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Note

High-performance liquid chromatography of adenine and hypoxanthine arabinosides

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Adenine arabinoside (ara-A)* is a purine nucleoside first synthesized in the early 1960s[1,2] and later isolated from culture filtrates of *Streptomyces antibioticus*[3]. It was reported to exhibit marked inhibitory activity against DNA viruses in cell culture[4–7]. Its antiviral activity has been extensively evaluated in a variety of animal systems[8] and utilized for therapeutic purposes in man[9]. The determination of ara-A and its principle metabolite, hypoxanthine arabinoside (ara-Hx) in biological fluids has been reported by high-performance liquid chromatography (HPLC) on cation- and anion-exchange resins[10–13]. In our earlier work, good separations of ara-A, ara-Hx, Hyp, Ino and internal marker (8-amino ara-A) were obtained with Aminex A-28 columns using 0.2 *M* sodium acetate at pH 7.4 as the mobile phase. However, when present, Ado was not separated from ara-A in this system. The separation of this epimeric pair required the use of an additional column of a strong cation-exchange resin such as Aminex A-6 or A-7.

The chromatographic separation of saccharides[14–16] and nucleosides[17,18] has been accomplished through borate complexing. In an effort to eliminate the use of a two-column system for the efficient separation of ara-A from its metabolites, the use of borate buffers was investigated in the Aminex A-28 system.

EXPERIMENTAL

Chemicals and reagents

Sodium borate decahydrate and acetic acid (reagent grade) were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). Sodium acetate (anhydrous analytical reagent) was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Ado, Ino, Hyp and Ado deaminase were obtained from Calbiochem (San Diego, Calif., U.S.A.). Ara-A, ara-Hx and 8-amino ara-A were obtained from Parke, Davis & Co. (Ann Arbor, Mich., U.S.A.). Stock solutions of 0.04 *M* sodium borate and 2 *M* sodium acetate were used to prepare mobile phase buffer solutions by appropriate dilution in distilled water. The pH was adjusted by the addition of 5% (0.83 *M*) acetic acid.

* Abbreviations used: ara-A = 9-β-D-arabinofuranosyladenine, arabinosyladenine, vidarabine; ara-Hx = 9-β-D-arabinofuranosylhypoxanthine, arabinosylhypoxanthine; 8-amino ara-A = 8-aminoarabinosyladenine; ara-AMP = arabinosyladenine 5′-monophosphate; Ado = adenosine; Ino = inosine; Hyp = hypoxanthine.

Equipment

A Series 4200 liquid chromatograph (Varian Assoc., Walnut Creek, Calif., U.S.A.) with a 254-nm fixed-wavelength UV detector and an A-25 recorder was used throughout this study. A Model 153 fixed-wavelength UV detector (Altex Scientific, Berkeley, Calif., U.S.A.) was used to provide dual column capability. Stainless-steel columns (15 cm × 0.37 I.D.) were slurry-packed with Aminex A-28 resin (Bio-Rad Labs., Richmond, Calif., U.S.A.) in distilled water. The Aminex A-28 resin was pre-equilibrated for 24 h in 2 *M* sodium acetate solution. The temperature was maintained at 60° to facilitate diffusion and to decrease the viscosity of the solvent. The flow-rate of the mobile phase was adjusted to 0.5 ml/min, which required a pressure of 700–1000 p.s.i. Samples were injected on-column by means of a syringe through a stop-flow injector.

Methods

Plasma samples were deproteinized by centrifugation through CF25 or CF50A Centriflo membrane cones (Amicon Corp., Lexington, Mass., U.S.A.) at 100 *g* for 60 min. Urine samples were filtered through No. 497 paper (Schleicher and Schüll, Keene, N.H., U.S.A.) and diluted with distilled water as required. The internal marker, 8-amino ara-A, was added to all samples provided that interference peaks were not present. Peaks were quantitated manually using peak-height measurements. Ara-A and ara-Hx concentrations in biological samples were determined from calibration graphs prepared by linear regression analysis of standards at four or five different concentrations.

RESULTS AND DISCUSSION

Anion-exchange techniques were selected for the separation of ara-A and ara-Hx in the presence of Ado, Ino and Hyp for several reasons. Firstly, except for an overlap of Ado and ara-A, all other components are separated in a single analytical system. Secondly, borate complexing provides an additional parameter for the effective separation of ribosyl and arabinosyl nucleosides. As borate complexing is most pronounced with sugars that contain *cis*-hydroxyl groups in a furanoid structure[19], the borate-diol of ribose shows a high affinity for strongly basic anion exchangers.

With 0.005 *M* sodium borate solution as the mobile phase, the effect of pH on the retention of ara-A, 8-amino ara-A, ara-Hx, Ado and Ino on Aminex A-28 is shown in Table I. Optimal separation of these components occurred at pH 6.3. Ado, ara-A and 8-amino ara-A have no ionic properties that can be manipulated in order to change their retentions in anion-exchange systems except at strongly alkaline pH. The apparent exception of Ado is attributed to the formation and exchange of the borate-complex anion. The retention of ara-Hx and Ino is based upon true anion exchange. In addition, the formation of a borate complex with Ino resulted in total resin retention in the pH range tested.

The effect of the molarity of the mobile phase on the retention of components at a constant pH of 6.3 is shown in Table II. Maximum separation of components was achieved at a borate concentration of 0.01 *M*. As expected for true anion exchange, the retention of ara-Hx was inversely proportional to borate concentration, while ara-A and 8-amino ara-A were not greatly affected. In contrast, the retention of Ado was

TABLE I

INFLUENCE OF pH ON THE RETENTION TIME OF NUCLEOSIDES ON AMINEX A-28

Mobile phase 0.005 *M* sodium tetraborate solution.

pH	*Retention time (min)*				
	ara-A	*8-amino ara-A*	*Ado*	*ara-Hx*	*Ino*
9.0	11.0	20.0	N.E.*	N.E.	N.E.
7.3	9.5	—	N.E.	N.E.	N.E.
7.0	7.0	—	28.0	N.E.	N.E.
6.3	6.0	8.0	10.0	17.5	N.E.
6.0	6.0	8.0	8.5	11.5	—
5.0	6.0	—	7.0	5.0	—

* N.E. = Not eluted from column.

directly proportional to borate concentration, as would be expected from increased borate-complex anion formation.

The chromatographic separation of nucleosides and Hyp on Aminex A-28 using a mobile phase consisting of 0.01 *M* sodium borate at pH 6.3 is shown in Fig. 1A. Although the separations were reproducible from day to day and the columns appeared to be stable, a gradual increase in the retention times of Ado, ara-Hx and Hyp was found with columns used over periods of weeks or months. The chromatographic profile on the same column after a 3-month period is shown in Fig. 1B. As freshly prepared columns operated satisfactorily, the gradual replacement of acetate with borate ion on the resin appeared to be a possibility. The addition of sodium acetate to the mobile phase restored the retention times of the above components to their initial values. Subsequently, a mobile phase consisting of 3 parts of 0.01 *M* sodium borate and 1 part of 0.01 *M* sodium acetate at pH 6.4 was employed.

The response of the UV detector to known amounts of ara-A and ara-Hx was linear throughout the range (0–20 μg/ml), as shown in Fig. 2. The reproducibility of the peak heights of ara-A and ara-Hx was established in a trial in which 10 replicate assays were run at a concentration of each component of 20 μg/ml. The coefficient of variation was 2.1% for ara-A and 7.4% for ara-Hx. Standard graphs prepared by the addition of known amounts of ara-A and ara-Hx to deproteinized plasma, urine or water showed no significant differences in recoveries.

The sensitivity of detection was enhanced by the low noise level of the chromatographic system and the relatively sharp chromatographic peaks. This permitted

TABLE II

INFLUENCE OF MOLARITY ON THE RETENTION TIMES OF NUCLEOSIDES ON AMINEX A-28

Mobile phase sodium tetraborate solution at pH 6.3.

Molarity	*Retention time (min)*				
	ara-A	*8-amino ara-A*	*Ado*	*ara-Hx*	*Hx*
0.005	6.0	8.0	10.0	17.5	—
0.01	6.5	9.0	12.5	16.5	19.0
0.02	6.8	9.5	15.2	13.0	—

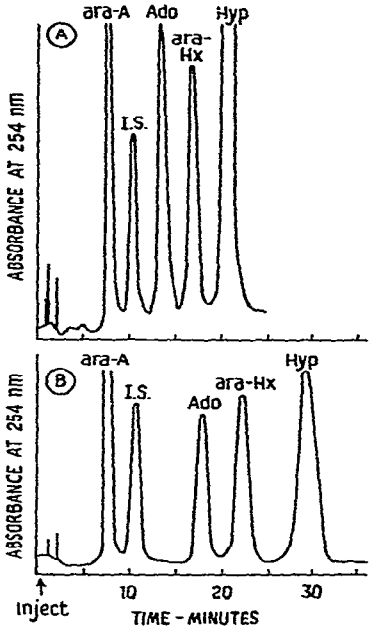
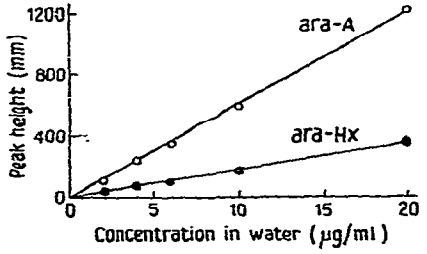


Fig. 1. HPLC of a nucleoside mixture on an Aminex A-28 column. (A) Initial separation; (B) separation after 3 months.

operation of the UV detectors at maximal sensitivity. Under these conditions, the lower limits of detection were about 2 ng for ara-A and 5 ng for ara-Hx. A typical chromatogram of human plasma taken 30 min after the start of an i.v. infusion of ara-A (10 mg/kg·day over a 12-h infusion period) is shown in Fig. 3. A 20-μl volume of internal marker (20 μg/ml in water) and 20 μl of 0.1 *M* sodium acetate buffer at pH 6.3 were added to 160 μl of deproteinized plasma and a 10-μl sample of this mixture



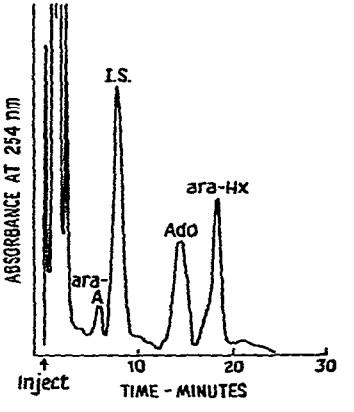


Fig. 2. Response curves for ara-A and ara-Hx.

Fig. 3. HPLC of deproteinized human plasma 30 min after start of i.v. infusion of ara-A (10 mg/kg·day).

was injected on the column. Ado and a small amount of ara-A were found in addition to the major peak representing ara-Hx.

Presumptive identification of ara-A and ara-Hx in biological samples was made by comparing the retention times of the peaks with those of known standards. Identification of ara-A was confirmed by use of an enzymic peak-shift technique[20]. A study in which human eyes were treated with a 3% ophthalmic solution of ara-AMP required the assay of the aqueous humor. A 20-μl sample was assayed directly without deproteinization because of limitations on the sample size; this produced an elevated base-line. A 5-μl volume of internal marker (10 μg/ml in water) and 5 μl of Ado deaminase (42 I.U./ml in water) were added to 20 μl of sample and the mixture was incubated at room temperature for 15 min. The results shown in Fig. 4 indicate the complete disappearance of the ara-A peak and a corresponding rise in ara-Hx produced by the deamination of ara-A. This technique is not applicable when co-vidarabine[21] or other inhibitors of Ado deaminase are added to freshly collected blood samples.

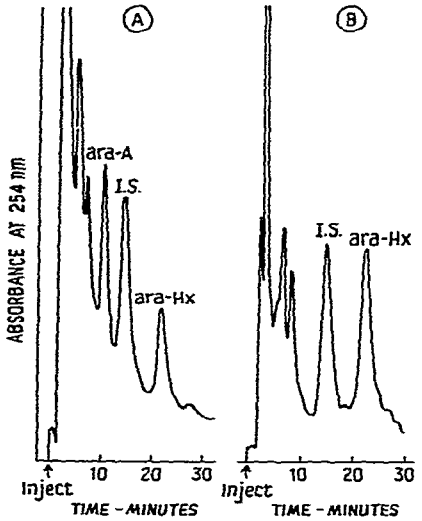


Fig. 4. HPLC of human aqueous humor following topical application of 3% ara-AMP ophthalmic solution. (A) Untreated sample; (B) after treatment with Ado deaminase.

CONCLUSION

By means of HPLC in a borate buffer system, ara-A and ara-Hx are separated from ribosyl nucleosides and determined quantitatively in blood and urine.

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