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High-performance liquid chromatographic method for analysis of 2′,3′-dideoxyinosine in human body fluids

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SUMMARY

A paired-ion high-performance liquid chromatographic method was developed to measure concentrations of 2′,3′-dideoxyinosine (ddI) in human plasma, urine and cerebrospinal fluid. Samples were prepared using a solid-phase extraction technique which allows for a five-fold concentration of the drug. 2′-Deoxyguanosine was added as an internal standard prior to the extraction. Recoveries for 2′-deoxyguanosine and ddI were 80 ± 15 and 85 ± 10%, respectively. Extracted samples were then injected onto a C_{18} column and eluted isocratically with a mobile phase containing 0.1% of the ion-pairing reagent, heptafluorobutyric acid, and 5% acetonitrile. The retention time was 7.4 min for 2′-deoxyguanosine and 8.4 min for ddI. The lower limit of detection for ddI is 0.1 μM. Using this technique the acid lability of ddI was demonstrated and the plasma concentration versus time profile from a patient receiving the drug was examined.

INTRODUCTION

2′,3′-Dideoxyinosine (ddI) is an analogue of the naturally occurring nucleoside, inosine. Along with dideoxyanalogues of adenosine, guanosine, cytidine and thymidine, ddI has been found to suppress the infectivity and the cytopathic effect of human immunodeficiency virus (HIV) in vitro [1–4]. ddI is currently being evaluated in clinical trials as a potential anti-AIDS drug.

The assay reported here was developed to quantitate ddI in the plasma, urine and cerebrospinal fluid (CSF) of children with AIDS receiving ddI as part of a phase I trial. This rapid and sensitive method uses solid-phase extraction to

isolate and concentrate the sample and paired-ion chromatography using a C_{18} column to elute the extracted sample isocratically with a mobile phase containing heptafluorobutyric acid. Compounds of interest are detected by UV spectrophotometry. The present report describes the methodology involved in this assay and presents results of a pharmacokinetic study in a child treated both with intravenous and oral ddI. The stability of ddI under conditions similar to the acid encountered in the stomach is also examined.

EXPERIMENTAL

Chemicals

Heptafluorobutyric acid was purchased from Pierce (Rockford, IL, U.S.A.). 2′-Deoxyguanosine was obtained from Sigma (St. Louis, MO, U.S.A.). ddI was provided by the Pharmaceutical Resources Branch, National Cancer Institute (Bethesda, MD, U.S.A.). Acetonitrile and methanol were HPLC grade from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Equipment

Chromatographic analysis was done on a Waters high-performance liquid chromatographic (HPLC) system consisting of a Wisp Model 710B injector, two Model 510 solvent-delivery pumps and a Model 490 programmable-wavelength detector. The recorder used was a dual-channel BBC Goerz Metrawatt SE 120. Sample preparation was done using a Baker 10 SPE vacuum manifold connected to an in-house vacuum system and a Meyer N-Evap analytical evaporator.

HIV precautions

Laboratory specimens were handled according to the guidelines set forth by the Centers for Disease Control (CDC) [5–7]. All samples were heated at 57°C for 45 min prior to extraction [8]. All subsequent sample manipulations were also performed in a biological safety cabinet using gloves and gown. Transfer of samples was done with plastic transfer pipets to reduce risk of puncture. Samples were centrifuged in isolation buckets.

Plasma preparation

C_{18} Sep-Pak cartridges were primed with 6 ml of methanol followed by 12 ml of distilled, deionized water. Samples of plasma were prepared by first heating to 57°C in a Sybron/Thermolyne heat block for 45 min (to inactivate HIV) followed by centrifugation at 400 *g* for 10 min. The internal standard (2′-deoxyguanosine, 10 mg per 100 ml of water) was added to achieve a final concentration of 0.5 μg/ml. Samples (usually 1 ml) were then loaded onto the

Sep-Pak cartridges and drawn through under vacuum at approximately 0.5 ml/min. After drawing through all liquid from the plasma, each cartridge was washed with 2 ml of water drawn through under vacuum at approximately 1 ml/min. Samples were then eluted with 2 ml of 100% methanol into test tubes (0.5 ml/min). Total evaporation of the methanol was achieved under a stream of nitrogen while heating in a water bath at 37°C. Samples were reconstituted in a volume of 200 μl of water and vortex-mixed for 30 s. After a final centrifugation for 3 min in an HBI microcentrifuge at 11 900 *g*, samples were analyzed by HPLC as described. Recovery of ddI and deoxyguanosine after extraction was investigated by comparison of an aqueous standard containing the same concentration of ddI as a plasma standard. After extraction of the plasma standard, the two were compared quantitatively on three replicates.

Urine preparation

Urine samples (1 ml) were processed in the same manner as the plasma except that the final reconstitution of each was done into 1 ml since the ddI concentration was usually high enough to not require concentrating the sample. In some cases a further dilution was necessary before analysis. The amount of urine processed is dependent on the dose of ddI the patient was given.

CSF preparation

CSF was also processed in the same manner as plasma except that only 1 ml of water was used to wash samples on the Sep-Pak prior to methanol elution, since the interferences in CSF are minimal. Under conditions where concentrating is not necessary, the CSF may be injected directly into the HPLC system without prior extraction.

HPLC method

The mobile phase, 5% acetonitrile in heptafluorobutyric acid (0.1%, v/v) in deionized, distilled water (v/v), was pumped at a flow-rate of 2.0 ml/min through a Waters NovaPak (10 cm × 8 mm I.D., 4 μm) cartridge in a Z-module (Waters Assoc.). Effluent was monitored at wavelengths of 252 and 260 nm. Under these conditions the deoxyguanosine eluted at 7.4 min and the ddI at 8.4 min. A wash cycle of 100% acetonitrile for 30 s after each run (beginning at 8.5 min) was programmed in to keep the interference from late-eluting peaks to a minimum. In the urine analysis it was necessary to adjust the acetonitrile to 3% because of the additional interfering peaks present. This resulted in an elution time of 19.6 min for ddI.

Acid lability of ddI

A solution containing 1.7 μM ddI in water was incubated at room temperature in 0.01 *M* hydrochloric acid. Aliquots (1 ml) were taken at regular intervals and the acid was neutralized by the addition of 10 μl of 1 *M* sodium hy-

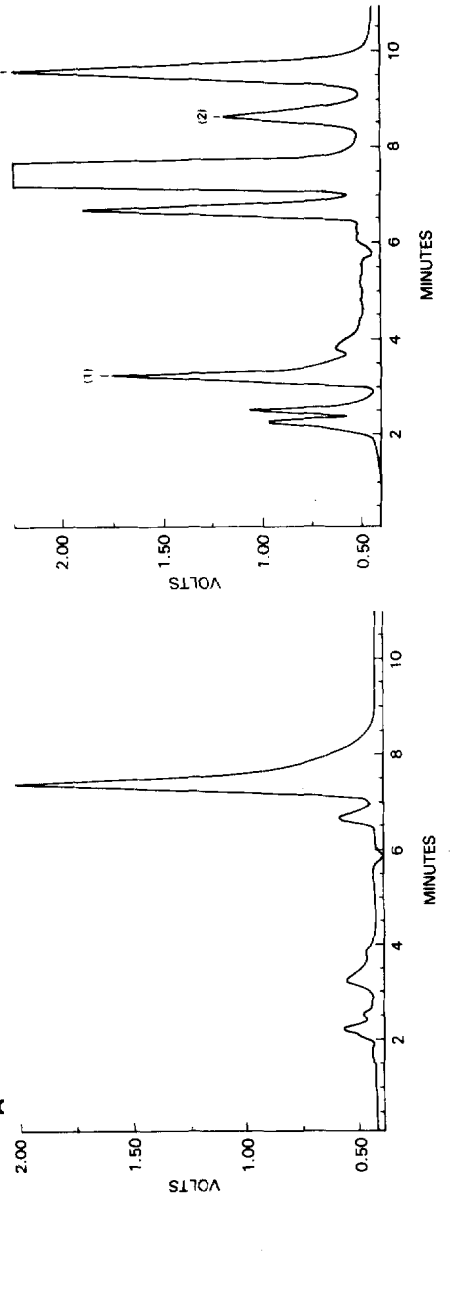

Fig. 1. Chromatograms of (A) blank patient plasma, (B) ddI and internal standard, deoxyguanosine, in patient plasma, (C) blank CSF and (D) CSF spiked with ddI. Chromatographic conditions as described in the Experimental section. Peaks: 1 = hypoxanthine (tentatively identified); 2 = 2′-deoxyguanosine; 3 = ddI.

droxide solution. For the zero time control, neutralizing sodium hydroxide was added to the 0.01 *M* hydrochloric acid before adding the ddI.

RESULTS AND DISCUSSION

The recovery of ddI, checked using 1.7 μM, and the internal standard, deoxyguanosine, checked using 1.9 μM, from spiked plasma was 80 ± 15 and $85 \pm 10\%$, respectively. Spot checks of low (0.212 μM) and high (13.6 μM) concentration standards demonstrated the same recovery throughout the range.

Fig. 1 illustrates actual chromatograms of blank patient plasma, ddI in patient plasma 1 h after receiving an intravenous dose, blank CSF and CSF spiked with ddI. 2′-Deoxyguanosine (peak 2) and ddI (peak 3) elute at 7.4 and 8.4 min, respectively. The lowest detectable amount of ddI is 20 pmol (0.1 μM if the sample is prepared as described above and 50 μl are injected). This allows for a signal-to-noise ratio greater than 3. Attempts to further concentrate or inject a larger volume usually result in the appearance of endogenous peaks which interfere with quantitation of the compounds of interest. 2′-Deoxyguanosine is a suitable internal standard since its recovery, stability and retention time are similar to that of ddI. As can be seen in Fig. 1B, an additional peak has appeared at 3.2 min. This peak coelutes with hypoxanthine, a degradation product of ddI, and has the same wavelength ratio at 252/260 nm as a hypoxanthine standard. Although we have not detected ddI in the CSF of patients receiving low doses of the drug, Fig. 1C and D illustrate that at higher doses this assay will have utility in quantitating ddI in CSF.

Fig. 2 shows a chromatogram of patient urine following a dose of ddI. The greater number of interfering peaks in urine required different conditions and a longer elution time to resolve ddI, as described in the Experimental section. The elution time under these conditions is 19 min.

The plasma concentration versus time profile for a five-year-old male patient treated with 60 mg/m^2 orally and 60 mg/m^2 intravenously is shown in Fig. 3. The peak ddI concentration 1 h after the end of the intravenous infusion was 3.7 μM and the peak concentration following the oral dose was 2.2 μM. Disappearance of this drug from plasma is very rapid and at this dose level ddI is not detectable in plasma after 4 h.

Triplicate standard curves were obtained using spiked plasma samples with concentrations of ddI ranging from 0.212 to 13.6 μM. Using reciprocally weighted linear regression analysis (forced through the origin) [9] the peak-height ratios of ddI to internal standard versus concentration gave a correlation coefficient of 0.998. The standard curve was linear over the entire concentration range tested.

In order to account for the lower levels of ddI observed when the drug is administered orally, the acid lability of ddI was studied by incubating the drug in 0.01 *M* hydrochloric acid (pH 2.0) at room temperature. Fig. 4 shows the

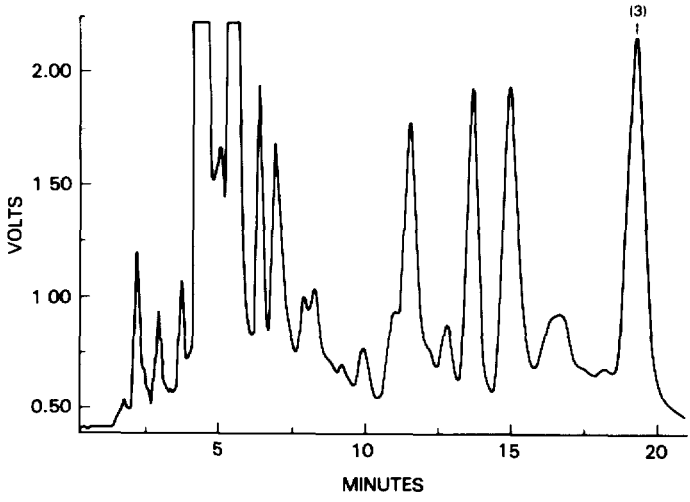

Fig. 2. Chromatogram of ddI in patient urine. Chromatographic conditions as described in the Experimental section. Peak 3 is ddI.

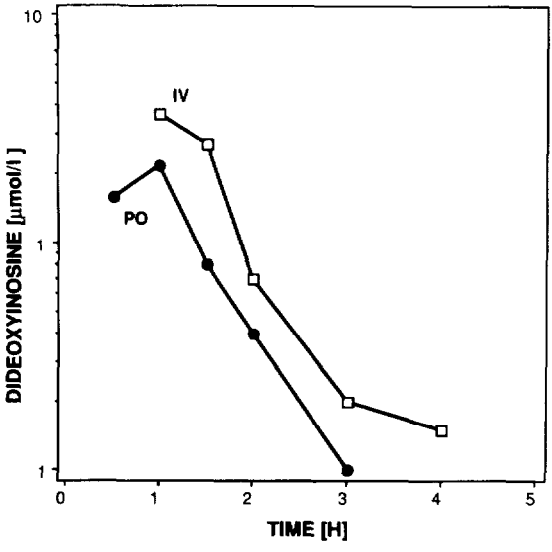

Fig. 3. Plasma concentration versus time profile of ddI in a typical patient after a 60 mg/m^2 intravenous dose (□) and after the same dose administered orally (●). Samples were drawn at the time intervals indicated.

profile of ddI degradation under these conditions. After less than 20 min at pH 2.0, more than half of the original ddI is hydrolyzed. Because of this rapid degradation of ddI under acidic conditions, it is very important that it be administered following an antacid when given orally. Eventually, it may be more

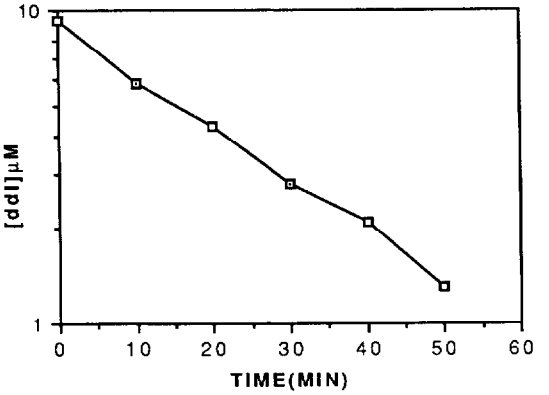

Fig. 4. ddI decomposition in 0.01 *M* hydrochloric acid (pH 2.0) over time.

convenient to have an enteric-coated formulation of the drug to alleviate this problem.

The assay described in this paper is a rapid and sensitive method of quantitating ddI in biological fluids using a simple extraction technique and isocratic elution. As demonstrated, it should prove useful in performing pharmacokinetic studies in patients receiving this drug. The optimal administration of ddI may eventually require therapeutic drug monitoring. Because of the rapid turnaround possible with this method, it could also be used for this purpose.

REFERENCES

1 H. Mitsuya and S. Broder, Proc. Natl. Acad. Sci., 83 (1986) 1911.
2 H. Mitsuya and S. Broder, Nature, 325 (1987) 773.
3 M.A. Johnson, G. Ahluwalia, M.C. Conelly, D.A. Cooney, S. Broder, D.G. Johns and A. Fridland, J. Biol. Chem., 263 (1988) 15354.
4 D. Cooney, G. Aluwahlia, H. Mitsuya, A. Fridland, M. Johnson, Z. Hao, M. Dalal, J. Balzarini, S. Broder and D.G. Johns, Biochem. Pharmacol., 36 (1987) 1765.
5 J.H. Richardson and W.E. Barkley (Editors), Biosafety in Microbiological and Biomedical Laboratories, Public Health Service, Washington, DC, 1984, DHHS Publication No. (CDC)84-8395.
6 CDC, HTLV-III/LAV: Agent Summary Statement, Mortality Morbidity Weekly Report, 35 (1986) 540.
7 CDC, Recommendations for Prevention of HIV Transmission in Health-Care Settings, Mortality Morbidity Weekly Report, 36 (Suppl. 2) (1987) 3S.
8 L. Resnick, K. Veren, S.Z. Salahuddin, S. Tondreau and P.D. Markham, J. Am. Med. Assoc., 255 (1986) 1887.
9 G.D. Knott, Comput. Programs Biomed., 10 (1979) 261.