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Note

Measurement of adenosine, inosine, and hypoxanthine in human plasma

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Adenosine has attracted much attention in recent years because of its possible role in vasodilation, neurotransmission, immune response and cyclic AMP formation [1, 2]. In addition, inosine and hypoxanthine were identified as endogenous ligands for benzodiazepine receptors [3] and were also shown to serve as a sensitive marker for tissue ischaemia [4, 5]. Several methods have been reported for the quantitation of adenosine [6—13], inosine [6—8, 13], and hypoxanthine [6, 8, 14, 15], using spectrophotometric [6, 7, 13, 15, 16], fluorometric [9, 10, 14], isotope dilution [9], and radioimmunological [11, 12] techniques. The reported plasma levels of the various purines, however, are highly variable. Part of this variability may be due to the fact that the metabolism of adenosine in blood is very rapid [13, 17]. Adenosine is avidly taken up by erythrocytes and is also degraded by plasma adenosine deaminase [18]. Thus, besides adequate methods for identification of each purine, reproducible measurements of plasma adenosine, inosine, and hypoxanthine require a reliable technique for blood collection.

This communication describes the quantitation of adenosine, inosine, and hypoxanthine in human plasma by high-performance liquid chromatography (HPLC) using the enzymatic peak shift method for verification. Furthermore, an effective technique for blood sampling is reported which almost completely prevents the further metabolism of adenosine and its degradative products during and after the sampling process.

EXPERIMENTAL

Chemicals and materials

Nucleosides, bases, adenosine deaminase (EC 3.5.4.4), and nucleoside phosphorylase (EC 2.4.2.1.) were obtained from Boehringer (Mannheim,

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F.R.G.). Dipyridamole (10 mg per 2 ml water) was purchased from Thomae (Biberach a.d. Riss, F.R.G.). Methanol and all other chemicals (analytical grade) were from Merck (Darmstadt, F.R.G.). Water was generated by a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) Econo-Columns (100 mm × 3 mm I.D.) and anion-exchange resin AG 1-X2 (acetate) were from Bio-Rad (Richmond, CA, U.S.A.). Reversed-phase Nucleosil C_{18} (particle size 7.5 μm) was from Macherey-Nagel (Düren, F.R.G.). Plastic syringes (2 and 5 ml) were from Becton and Dickinson (Rutherford, NJ, U.S.A.).

Apparatus

Chromatographic analysis was carried out with a high-performance liquid chromatograph Model 5000 (Varian Assoc., Palo Alto, CA, U.S.A.) using home-made columns (300 mm × 4 mm I.D.) packed with reversed-phase Nucleosil C_{18}. Column temperature was maintained at 29°C using a thermostat (Haake Model FE). Absorbance changes were measured at 254 nm and recorded with a Model 56 two-pen recorder (Perkin Elmer, Norwalk, CT, U.S.A.).

Sample preparation

Blood was drawn using a specially developed syringe system (Fig. 1). A 5-ml "collecting" syringe was mechanically coupled with the 2-ml syringe containing 2 ml of an ice-cold "stopping" solution (mmol/l: dipyridamole, 0.2; NaCl, 154; KCl 5.6; $NaHCO_3$, 5.9; glucose, 5.6; $CaCl_2$, 2.15; Na_2HPO_4, 0.82; NaH_2PO_4, 0.17). Blood was drawn by pulling the plunger of the collecting syringe which caused immediate mixing of the aspirated blood with the stopping solution. Due to the dimensions of the syringes a constant mixing ratio of 1:1 was achieved. Following collection, blood was centrifuged at 8000 *g* for 2 min (Eppendorf table centrifuge). A 2-ml aliquot of the supernatant was removed, deproteinised with 100 μl of 60% perchloric acid and centrifuged (8000 *g*, 2 min) to remove the protein precipitate.

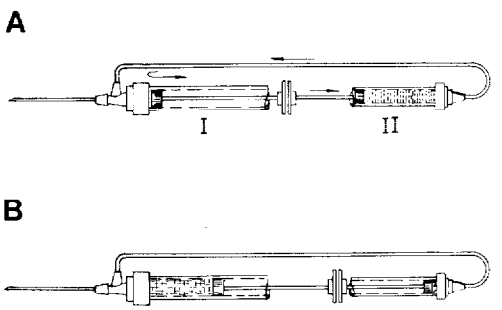

Fig. 1. Syringe system used for collecting blood. Syringe I (collecting syringe) was mechanically coupled to syringe II containing ice-cold 2 m*M* dipyridamole in Locke solution (stopping solution). Pulling the plunger of the collecting syringe causes the immediate mixing of blood with stopping solution at a constant ratio. (A) Before collection of blood; (B) after collection of blood.

Chromatography

Neutralisation of each sample was carried out by applying 1.5 ml of the acid extract on to columns (0.5 × 10 cm) filled with an AG 1-X2 anion-exchange

resin (acetate) equilibrated with water. Columns were washed with 9 ml of 5 m*M* acetic acid and the total column eluate was lyophilised. Residue was taken up in 250 μl of water of which 200 μl were injected onto the C_{18} column, equilibrated with water (flow-rate 1 ml/min). Following sample application a linear ascending gradient was started reaching 23% methanol after 25 min. Fractions containing adenosine, inosine, and hypoxanthine (fractions I, II, and III in Fig. 2A) were individually collected and evaporated to dryness. Compounds were identified by their retention time and coelution with standards. Enzymatic shifts were performed in each case according to the following principles:

$$\text{Adenosine} \xrightarrow{\text{adenosine deaminase}} \text{Inosine} + NH_3$$

$$\text{Inosine} \xrightarrow[(P_i)]{\text{nucleoside phosphorylase}} \text{Hypoxanthine} + \text{ribose-1-phosphate}$$

$$\text{Hypoxanthine} \xrightarrow[(\text{ribose-1-phosphate})]{\text{nucleoside phosphorylase}} \text{Inosine} + P_i$$

The evaporated column eluates were taken up either in 400 μl of water (adenosine samples), 400 μl of 2.5 m*M* phosphate buffer, pH 7.4 (inosine samples), or 400 μl of 10^{-3} *M* ribose-1-phosphate solution (hypoxanthine samples). Peak shift was initiated by the addition of 5 μl of adenosine deaminase (3.3×10^5 U/l) or 5 μl of nucleoside phosphorylase (2×10^4 U/l). All fractions were incubated for 30 min at 37°C, subsequently treated for 2 min at 100°C and then lyophilised. For rechromatography, each sample was taken up in 250 μl of water of which 200 μl were applied onto a C_{18} reversed-phase column as described above (Fig. 2B—D). In order to destroy completely enzymatic activity transferred to the C_{18} material, the columns were occasionally washed with 600 μl of 0.1 *M* hydrochloric acid.

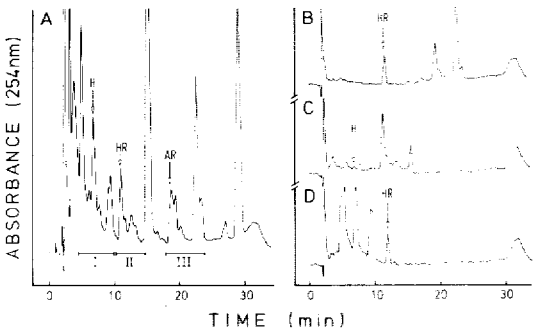

Fig. 2. Representative chromatograms of human plasma following sample preparation as described. (A) Original chromatogram showing retention of hypoxanthine (H), inosine (HR), and adenosine (AR). The different purine compounds were collected in fractions I, II, and III. In the case of adenosine, samples were treated with adenosine deaminase, in the case of inosine and hypoxanthine with nucleoside phosphorylase. (B) Rechromatography of adenosine (fraction III) yielding inosine (HR). (C) Rechromatography of inosine (fraction II) yielding hypoxanthine (H). ((D) Rechromatography of hypoxanthine (fraction I) yielding inosine (HR). For details see Experimental.

Calculation

Plasma levels of each purine were calculated according to the following formula: $c = \frac{m \times P_{sa} \times i}{P_{st} \times d}$

c = concentration of purine in plasma (μM)
m = amount of the injected standard (μmol)
P_{sa} = peak height of the sample (cm)
i = dilution factor (given the conditions described above: $i = 0.64$)
P_{st} = peak height of the standard (cm)
d = volume of plasma from which analysis was performed where

$d = \frac{1.43 \times (1 - h)}{(2 - h)}$ (h = haematocrit)

Values reported are not corrected for losses incurred during the different steps of the analysis.

RESULTS AND DISCUSSION

A new device was developed (Fig. 1) which permits the reliable sampling of blood for the measurement of adenosine and its degradative products inosine and hypoxanthine in plasma. It is based on the immediate mixing of blood with a solution containing dipyridamole (stopping solution) directly at the site of sample collection. Rapid inactivation of adenosine metabolism is absolutely necessary, since the half-life of adenosine in human blood is shorter than 10 sec and most of the nucleoside is removed within less than 30 sec (Fig. 3). Addition of dipyridamole almost completely prevented the disappearance of adenosine, and more than 90% of this nucleoside is still present in plasma after 5 min. Similar results were obtained with inosine; however, the loss of inosine from freshly drawn blood was much less dramatic.

The major route of adenosine removal in blood is by uptake into erythrocytes and incorporation into adenine nucleotides by the action of low K_m adenosine kinase [13, 17]. Furthermore, adenosine is removed by degradation to inosine and the enzyme responsible for this action, adenosine deaminase, is present in erythrocytes as well as in plasma [17]. In view of the rapid metabolism of adenosine, dipyridamole exhibits several features which make it particularly suitable for the sampling of human blood. It is well established that dipyridamole is a potent inhibitor of nucleoside transport in almost all tissues studied [17]. Dipyridamole was also shown to inhibit adenosine deaminase, however, only at higher concentrations [18]. Furthermore, this drug inhibits platelet aggregation [19] and thus the liberation of ADP during the release reaction. This latter action might be of particular importance, since degradation of ADP by cellular ectonucleotidases could give rise to the formation of adenosine during the sampling process. We have tested the possibility of adenosine being derived from extracellular adenine nucleotide breakdown by adding to the stopping solution 10^{-5} M α,β-methylene adenosine 5′-diphosphate (AOPCP), a potent inhibitor of ecto-5′-nucleotidase [20]. In two experiments we found no difference to the values given in Table I. Therefore, under our conditions, nucleotide breakdown in plasma during sampling does not contribute to the level of measured adenosine.

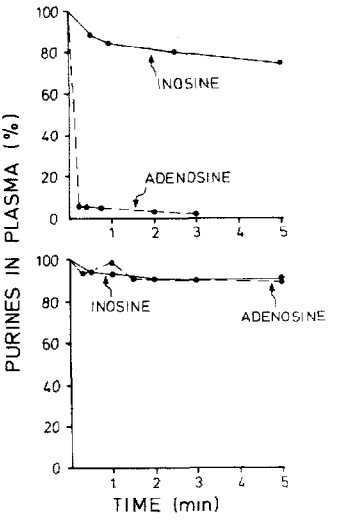

Fig. 3. Disappearance of adenosine and inosine in human plasma when blood was collected in the absence (upper panel) or presence (lower panel) of 0.2 m*M* dipyridamole. Blood was drawn from the cubital vein of a healthy individual using the syringe system shown in Fig. 1. The decrease in plasma purine levels was monitored by adding [8-^{14}C]adenosine (spec. act. 58 mCi/mmol, 10^5 cpm per tube), or [8-^{14}C]inosine (spec. act. 50 mCi/mmol, 2×10^5 cpm per tube) to syringe II (see Fig. 1). Following collection, blood was incubated for the time indicated. Sample preparation and separation by HPLC was the same as described under Experimental.

TABLE I

PLASMA LEVELS OF ADENOSINE, INOSINE, AND HYPOXANTHINE OF HEALTHY VOLUNTEERS

Blood was drawn from the cubital vein using the syringe system shown in Fig. 1. Analysis of purines was by HPLC (Fig. 2).

Subject	Adenosine (μM)	Inosine (μM)	Hypoxanthine (μM)
H.B.	0.404	0.099	0.502
J.O.	0.097	0.056	0.437
J.S.	0.340	0.113	0.315
B.B.	0.194	0.071	0.372
M.K.	0.388	0.099	0.388
$\bar{x}$ ± S.E.M.	0.285 ± 0.06	0.088 ± 0.01	0.403 ± 0.03

Values reported in the literature for adenosine, inosine, and hypoxanthine are highly variable. In the case of adenosine, they range between 0.07 and 1 μM [12, 21, 22], for inosine they are 0.7—0.9 μM [5, 23], while hypoxanthine is reported to be 0.4—1.3 μM [5, 15]. Reasons for this variability are not readily apparent in each case; however, a major source of error may be the conditions of blood sampling. Values for the various purines determined by us in plasma of healthy individuals by HPLC are shown in Table I. Recovery of adenosine and inosine during the different steps of analysis was 88% ± 2,67% ± 1, and 73% ± 1 ($\bar{x}$ ± S.E.M., $n = 5$), respectively.

The half-life of adenosine in whole dog blood was reported to be 3—6 min

[13]. These values, however, cannot be directly compared with our results, since micromolar amounts of adenosine were added to whole blood in these experiments. In our study, the disappearance of adenosine was investigated using only tracer amounts of labelled adenosine which mixed with adenosine endogenously present. Thus, the half-life reported here refers to the normal plasma level of adenosine.

We found it was necessary to identify adenosine, inosine, and hypoxanthine positively in every sample by enzymatic peak shift since the primary chromatogram (Fig. 2A) contained such a multitude of unidentified substances and the pattern of the chromatogram was not consistent between the individuals studied. Furthermore, in some but not all volunteers, ultraviolet-absorbing substances co-chromatographed with the purines of interest. It is conceivable that this interindividual variability may become of particular importance when plasma purines are measured under pathological conditions or at different nutritional states.

In summary, our method for collecting blood and compound identification permits the accurate measurement of adenosine, inosine, and hypoxanthine concentrations in plasma. Due to the syringe system used, which is mechanically coupled, this sampling method is also safe for use with humans.

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