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Note

Determination of inosine and adenosine in human plasma using high-performance liquid chromatography and a boronate affinity gel

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The determination of nucleosides from biological fluids by the reversed-phase partition [1—6] or ion-exchange [7—12] mode of high-performance liquid chromatography (HPLC) has been the subject of much recent research. The direct analysis of plasma nucleosides has in our experience been especially difficult because of the extremely low, variable, endogenous levels in the normal human population, and the tendency for the compound of interest to be obscured by other components found in plasma.

Uziel et al. [3] offered a solution to the selectivity aspects of nucleoside analysis through the pre-treatment of urine with an affinity gel which contains a boronate group (now offered commercially as Affi-Gel® 601). We have found this material to be extremely useful for the pre-fractionation and concentration of plasma nucleosides prior to injection in HPLC, thus greatly increasing sensitivity as well as selectivity. The normal human plasma levels of at least two nucleosides, inosine and adenosine, were reliably measured in all subjects examined using this boronate affinity gel. The identity and absolute quantitative value of these two compounds has been verified by the enzymatic peak-shift method.

METHODS

Instrumentation

A Varian Aerograph 4100 high-pressure liquid chromatography system with a Rheodyne Model 7120 loop injector for sample introduction and a UV absorbance detector which monitors at 254 nm was used throughout the study. A 250 × 4.6 mm stainless-steel column was packed with Spherisorb 5 μm ODS reversed-phase material (Spectra Physics, Santa Clara, Calif., U.S.A.). Packing was done from a butanol slurry using a DST-100 constant-pressure pump (Haskell Engineering, Burbank, Calif., U.S.A.) operating at a delivery pressure of

6000 p.s.i. An LS-8000 liquid scintillation counter (Beckman Instruments, Fullerton, Calif., U.S.A.) was used for ^{14}C counting.

Boronate affinity gel column

Affi-Gel® 601 (Bio-Rad Labs., Richmond, Calif., U.S.A.), a boronate affinity gel, was used for the batch separation. The manufacturer's specifications are 100—200 mesh, 100 mequiv. boronate per g dry wt., 6 ml settled bed volume per g packing material. The Affi-Gel 601 (0.25 g) was weighed out and allowed to swell overnight in 0.25 *M* ammonium acetate (pH 8.8). This was poured into a 0.7 × 4 cm column. The column was stored at 4° when not in use. After 4 months of intermittent use, the columns have shown no deterioration under the conditions of this assay, although they have occasionally been repacked when air spaces developed.

Sample preparation

A 5-ml volume of blood was drawn via venipuncture, and immediately plunged into an ice-bath. The plasma was separated at 500 *g* at 4°, and then ultrafiltered through a 2100 CF 50 membrane cone (Amicon, Lexington, Mass., U.S.A.) at 4°. Recoveries of inosine and adenosine through the ultrafiltration were quantitative.

Plasma samples were obtained as described from five male and one female normal subjects between the ages of 29 and 45 years. The blood samples were obtained in the morning with no restriction on breakfast eating habits.

Chromatography

To 1 ml of ultrafiltrate, 0.1 ml of 2.5 *M* ammonium acetate (pH 8.8) was added. This mixture was then applied to the top of a boronate affinity gel column, and the sample was washed on with 1 ml of 0.25 *M* ammonium acetate (pH 8.8). The column was then washed further with 7 ml of the same buffer. Elution of the nucleosides was carried out with 6 ml of 0.1 *M* formic acid as used by Davis et al. [4] for urinary nucleoside separation using a synthesized boronate affinity gel.

The 6-ml solution of 0.1 *M* formic acid eluate was lyophilized to dryness, and the residue dissolved in 200 µl of water, resulting in a nucleoside concentration equal to five times that in the original plasma. Fifty to one hundred microlitres of this solution were then injected onto the reversed-phase HPLC system described above, using a solvent system of 0.05 *M* H_3PO_4 in water, adjusted to pH 3.05 with sodium hydroxide.

A chromatogram of normal human plasma is shown in Fig. 1.

Reagents

Ammonium acetate (A.C.S.; J.T. Baker, Phillipsburg, N.J., U.S.A.), and formic acid (reagent, A.C.S.; M.C. & B., Norwood, Ohio, U.S.A.) were used in the preparation of solutions for analysis. [8-^{14}C]Inosine was purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and had an activity of 35 mCi/mmole. Radiopurity was assayed by thin-layer chromatography (silica gel; *n*-butanol—2 *N* NH_4OH, 10:2) and found to be greater than 98%. [8-^{14}C]-Adenosine was purchased from New England Nuclear (Boston, Mass., U.S.A.) and had an activity of 54.7 mCi/mmole and a manufacturer's specification for radiopurity greater than 97%.

Inosine (Ajinomoto Co., Japan) and adenosine (Grade A; Calbiochem, La Jolla, Calif., U.S.A.) were dissolved in water at varying concentrations and used in the preparation of standard curves.

Purine nucleoside phosphorylase (EC 2.4.2.1), a product of Boehringer (Mannheim, G.F.R.), had an activity of 25 units/mg in a suspension of 1 mg/ml and was used for the enzymatic peak-shift measurements.

RESULTS

Fig. 1 illustrates a UV absorbance pattern of boronate-treated plasma on the HPLC reversed-phase column. Peaks marked INO and ADO are identical in retention time to inosine and adenosine, respectively. To verify the identity of these peaks, duplicate injections of the sample were made and the pooled fractions corresponding to the peaks were collected and adjusted to pH 7—7.4. Three microliters of the purine nucleoside phosphorylase solution were added and allowed to digest for 1 h. The fractions were lyophilized, reconstituted to 0.2 ml, and reinjected. Quantitative conversion to the corresponding base (hypoxanthine or adenine) and complete elimination of the original peak was found in all samples tested. Although other small peaks can be found that correspond to various nucleosides, the concentrations represented are too low to allow the use of this quantitative peak-shift technique.

Six subjects were examined for normal circulating levels of inosine and adenosine, and the results, in μg/ml plasma, are shown in Table I.

Precision of the assay

Sources of error in the assay may be divided into two categories. The first is variation associated with application of the sample onto the boronate column, and collection of the acid fraction, lyophilization and concentration. The

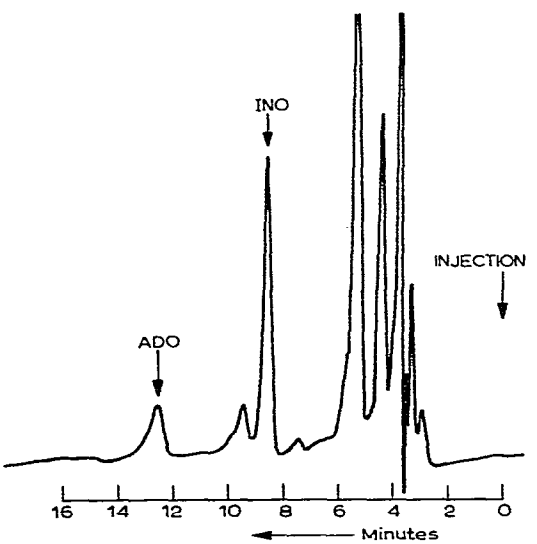

Fig. 1. Chromatogram of boronate-treated normal human plasma (subject J.C.); conditions as indicated in text. Injection volume was 75 μl, attenuation 0.04 a.u.f.s.

second source of error is the chromatographic analysis and quantitation by HPLC.

The Affi-Gel 601 boronate column efficiency was evaluated for both adenosine and inosine in plasma. Pooled, normal human plasma ultrafiltrate was spiked with [^{14}C]inosine to a concentration of 0.77 μg/ml or [^{14}C]adenosine at 0.49 μg/ml. The ultrafiltrate was treated as described under Chromatography, applied to the boronate column and the acid fraction counted for ^{14}C determination. Inosine and adenosine determinations were carried out in quadruplicate with recoveries in excess of 90%, as shown in Table II.

TABLE I

NORMAL CIRCULATING LEVELS OF ADENOSINE AND INOSINE

Subject	Adenosine (μg/ml)	Inosine (μg/ml)
S.K.G.	0.052	0.094
J.M.	0.034	0.13
R.N.	0.030	0.18
LV	0.087	0.31
T.G.	0.018	0.15
J.C.	0.149	0.60

TABLE II

RECOVERIES OF ADENOSINE AND INOSINE FROM THE BORONATE COLUMN

Compound	Mean recovery (%)	Standard deviation (%)
Adenosine	94.4	2.5
Inosine	92.1	2.5

TABLE III

PRECISION OF HPLC ASSAY

Subject	Inosine			Adenosine*		
	μg/ml	Mean	S.D. (%)	μg/ml	Mean	S.D. (%)
S.G.	0.093	0.0936	2.2	0.052	0.052	2.2
	0.092			0.052		
	0.096			0.050		
J.M.	0.134	0.131	6.1	0.033	0.034	5.1
	0.122			0.033		
	0.137			0.036		
R.N.	0.186	0.183	2.8	0.031	0.030	12
	0.186			0.033		
	0.177			0.026		

*Concentrations are calculated to original concentration in plasma.

The precision of the HPLC assay was estimated by three different repetitive injections of the boronate column concentrate from three subjects. The results of these determinations are given in Table III and demonstrate a mean standard deviation of 3.7% for inosine and 6.4% for adenosine.

The overall precision of the assay was estimated by dividing the original plasma of the three subjects into two aliquots (six samples). Each aliquot was concentrated from the boronate column and analyzed in duplicate by HPLC. The average coefficient of variation for the inosine assay was 13% and 7% for the adenosine assay.

DISCUSSION

Plasma levels of nucleosides are of potential interest in a number of disease states. It has already been noted by Hartwick and Brown [1] that an increased level of circulating adenosine is found in the plasma of patients suffering from adenosine deaminase (ADA) deficiency. In that study [1] levels of adenosine in normal patients were not found. This result could be explained by a lack of sufficient sensitivity when a simple direct injection method is used. It is of interest to note that elevated adenine levels had previously been found in the red blood cells of ADA-deficient patients [13].

Nucleoside levels are also of interest in the study of Lesch-Nyhan disease, in which hypoxanthine—guanine phosphoribosyltransferase activity is severely reduced by an inborn error of purine metabolism [14]. Various nucleosides have been considered as possible therapeutic treatments.

Some workers [3—6] have already exploited the boronate affinity gel as a preliminary extraction procedure for modified nucleosides in urine and serum. Excretion patterns of these compounds are being studied as possible cancer markers [3—5] and the patterns may also be useful in evaluating the effects of chemotherapy on cancer [5, 6].

The method reported here will offer an expanded range of applications directly related to its increased sensitivity and selectivity over direct-injection, reversed-phase HPLC column methods. Use of this procedure will enable drug metabolism studies to be carried out since endogenous circulating levels of nucleosides will now be detectable.

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