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SIMULTANEOUS ANALYSIS OF 1-β-D-ARABINOFURANOSYLCYTOSINE, 1-β-D-ARABINOFURANOSYLURACIL AND SODIUM SALICYLATE IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic column switching system is described for the rapid and complete separation of 1-β-D-arabinofuranosylcytosine (Ara-C), 1-β-D-arabinofuranosyluracil (Ara-U) and sodium salicylate using an internal standard of sodium cefmetazole. The system is highly selective and separates these compounds from interfering compounds commonly in biological matrices. The system was tested by following the pharmacokinetics of Ara-C after rectal administration in the presence of sodium salicylate which is an aid to drug absorption. The chromatographic system is also suitable for monitoring levels of Ara-C and its metabolite Ara-U after intravenous administration of Ara-C.

INTRODUCTION

1-β-D-Arabinofuranosylcytosine (Ara-C) is administered by intravenous or subcutaneous routes for the treatment of acute myelocytic leukemia and acute lymphocytic leukemia [1—4]. After intravenous injection, plasma Ara-C exhibits biphasic pharmacokinetics. There is an initial rapid metabolism

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of Ara-C by deamination to 1-β-D-arabinofuranosyluracil (Ara-U) followed by a slow elimination phase ($t_{½}$ = 2—2.5 h) [5, 6]. The initial deamination of Ara-C by pyrimidine neucleoside deaminase (cytidine aminohydrolase) occurs rapidly with a half-life of ca. 11 min, and is a saturable process [7, 14]. Thus to determine the pharmacokinetics and bioavailability of Ara-C, it is necessary to monitor both Ara-C and Ara-U levels in the blood.

We have chosen to administer Ara-C via a novel route, the rectum, using adjuvant enhanced absorption technology that has been developed in our laboratories. The adjuvant of choice for this work was sodium salicylate, and the vehicle of administration was a rectal suppository. Previous analytical methods have suffered from at least one of the following disadvantages: relative insensitivity, complex preparation, lack of specificity or long elution times. The methods have included biological assay using tissue cultures [8, 9], radiolabelling of Ara-C [7, 8], paper chromatography [7, 10] or high-performance liquid chromatography (HPLC) [6, 11, 12].

EXPERIMENTAL

Reagents

Cytidine, cytosine, uridine, Ara-U, formic acid (95—97%) and sodium salicylate (99+%) were obtained from Sigma (St. Louis, MO, U.S.A.). Witepsol S55 was obtained from Kay-Fries (Montvale, NJ, U.S.A.). Sodium cefmetazole was obtained from Merck Sharp and Dohme (West Point, PA, U.S.A.). Ammonium hydroxide was obtained from Fischer (Pittsburgh, PA, U.S.A.). Ara-C was obtained from Upjohn (Kalamazoo, MI, U.S.A.). Tetrahydrouridine (THU) was obtained from Calbiochem-Beliring (La Jolla, CA, U.S.A.).

HPLC equipment

The plan and key for the system are illustrated in Fig. 1. Pumps 1 and 2 were Waters Model M45; pump 3 was an Eldex Model A-60-5, and pump 4 was a Fluid Metering lab pump. All three valves (Rheodyne Model 7001) were activated via Rheodyne Model 7000 controllers, and all events were controlled by a Waters system controller. The UV detector was a dual-channel Waters

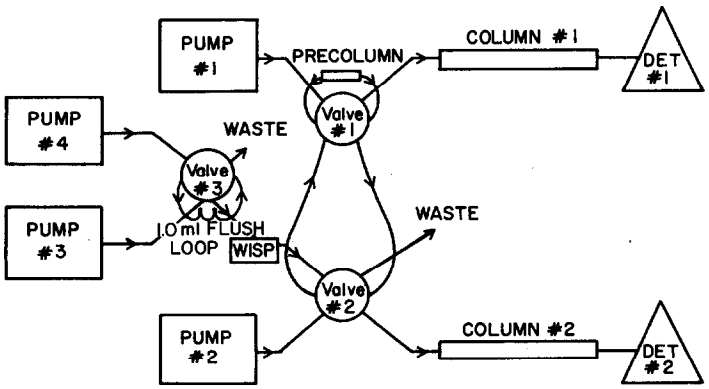

Fig. 1. Plan and key of the HPLC system used.

Model 440 absorbance detector set at 254 nm. The data were recorded on a Waters data module. Samples (20 μl) were injected using a Waters WISP 710B. The pre-packed columns were obtained from Ranin and contained a 5-μm particle size reversed-phase C_{18} material (Ranin OD-MP RP-18). The precolumn was a Ranin OD-MP RP-18 Spheri 5 of 30 mm × 4.6 mm. Column 1 consisted of two Ranin OD-MP RP-18 Spheri 5, 100 mm × 4.6 mm, and column 2 consisted of one Ranin OD-MP RP-18 Spheri 5, 100 mm × 4.6 mm. Pump 4 was activated by a Waters system controller.

Sample preparation

Biological samples. Blood samples (300 μl) were collected in serum collecting tubes containing 5 μg of THU to eliminate deamination of Ara-C [5, 6]. The tubes were centrifuged for 10 min at 900 *g* and 100 μl of serum were removed. An internal standard, sodium cefmetazole, was added to the serum (25 μl containing 0—5 μg of sodium cefmetazole). The serum was then deproteinated using 200 μl of acetonitrile and centrifuged for 5 min at 900 *g*. The supernatant was collected and evaporated to dryness under a nitrogen gas stream. The sample was reconstituted with 100 μl of water then analyzed by HPLC.

Standard samples. Standard aqueous solutions containing Ara-C, Ara-U and sodium salicylate were prepared daily. Aliquots of the standard solutions were added to blank pooled blood samples containing THU. The spiked standard blood samples were prepared for HPLC analysis in identical fashion to the biological samples.

HPLC conditions

The conditions are summarized in Table I for the solvents, flow-rates and pressures, and Table II for the events sequence. The key in each table is to be used in conjunction with Fig. 1. The starting conditions are valves 1, 2 and 3 in a clockwise position causing pump 1 to bypass valve 1 and elute from column 1; pump 2 to bypass valve 2 and elute from column 2; pump 3 to bypass valve 3 through valves 2 and 1 and then to precolumn and to waste; pump 4 was off but connected to bypass valve 3 to waste.

TABLE I

SOLVENT SYSTEMS, FLOW-RATES AND PRESSURES REFERRED TO IN FIG. 1 AND TEXT

Pump No.*	Flow-rate (ml/min)	Pressure (bars)	Solvent system and code
1	2	140	0.017 *M* ammonium hydroxide—formic acid buffer adjusted to pH 3.0 with formic acid (I)
2	2	70	0.0068 *M* ammonium hydroxide—0.01 *M* formic acid—20% acetonitrile (II)
3	2		0.017 *M* ammonium hydroxide—formic acid buffer adjusted to pH 3.0 with formic acid (I)
4	2		acetonitrile (III)

*See Fig. 1.

TABLE II

EVENT SEQUENCE CONTROLLED THROUGH SYSTEMS CONTROLLER (SEE FIG. 1)

Event	Time (min)	Process*	Effect
Inject	0.0	WISP injection	Sample through valves 1, 2 and precolumn to waste
Pulse 1	0.3	Valve 1 CCW	Precolumn in for system 1 (column 1 and solvent 1)
Pulse 3	0.4	Valve 2 CCW	System 2 flow through bypass port of valve 1
Pulse 2	1.0	Valve 1 CW	Precolumn out of system 1 and into system 2
Pulse 4	3.0	Valve 2 CW	Precolumn out of system 2; solvent II through precolumn from pump 3 to waste
Pulse 5	3.1	Valve 3 CCW	Flush loop through precolumn to waste
Pulse 6	4.1	Valve 3 CW	Flush loop out of precolumn loop
on 7	4.2	power on pump 4	Reload flush loop with solvent III
off 7	4.7	power off pump 4	Reload complete

*CW = clockwise, CCW = counterclockwise.

The column switching was designed so that the fraction containing Ara-C and Ara-U was transfered from the precolumn to column 1, and the fraction containing sodium salicylate and sodium cefmetazole was transfered from the precolumn to column 2. It was also necessary to alter solvent systems to elute sodium salicylate and sodium cefmetazole. Initially, a 20-μl sample was injected and eluted onto the precolumn with solvent I; the rapidly eluting components that interfere with the assay were eluted to waste. When this was complete, the precolumn outflow was switched to column 1. The fraction containing Ara-C and Ara-U was then eluted onto column 1 and resolved using solvent system I. After transfer from the precolumn was completed, the precolumn outlet was switched to column 2. Column 1 was then eluted with solvent system I using pump 1. The eluent was monitored by UV spectroscopy at a wavelength of 254 nm (see Figs. 2A, 3A, 3B and 4). The precolumn after switching was eluted with solvent system II to elute sodium salicylate and sodium cefmetazole onto column 2. When the transfer was completed, the precolumn was switched to waste and flushed with solvent system III (from pump 4) to remove any of the sample compounds remaining on the precolumn. Column 2 was eluted with solvent system II using pump 2. The eluent was monitored by UV spectroscopy at 254 nm (see Figs. 2B, 3C and D). When flushing with solvent III was completed, the precolumn was reconditioned by eluting with solvent system I in preparation for the next injection.

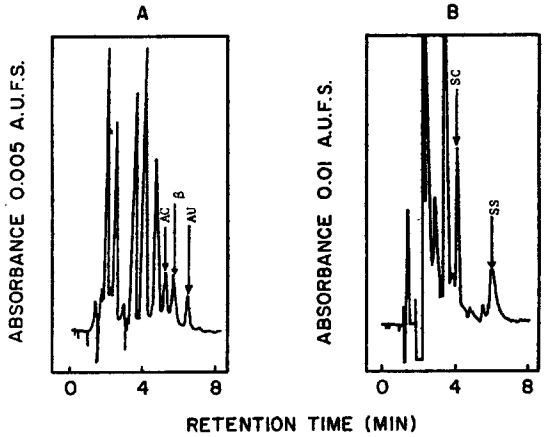

Fig. 2. High-performance liquid chromatograms of pooled serum samples spiked with (A) Ara-C (AC) 2 μg/ml and Ara-U (AU) 0.5 μg/ml for column 1; and (B) sodium salicylate (SS) 20 μg/ml and sodium cefmetazole (SC) 5 μg/ml for column 2.

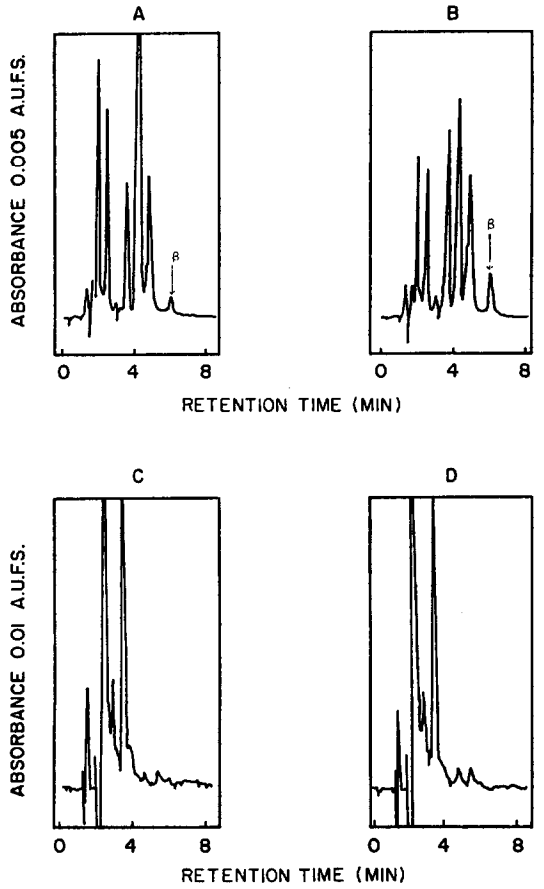

Fig. 3. High-performance liquid chromatograms of blank serum samples from two dogs (A) and (C) for column 1, (B) and (D) for column 2.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 2A (for column 1) and 2B (for column 2) show typical chromatograms of pooled, spiked serum samples containing Ara-C (2 μg/ml). Ara-U (0.5 μg/ml), sodium salicylate (20 μg/ml) and sodium cefmetazole (5 μg/ml). Peak β (Fig. 2A), which elutes between the Ara-C and Ara-U peaks, varies in height from dog to dog (see Fig. 3A and B). A similar variation in peak heights of inter-animal HPLC traces for an Ara-C/Ara-U system has been reported by Linssen et al. [6]. In their study, the variable peak had the same elution properties as Ara-U. Fig. 3A—D shows blank serum samples from two dogs. Fig. 3A and B are chromatograms of column 1 and Fig. 3C and D are chromatograms of column 2. The blank serum chromatogram in Fig. 3A—D shows no interfering substances with the same eluting properties as Ara-C, Ara-U, sodium salicylate and sodium cefmetazole. In the development of the assay, the pH of the Ara-C/Ara-U column solvent system is critical. At pH 3.0 ± 0.1, good resolution was obtained between Ara-C and peak β. Fig. 4 shows the chromatogram from column 1 of a mixture containing cytosine (I), cytidine (II), uridine (III), Ara-C (IV) and Ara-U (V) which have caused interference with Ara-C and Ara-U peaks on other chromatographic systems [6, 7, 11].

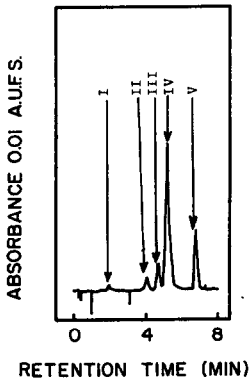

Fig. 4. High-performance liquid chromatogram from column 1 of serum samples spiked with (I) cytosine (0.3 μg/ml), (II) cytidine (0.3 μg/ml), (III) uridine (0.3 μg/ml), (IV) Ara-C (5 μg/ml) and (V) Ara-U (1 μg/ml).

Calibration curves and accuracy

Spiked standard samples were analyzed before each batch of biological samples to check for any changes in chromatographic characteristics. None were detected in the study. As an internal standard was used, peak height ratios (PHR), with respect to the internal standard, were used for Ara-C, Ara-U and sodium salicylate. A plot of Ara-C PHR vs. concentration in the range 0.125—8.000 μg/ml serum (n = 3 at each of the eight points on the curve) gives a linear correlation coefficient of 0.9951 and passes through the origin. The relative coefficient of variation at 0.125 μg/ml was 15% and at 8.000 μg/ml was 0.3%. The percent recovery was 98 ± 1% at 4 μg/ml (n = 3) with respect to aqueous solutions.

A plot of Ara-U PHR vs. concentration in the range 0.0625—2.0000 μg/ml serum (n = 3 at each of the seven points on the curve) gives a linear correlation coefficient of 0.9972 and passes through the origin. The relative coefficient of variation at 0.0625 μg/ml was 2.1% and at 2.0000 μg/ml was 0.6%. The percent recovery was 108 ± 0.2% at 1.0 μg/ml (n = 3) with respect to aqueous solutions.

For sodium salicylate, PHR vs. concentration in the range 1.25—80 μg/ml serum (n = 3 at each of the eight points on the curve) gives a linear correlation coefficient of 0.9940 and passes through the origin. The relative coefficient of variation at 1.25 μg/ml was 17% and at 80.0 μg/ml was 0.02%. The percent recovery was 113 ± 0.4% at 40 μg/ml (n = 3) with respect to aqueous solutions.

Biological data

Figs. 5 and 6 show the serum levels of Ara-C/Ara-U and sodium salicylate, respectively, after the administration of a suppository containing Witepsol S55 (696 mg), Ara-C (32 mg), water (262 μl) and sodium salicylate (322 mg) to beagle dogs (n = 6). Ara-C is rapidly absorbed to a maximum serum level after 20—30 min. Ara-U levels increase slowly in the serum to a plateau value which is maintained from 30—120 min. Sodium salicylate reaches its peak serum level after 90 min. The prolonged serum levels of Ara-C via the rectal route may provide a viable alternative to slow infusion therapy currently in use. The pharmacokinetic implications of this study will be reported more extensively at a later date [13].

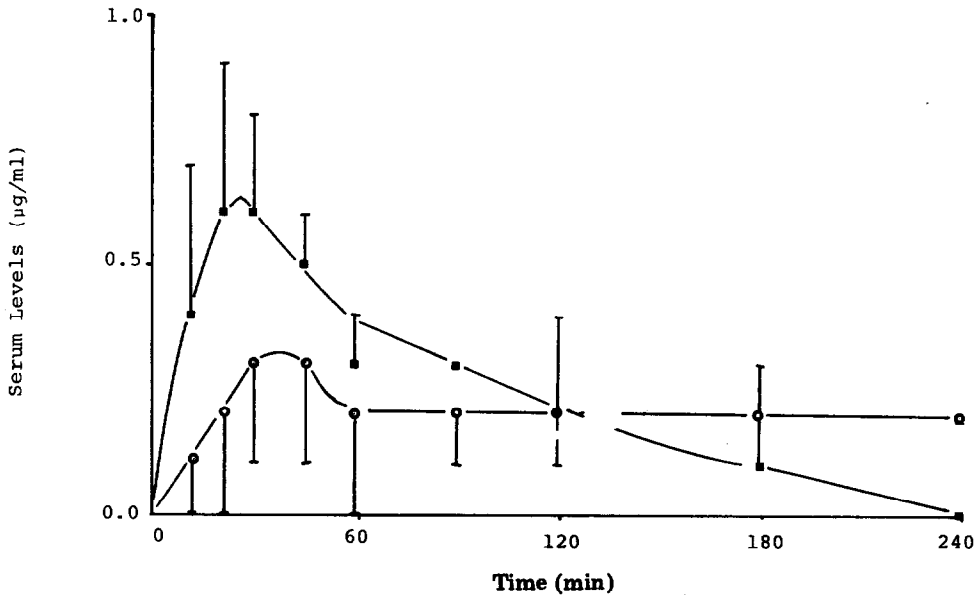

Fig. 5. Serum levels of Ara-C (▪) and Ara-U (○) after rectal administration of a suppository containing Ara-C and sodium salicylate.

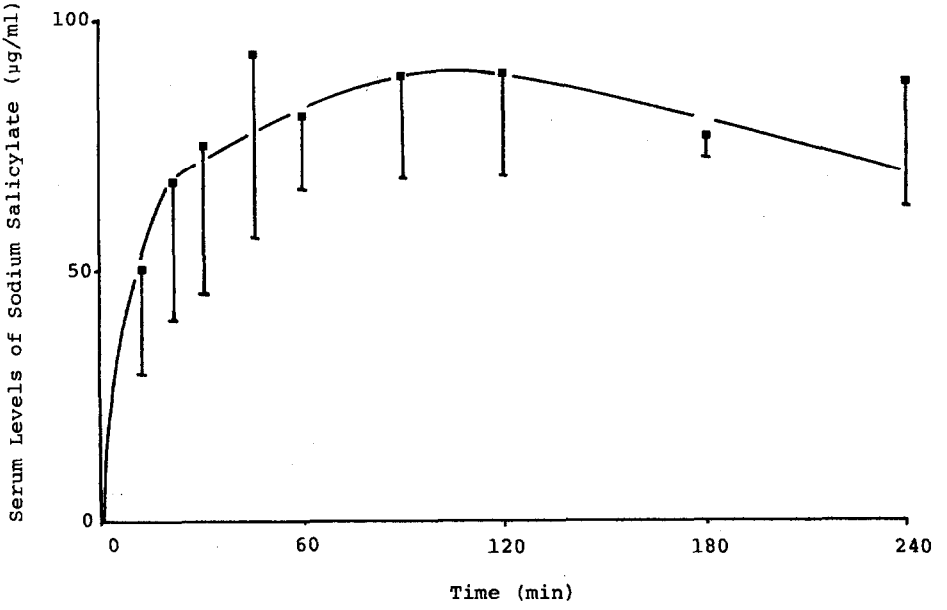

Fig. 6. Serum levels of sodium salicylate after rectal administration of a suppository containing Ara-C and sodium salicylate.

CONCLUSIONS

A method has been described for the analysis of Ara-C, Ara-U and sodium salicylate with the following advantages over other published methods: decreased analysis time due to a rapid elution of compound enabling analysis of a large number of samples per day (ca. 77 samples per 9-h day); selectivity with respect to interferences; small sample size; minimal sample preparation; simultaneous analysis of Ara-C, Ara-U and sodium salicylate; and ready applicability to Ara-C and Ara-U monitoring for intravenous therapy currently in use.

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