS, III and R. E. BEACH

Division of Nephrology, Department of Internal Medicine, University of Texas, Medical Branch, Galveston, TX 77550 (U.S.A.)

and

C. R. BENEDICT*

Division of Cardiology, Department of Internal Medicine, University of Texas, Health Science Center at Houston, Houston, TX 77030 (U.S.A.)

ABSTRACT

This paper describes a simple and sensitive high-performance liquid chromatographic method for measuring adenosine, inosine and hypoxanthine in cell suspensions. The method involves direct injection of the filtered sample on a microbore C_{18} reversed-phase column with UV detection at 259 nm. The mobile phase consisted of 125 m*M* potassium dihydrogenphosphate, 1.0 m*M* tetrabutylammonium hydrogensulfate, 1.5% acetonitrile and 20 m*M* triethylamine, pH 6.5. The minimum detectable amounts (signal-to-noise ratio of 3:1) were 2.0 pmol of adenosine, 2.5 pmol inosine and 3.5 pmol of hypoxanthine. The limits of quantitation were 2.9 ± 0.2 pmol for adenosine, 4.2 ± 0.3 pmol for inosine and 4.9 ± 0.4 pmol for hypoxanthine. This method was used to quantitate adenosine release by dispersed rat renal outer medullary cells (tubules) under conditions of normoxia and hypoxia.

INTRODUCTION

Adenosine (Ado) is a potent extracellular messenger in a variety of systems. It causes vascular smooth muscle relaxation [1], regulates regional blood flow in brain [2], inhibits norepinephrine release from sympathetic nerve endings [3] and in the kidney alters renal hemodynamics and renin secretion [4–6]. The increase in our knowledge of the actions of Ado has been accompanied by the elucidation of the biochemical pathways for Ado metabolism. It is known that Ado can be formed intra- or extra-cellularly and that both adenosine monophosphate and S-adenosylhomocysteine can serve as the immediate precursors for Ado [7].

These findings have lead to attempts at delineating the specific sites of Ado production and its regulation in various organs under conditions of hypoxia or ischemia. Adenosine and its enzymatic breakdown products inosine (Ino) and hypoxan-

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thine (Hyp) have been previously measured predominantly by high-performance liquid chromatography (HPLC) with UV detection [8–13]. However, these methods are of limited usefulness for studying the metabolic pathways of Ado in isolated cells or cell suspensions as they lack reliability to quantitate Ado, Ino and Hyp at a concentration range of 3–5 pmol. An isocratic method is described for the determination of Ado, Ino and Hyp by the use of reversed-phase HPLC on a 2.1-mm I.D. C_{18} column with UV detection at 259 nm. Adenosine production by renal outer medullary cells under conditions of normoxia and hypoxia was measured by this method to demonstrate its sensitivity and precision.

METHODS

HPLC

The system consisted of Waters 6000A (Milford, MA, U.S.A.) pump with microflow modifications, a Waters U6K injector with 10-μl injection loop and a Kratos Spectroflow 783 UV detector (Kratos Analytical, Ramsey, NJ, U.S.A.) equipped with a 2.4-μl flow cell operated at 259 nm. A Brownlee RP-18 column (220 × 2.1 mm I.D.) attached to a Brownlee RP-18 (15 × 2.1 mm) guard precolumn was used for chromatographic separations (Santa Clara, CA, U.S.A.). To obtain high sensitivity and to minimize the peak broadening the connection tubings were kept as short as possible, the microbore manifold was installed on the detector to connect the column directly to the detector cell and a 2.4-μl analytical flow cell was used. Data were collected and peaks integrated using IBM PC/AT and Nelson Analytical 2600 Series chromatography software (Cupertino, CA, U.S.A.).

The chemicals used were of HPLC/analytical grade. Acetonitrile was obtained from Aldrich (Milwaukee, WI, U.S.A.). Potassium dihydrogenphosphate, potassium hydroxide, tetrabutylammonium hydrogensulfate (TBAHS) and triethylamine (TEA) were obtained from Fluka (Ronkonkoma, NY, U.S.A.). Ado, Ino and Hyp standards were obtained from Sigma (St. Louis, MO, U.S.A.). Collagenase, hyaluronidase and dipyridamole were also obtained from Sigma. *erythro*-9-(2-Hydroxy-3-nonyl)adenine (EHNA) was a gift from Wellcome Research Labs. (Research Triangle Park, NC, U.S.A.). Milli-Q Water (Millipore, Bedford, MA, U.S.A.) was used for preparation of the mobile phase. Kidneys were obtained from male Spraque-Dawley rats (Taconic Farms, Germatown, NY, U.S.A.), 250–350 g, that received standard rat chow (Purina, St. Louis, MO, U.S.A.) and tap water *ad libitum*.

Mobile phases contained 125 m*M* potassium dihydrogenphosphate, 1.5% (v/v) acetonitrile, 20 m*M* triethylamine and 1.0 m*M* TBAHS (pH 6.5) and adjusted to pH 6.5 with 8 *M* potassium hydroxide. This solution was filtered using a 0.45-μm nylon filter (Ranin Instruments, Woburn, MA, U.S.A.) and degassed under vacuum with sonication for 10 min before use. Chromatography was carried out at room temperature using a mobile phase flow-rate of 0.5 ml/min and sample injections of 10 μl. Generally, equilibration of the column with stable chromatographic conditions was assumed when a steady baseline was observed at 0.002 a.u.f.s. with a rise time of 0.1 s for at least 60 min. Standard curves for Ado, Ino and Hyp prepared in the incubation media were constructed for each assay. The standards encompassed a range from 3 to 50 pmol. At the end of each day the column was run overnight with methanol to elute any retained materials.

Suspensions of outer medulla

Suspensions of outer medulla (tubules) (OM) were prepared by the method of Chamberlin *et al.* [14], with minor modifications. Animals were anesthetized by intraperitoneal pentobarbital, 50 mg/kg. The kidneys were perfused retrograde through the aorta with a hypertonic mannitol solution (solution A: NaCl, 115 m*M*; $NaHCO_3$, 25 m*M*; NaH_2PO_4, 0.2 m*M*; KCl, 8.0 m*M*; $CaCl_2$, 0.25 m*M*; $MgSO_4$, 1.0 m*M*; sodium lactate, 5.0 m*M*; L-alanine, 1.0 m*M*; glucose, 5.0 m*M*; dextran, 0.3%; and mannitol, 25 m*M*) at 4°C until the kidneys were were completely blanched of blood and brisk urine flow was established. The kidneys removed, longitudinally bisected and the inner strip of the outer medulla dissected free and minced. The minced tissue was digested at 37°C for three 10-min periods in solution A that contained 110 U/ml collagenase Type IV and 297 U/ml hyaluronidase. The digested solution was stirred continuously and bubbled with oxygen–carbon dioxide (95:5). Following each of the first two digestion periods, the digested material was filtered through a 105-μm polypropylene filter (Spectrum, Los Angeles, CA, U.S.A.) and the undigested tissue was resuspended in fresh enzyme solution. At the end of the third digestion period, the suspension was aspirated through an 18-gauge needle to separate completely any remaining tissue aggregates. This tissue was washed with incubation fluid (NaCl, 115 m*M*; $NaHCO_3$, 25 m*M*; NaH_2PO_4, 2.0 m*M*; KCl, 5.0 m*M*; $CaCl_2$, 1.0 m*M*; $MgSO_4$, 1.0 m*M*; sodium lactate, 5.0 m*M*; glucose, 5.0 m*M*; dipyridamole, 0.02 m*M*; and EHNA, 0.01 m*M*) examined with a light microscope to insure a homogeneous tubule population, and kept at 4°C as the OM suspension. Alternatively, some suspensions were washed with the incubation fluid which did not contain the dipyridamole and EHNA in order to determine the need for the inhibitors of adenosine metabolism. An aliquot of the suspension was assayed for protein concentration by the Lowry method [15] for standardization of Ado release. The protein concentration in these suspensions were between 0.6 mg to 3 mg/ml.

Incubation protocol

Incubation fluid 0.8-ml aliquots, containing dipyridamole and EHNA was preequilibrated for at least 20 min with 0% or 8% oxygen and 5% carbon dioxide (balance nitrogen). Following this equilibration period, 0.1 ml of OM suspension (0.06 to 0.3 mg of protein) was added to each aliquot and incubated for 0.5 or 8 min while being bubbled with either 0% or 8% oxygen gas mixtures. The incubation fluid was rapidly filtered through 0.45-μm HA syringe filters (Millipore) to separate the supernatant from the medullary tissue. In addition, to determine whether Ado release occurred prior to incubation, an aliquot of the suspension (0.1 ml) was added directly into a syringe containing 0.8 ml incubation fluid and immediately filtered. No Ado could be detected in these samples ($n=8$). The experimental samples were stored at −70°C for less than two weeks until quantitated by HPLC. The amount of Ado released was expressed in terms of the amount of protein present in the total sample (suspension) (pmoles Ado/μg protein).

Oxygen tensions in the suspensions were measured by rapidly aspirating the incubation fluid into sealed glass syringes and analyzing on a Corning blood gas analyzer Model 165/2 (Medfield, MA, U.S.A.) calibrated using 0 and 20% oxygen standards. Oxygen tension measured in fluid equilibrated with nominally 0% oxygen was 16 mmHg (2.2%) and at 8% oxygen was 61 mmHg (8.2%). The small differences

between measured and nominal values presumably reflect gain or loss of oxygen from the air due to the open system. Nominal values are used in the text for simplicity.

Statistical analysis

All the result are expressed as mean ± 1 S.D. Adenosine concentrations from OM suspensions were analyzed by ANOVA for the effect of time and oxygen concentrations, and the differences between means were compared by Newmann–Keuls multiple comparison procedure [16]. Differences between means were considered significant at the $p < 0.05$ level.

RESULTS

When an Ado, Ino and Hyp standard mixture prepared in the mobile phase was injected, Ado eluted at 10.2 min with a capacity factor, $k' = 8.8$, Ino eluted at 3.8 min with a $k' = 2.6$ and Hyp eluted at 1.9 min with a $k' = 0.8$ (Fig. 1A). Similar results were obtained by injecting standards prepared using incubation fluid or cell suspen-

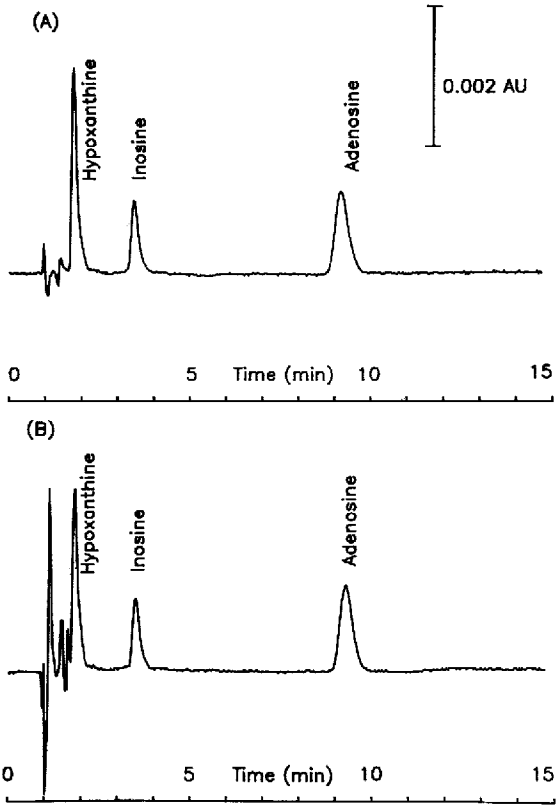

Fig. 1. Chromatogram showing the retention time for Ado (22.45 pmols), Ino (22.37 pmol) and Hyp (44.08 pmol). Mobile phase: 125 m*M* potassium dihydrogen phosphate, 1.5% (v/v) acetonitrile, 20 m*M* triethylamine and 1.0 m*M* TBAHS (pH 6.5). Flow-rate: 0.5 ml/min. (A) Standard prepared in mobile phase. (B) Standard prepared in incubation fluid or cell suspension media (Solution A).

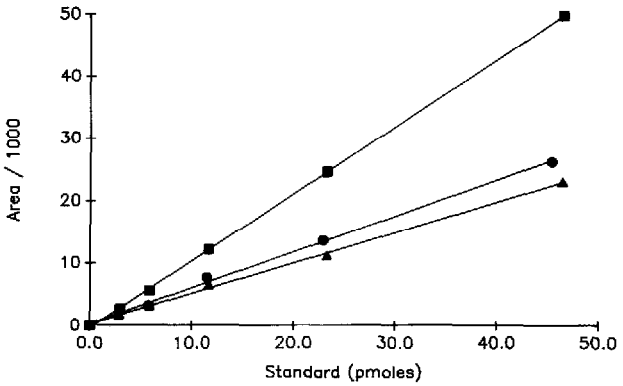

Fig. 2. Standard curve for Ado (■), Ino (▲) and Hyp (●) from 3 to 50 pmol ($r = 0.99$, $p < 0.001$ for all three compounds) ($n = 4$ with C.V. < 3% for all three compounds).

sion media (solution A) (Fig. 1B). The standard curve was linear throughout the range tested ($r = 0.99$; $p < 0.001$) (Fig. 2). The minimum detectable amounts (at a signal-to-noise ratio of 3:1) were 2.0 pmol of Ado, 2.5 pmol Ino and 3.5 pmol Hyp. The limits of quantitation were 2.9 ± 0.2 pmol for Ado, 4.2 ± 0.3 pmol for Ino and 4.9 ± 0.4 pmol Hyp. The interassay coefficient of variation (C.V.), was determined by injecting 5.0 ng of Ado (22.45 pmol), Ino (22.37 pmol) and Hyp (44.08 pmol) added to four different preparations of incubation fluid on four different days. The C.V. of the integrated areas was less then 4% for all three compounds. The purity of the detected Ado was determined by two methods: (1) The peak areas were increased in direct proportion to exogenously added Ado, Ino and Hyp; (2) absorptions at 259 and 280 nm were compared for all three compounds. The mean (± S.D.) $area_{259}/area_{280}$ ratios for all three compounds were similar between the sample peaks (6.30 ± 0.15 for Ado, 7.76 ± 0.18 for Ino and 22.5 ± 0.85 for Hyp) and the injected authentic standards (6.38 ± 0.12 for Ado, 7.70 ± 0.19 for Ino and 23.1 ± 0.73 for Hyp). These results indicate that there was negligible interference by impurities or components in the incubation media.

Chromatographic parameters were examined to improve peak separation, especially Hyp without significantly increasing the analysis time for each sample. Fig. 3

TABLE I

THE EFFECT OF GRADED OXYGEN TENSION ON ADENOSINE RELEASE IN SUSPENSIONS OF RAT OUTER RENAL MEDULLA

Nominal oxygen concentration (%)	Adenosine relaese (pmol of adenosine/μg protein)	
	Time of incubation (min)	
	0.5	8
8	0.78 ± 0.30 ($n = 7$)	2.69 ± 0.34 ($n = 13$)
0	1.46 ± 0.37 ($n = 5$)	5.24 ± 0.78[a] ($n = 8$)

[a] $p < 0.05$ compared to 8 and 0% oxygen, ANOVA with Dunnett's multiple comparisons test [16].

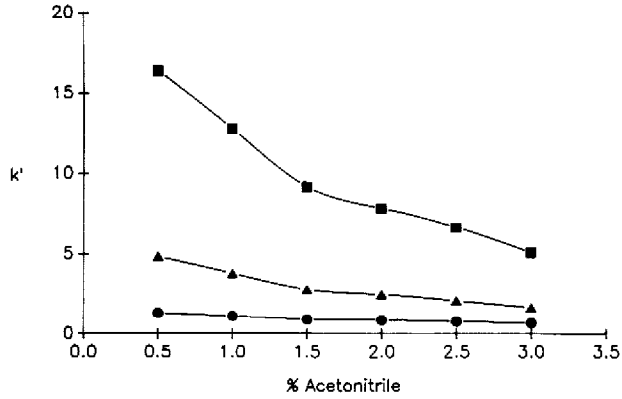

Fig. 3. Change in capacity factors for Ado (■), Ino (▲) and Hyp (●) with variations in acetonitrile concentration. Other components: 125 m*M* potassium dihydrogenphosphate, 1.0 m*M* TBAHS, 20 m*M* triethylamine (pH 6.5). Flow-rate: 0.5 ml/min.

shows the effect of change in acetonitrile percentage on k' of Ado, Ino and Hyp. Fig. 4 shows the effect on k' of Ado, Ino and Hyp with different concentrations of TBAHS.

Adenosine release was measured in OM suspensions incubated with 0 (hypoxic) or 8% (normal medullary) oxygen concentrations for 0.5 or 8 min (Table I). Ado concentrations after 8 min were significantly greater in incubation fluid of suspensions maintained with 0% oxygen than in fluid from suspensions 8% oxygen. In samples were EHNA and dipyridamole were not added, the Ado concentration was significantly reduced from 5.24 ± 0.78 pmol/μg of protein to 2.69 ± 0.83 pmol/μg of protein ($p < 0.05$) and Hyp (1.75 ± 0.40 pmol/μg of protein) and Ino (0.42 ± 0.01 pmol/μg of protein) were detected.

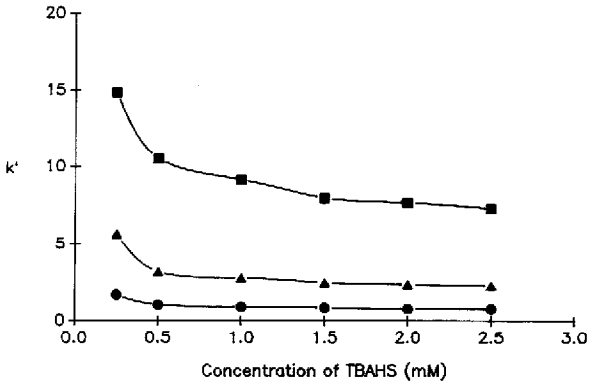

Fig. 4. Change in capacity factors for Ado (■), Ino (▲) and Hyp (●) with variations in TBAHS concentration. Other components: 125 m*M* potassium dihydrogenphosphate, 1.5% (v/v) acetonitrile, 20 m*M* triethylamine (pH 6.5). Flow-rate: 0.5 ml/min.

DISCUSSION

It is well known that application of gradient elutions to routine quantitative analysis can impair reproducibility of peak retention times since changes in the column equilibria between analyses easily results in peak shifting [17,18]. Therefore for the simultaneous measurement of Ado, Ino and Hyp, a highly sensitive isocratic, reversed-phase method was developed by optimization of the concentrations of ion-pairing reagent and percentages of acetonitrile used.

A low concentration of the ion-pairing reagent TBAHS was used to enhance peak separation and to eliminate peak asymmetry seen with mobile phases prepared with acetonitrile alone. Tetrabutylammonium ions (TBA) bind to the stationary phase of the C_{18} column due to hydrophobic interactions between the aliphatic groups of the packing material with the butyl groups of TBA. Consequently, positively charged groups arise on the stationary phase which should greatly modulate the retention of nucleotides (due to strong interactions with phosphate groups) and only slightly modulate the retention nucleosides (due to weaker interactions with oxygens or nitrogens with lone pair electrons) [19]. The use of ion-pairing reagent effectively reduced the strong hydrophobic interactions of the C_{18} stationary phase with the nucleosides. This improved peak symmetry and allowed better peak separations (elimination of tailing). In agreement with this, we found that increasing the concentration of TBAHS produced a decrease in the k' for Ado and Ino, and a smaller decrease in the k' for Hyp.

As the concentration of acetonitrile was increased, the k' values for Ado, Ino and Hyp decreased. Predictably, as the hydrophobicity of the solvent increased the nucleosides eluted earlier. No significant effect on retention times was seen at different pH value encompassing a range of 5.5 to 7.5, and the selected pH of 6.5 did not alter the retention characteristics of the column even after several months of use ($>$ 1000 sample injections).

It is known that underivatized surface silanols may react with solutes. Basic compounds often suffer from severe peak asymmetry and therefore poor column efficiency due to strong interactions of the nitrogen lone pair electrons with free silanol groups [19,20]. The effect of these silanol groups was modified with triethylamine (TEA) which was added to mobile phases as a silanol blocking or masking agent. When TEA was not added to the mobile phase, tailing of the peaks were observed suggesting that the column was not fully masked [19]. Tailing was not observed when 20 m*M* TEA was used.

Using this method, we examined the effects of hypoxia on Ado production by the outer medulla by measuring the Ado release during incubation of the OM suspension at two different *in vitro* oxygen contents. The oxygen concentrations used in the present studies are physiologically relevant because *in vivo* oxygen tensions previously determined in the renal medulla under control conditions have ranged from 30–60 mmHg [21]. This range is comparable to the oxygen tensions (61 mmHg) achieved in the present study in suspensions equilibrated with 8% oxygen. As shown in Table I, reduction to 16 mmHg (0% oxygen), a value comparable to that reported during hypoxia [19] was associated with an increase in Ado release.

In summary, a highly sensitive, isocratic HPLC method is described for the concurrent determination of Ado, Ino and Hyp in a suspension of OM cells. Using

this method Ado release by the cells in the renal outer medulla was demonstrated. When dipyridamole and EHNA was omitted in the incubation media, a major portion of the Ado released was metabolized to Ino and Hyp. The Ado release is increased by *in vitro* hypoxic conditions that are similar to those that are expected *in vivo* in the renal medulla during ischemic injury. This increased Ado release by hypoxia may have physiologic actions that may protect the medullary nephrons from ischemic injury by increasing renal medullary blood flow due to its vasodilatory effects.

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