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Note

High-performance liquid chromatographic analysis of hypoxanthine arabinoside in plasma

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Adenine arabinoside (ara-A) is an antiviral agent that has been used systemically in the treatment of herpes simplex and herpes zoster infections. In the body, ara-A is eliminated primarily by deamination to hypoxanthine arabinoside (ara-Hx). Following therapeutic doses, concentrations of ara-A in plasma are too low to accurately determine by conventional analysis, whereas concentrations of ara-Hx, being ten-fold higher, are often measured.

The determination of concentrations of ara-Hx in plasma has proven extremely difficult because the compound is structurally similar to many endogenous nucleosides that are found in relatively high concentrations in plasma. This structural similarity results in considerable difficulty in the chromatographic resolution of ara-Hx from potentially interfering endogenous compounds. Also, as with the endogenous nucleosides, ara-Hx is a polar molecule and cannot readily be extracted from plasma into organic solvents. As a result, it is necessary to inject plasma or deproteinized plasma directly on column, resulting in decreased column life expectancy as well as the appearance of many potentially interfering peaks from the endogenous nucleosides.

Few high-performance liquid chromatographic (HPLC) procedures for measuring concentrations of ara-Hx in plasma have been published, for the reasons just outlined [1—4]. All of the published procedures utilize ion-exchange chromatography which has important disadvantages compared to other HPLC separation techniques, including the need for column temperatures above ambient temperature to aid resolution, and the very short life expectancy of most ion-exchange columns.

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This paper describes a sensitive and selective HPLC procedure for the analysis of ara-Hx in plasma using conventional reversed-phase chromatography. A pre-column sample preparation procedure is incorporated to rid the plasma of many interfering substances. Previous assays have not employed a method for sample clean-up, resulting in interferences during chromatography. The method has been used in our laboratories to measure ara-Hx concentrations in plasma after therapeutic doses of adenine arabinoside monophosphate (ara-AMP). Data are presented in one patient following therapeutic doses of ara-AMP.

EXPERIMENTAL

Materials

Ara-Hx was graciously supplied by Warner-Lambert/Parke-Davis (Ann Arbor, MI, U.S.A.). Standard solutions were prepared in water at concentrations of 10 and 100 μg/ml. Methanol (distilled-in-glass) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The water used throughout was glass-distilled. Sample preparation cartridges (C_{18} Sep-Pak®) were purchased from Waters Assoc. (Milford, MA, U.S.A.). All cartridges were pre-washed within 4 h of use with 10 ml of methanol followed by 10 ml of water using 10-ml glass syringes. The cartridges were then dried by flushing through four 10-ml syringe volumes of air.

Sample preparation

Plasma (2 ml) was deproteinized by the addition of 0.8 ml of 10% trichloroacetic acid. The mixture was vortexed for 10 sec and centrifuged for 10 min at approximately 1000 *g*. Subsequently, 1.2 ml of deproteinized plasma was transferred to a 10-ml capacity culture tube and brought to pH 2 with 0.5 *M* sodium hydroxide. The pH was checked by pH paper. The resultant mixture was then loaded onto a pre-washed sample preparation cartridge with a tuberculin syringe. Water, pH 5.5 (6 ml) was then pressed through the cartridge with a 10-ml glass syringe, then 4 ml of water (pH 10 with 1 *M* sodium hydroxide) were passed through the cartridge and collected. The collected pH-10 water was then neutralized with hydrochloric acid to a pH of about 7 and 100 μl were injected onto the chromatograph.

Apparatus

The modular high-performance liquid chromatograph consisted of a constant-flow pump (Model U6K, Waters Assoc.), a variable-wavelength absorbance detector (Model 450, Waters Assoc.) set at 254 nm, and a strip-chart recorder (Model 9176, Varian Instruments, Palo Alto, CA, U.S.A.). The column was a μBondapak C_{18} column (250 mm × 3.9 mm I.D.; Waters Assoc.).

Chromatographic conditions

The mobile phase was 15% methanol in water. Prior to mixing, sodium borate was added to both the methanol and water to achieve a concentration of 0.02 *M*. In addition, the water containing the sodium borate was acidified to pH 6.5 with hydrochloric acid. The flow-rate of the mobile phase was 1.5 ml/min resulting in a column pressure of 17 MPa. Overnight the flow-rate was reduced to 0.1 ml/min.

Calibration

The assay was calibrated by analyzing 2.0-ml aliquots of blank plasma to which 0.1—10 μg/ml of ara-Hx had been added. These standards were extracted and chromatographed identically to samples containing unknown quantities of ara-Hx. For each sample, the peak height of ara-Hx was measured and plotted against concentration to determine the linearity of the extraction and detector response for ara-Hx. In addition, each peak height was divided by the concentration of ara-Hx in that sample to give a normalized peak height. These normalized peak heights were averaged and the mean value was used to determine the amounts present in samples containing unknown concentrations of ara-Hx. Precision of the assay was estimated by calculating the coefficient of variation (C.V.) for each set of normalized peak heights.

Reproducibility

Aliquots (2 ml) of normal human plasma were spiked with 0.5, 1.0, 5.0 and 10.0 μg/ml ara-Hx and assayed in quadruplicate using the method described. These samples were extracted and chromatographed as described above. Absolute peak heights were measured and the coefficients of variation were calculated for each concentration.

Recovery

Aliquots (2 ml) of plasma were spiked with known quantities of ara-Hx (0.5 and 5.0 μg/ml). After the samples were prepared and chromatographed as described, the peak heights for ara-Hx were compared with the peak heights obtained when a standard containing either 0.5 or 5.0 μg/ml ara-Hx was injected directly onto the column. The recoveries were normalized for volume changes that occurred during the sample preparation procedures.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of a blank plasma sample and a plasma sample containing approximately 2.0 μg/ml ara-Hx that were prepared according to the sample preparation procedure are shown in Fig. 1, together with a blank plasma sample that was not prepared. Plasma was collected from a single subject before and after administration of ara-AMP. As shown, the preparation procedure removed most of the peaks which could potentially interfere with the determination of ara-Hx. The retention time for ara-Hx under these conditions was approximately 4 min, but varied with changes in the composition of the mobile phase. Changes in composition of the mobile phase were necessary in some patients, to resolve ara-Hx from interfering peaks (see Discussion).

Calibration

A typical calibration curve relating peak height to concentration was linear for ara-Hx over the range of concentrations studied. The coefficients of variation for the estimation of ara-Hx averaged 9.3%. A standard curve was assayed with each set of samples containing unknown quantities of ara-Hx. The limit of detection of a prepared standard of ara-Hx, defined as five times baseline noise,

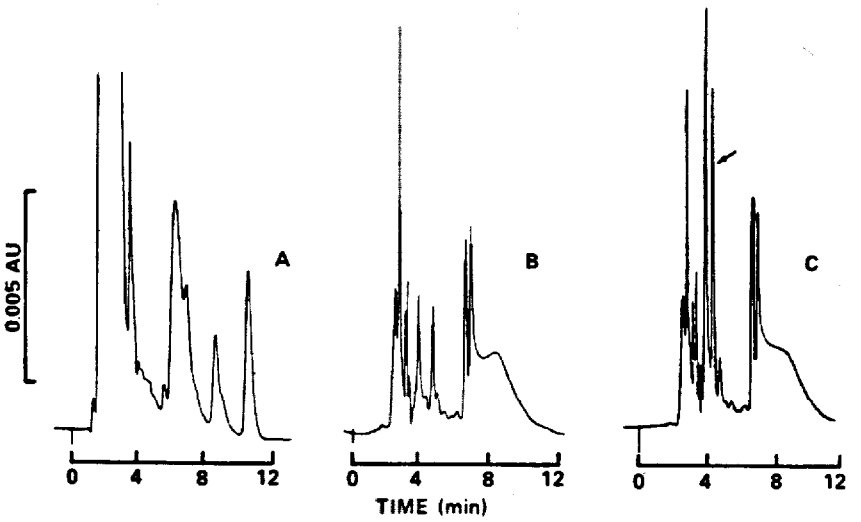

Fig. 1. Chromatograms of (A) blank plasma sample and two plasma samples (B and C) that were prepared according to the sample preparation procedure. Plasma samples were from a single subject who was treated with ara-AMP. (B) Represents plasma containing no ara-Hx (before drug administration), and (C) represents plasma from the same patient, containing approximately 2.0 μg/ml ara-Hx.

was 0.05 μg/ml. Again, this varied depending upon the presence of interfering peaks.

Reproducibility

Data showing the reproducibility of the assay are summarized in Table I. For any given concentration the determination of ara-Hx was highly reproducible. There was, however, a slight tendency of the average normalized peak height to decrease with increasing concentration.

Recovery

The total amount of ara-Hx recovered from the assay procedure was 34.5 ± 3.3% for 0.5 μg/ml, and 31.2 ± 3.1% for 5.0 μg/ml. These data represent the average of four determinations in each case.

TABLE I

REPRODUCIBILITY OF RECOVERY OF ARA-Hx FROM SPIKED PLASMA SAMPLES

Concentration (μg/ml)	Normalized peak height* (mm/μg)	Coefficient of variation* (%)
0.5	88.6	3.9
1.0	88.5	4.4
5.0	82.0	3.2
10.0	75.1	3.4

*Coefficient of variation of four determinations.

Patient data

Plasma concentrations of ara-Hx obtained in a single patient are presented in Fig. 2. The patient received 5 mg/kg ara-AMP intramuscularly every 12 h for three days followed by a single intravenous dose of 5 mg/kg ara-AMP. The plasma concentrations shown in Fig. 2 were obtained from plasma samples collected after the final intramuscular and the intravenous doses. As shown, the plasma concentration after both doses declined roughly in parallel with a terminal half-life of about 2 h.

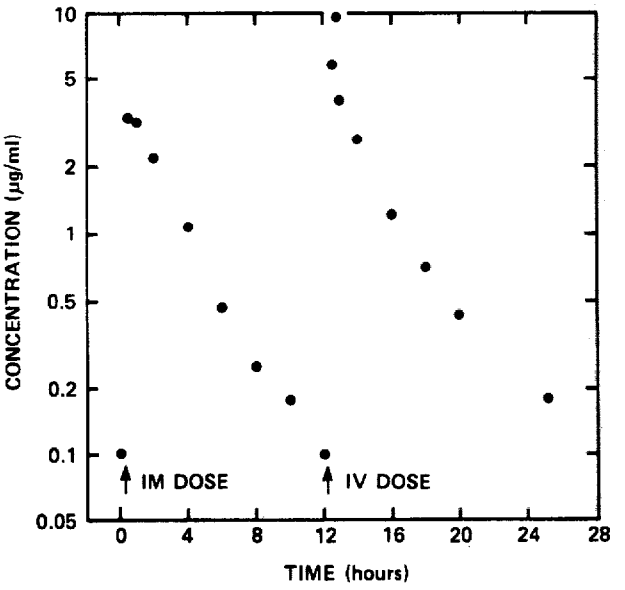

Fig. 2. Plasma concentrations of ara-Hx in a single patient after receiving ara-AMP intramuscularly (IM) and intravenously (IV) (5 mg/kg per 12 h). The points before the arrow indicate concentrations of ara-Hx just before drug administration.

DISCUSSION

Unlike other published procedures for ara-Hx that have employed ion-exchange chromatography [1—4], this assay uses conventional reversed-phase chromatography. Hence, column life is longer and the chromatography can be conducted at ambient temperature. In this study, a single column was used daily for six months before replacement was necessary.

Another important difference between this and the previously published HPLC procedures, in which deproteinized plasma is injected directly on column [1—4] is the incorporation of a sample preparation procedure. As shown in Fig. 1, the sample preparation procedure resulted in a substantial decrease in background noise, permitting the determination of ara-Hx at low concentrations. The volume and pH of the wash and elution steps were adjusted to achieve maximum signal-to-noise ratio. Maximum signal-to-noise ratio was achieved when recovery of ara-Hx was 32%, ie., at higher recoveries interfering substances ("noise") were present in even greater amounts in comparison with ara-Hx. The 32% recovery, although low, was reproducible and sufficiently sensitive to detect ara-Hx at concentrations as low as 0.1 μg/ml (Fig. 2).

The assay has been used to analyze plasma concentrations of ara-Hx after therapeutic doses of ara-A or ara-AMP and may be used with approximately equivalent sensitivity to assay ara-Hx concentrations from either 1 or 2 ml of plasma. Depicted in Fig. 2 are the plasma concentrations of ara-Hx obtained in a single subject using the method presented in this paper. There are many water-soluble endogenous compounds in plasma that absorb UV light at 254 nm and may potentially interfere with the determination of ara-Hx. However, resolution of ara-Hx from these peaks was obtained by varying the percent methanol in the mobile phase from about 12 to 18%, which changed the retention time of ara-Hx.

In summary, a sensitive, selective HPLC procedure for determining the concentration of ara-Hx in plasma is of considerable value in studies of the efficacy and toxicity of ara-A or ara-AMP. The procedure described here differs from previously published methods in that it incorporates a sample preparation step and uses conventional reversed-phase chromatography. The method has been used successfully to analyze concentrations of ara-Hx in plasma after therapeutic doses of ara-AMP.

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