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Determination of free serum didanosine by ultrafiltration and high-performance liquid chromatography

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method was developed to determine free didanosine concentrations in human serum. An ultrafiltration technique was used to recover didanosine from the samples. Didanosine was analyzed using a 150 mm × 3.9 mm I.D. Nova-Pak phenyl column and a mobile phase of 0.02 *M* sodium citrate (pH 5)–isopropanol (97.5:2.5, v/v) with detection set at 250 nm. Linearity was verified from 25 to 3000 ng/ml. The limit of detection at a signal-to-noise ratio of 3 was 25 ng/ml. The mean recovery of didanosine added to serum at 50, 100, 250 and 750 ng/ml was 97.4%, 97.3%, 92.9% and 95.4%, respectively. A within-day variation of 3.6% at 50 ng/ml and 1.7% at 250 ng/ml, and a day-to-day variation of 9.3% at 50 ng/ml and 3.6% at 230 ng/ml were found. Stability studies indicated that didanosine is stable in serum for at least 8.5 months at 20°C, 4°C and −20°C.

1. Introduction

Didanosine, a recently developed reverse transcriptase inhibitor nucleoside analogue, has been used in the treatment of human immunodeficiency virus (HIV) infection.

In December of 1993, the state-of-the-art conference recommended didanosine therapy as an alternative to zidovudine in cases of therapeutic failure or intolerance, and as an optional choice in the absence of intolerance or clinical failure but evidence of further immunodeficiency [1].

Usually, a weight-normalized dose is used to measure the exposure of an individual patient to didanosine. However, in light of physiologic disturbances in patients with advanced HIV disease, and considering the high variability in didanosine absorption due to its acid-labile nature, the routine measurement of drug exposure in each patient using his individual pharmacokinetic parameters could be extremely helpful in determining dosage. It has been demonstrated that the average steady-state concentration (C_{ssav}) of didanosine is positively correlated with its antiviral activity [2].

All the analytic assays developed to determine didanosine concentration in serum are based on liquid chromatography. The majority of these methods use solid-phase extraction procedures for sample preparation [3–7], except for one that uses a precipitation technique [8].

This paper describes an isocratic reversed-

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phase high-performance liquid chromatographic (HPLC) method to determine serum-free didanosine concentrations using ultrafiltration as the pre-treatment step. The procedure was evaluated in a clinical setting to determine its usefulness in monitoring serum levels in patients receiving didanosine treatment.

2. Experimental

2.1. Standards and reagents

Didanosine was kindly provided by Bristol-Myers Squibb (Princeton, NJ, USA). Trisodium citrate dihydrate GR ($C_6H_5Na_3O_7 \cdot 2H_2O$) was purchased from Merck (Darmstadt, Germany), isopropanol from Romil Chemicals (Loughborough, UK) and the Ultrafree-MC filters (low binding regenerated cellulose PL, 10 000 nominal molecular mass limit) from Millipore (Bedford, MA, USA). All chromatographic solvents were of HPLC grade.

2.2. Instrumentation

The HPLC system used (Kontron Instruments, Milan, Italy) was comprised of a Model 325 pump, a Model 465 autosampler and a Model 432 variable wavelength UV detector. The chromatograph incorporates Kontron data system 450 multitasking version 3.40 software.

2.3. Chromatographic conditions

A modification of the method described by Hartman et al. [4,5] was used. The column (150 mm × 3.9 mm I.D.) was packed with Nova-Pak Phenyl (Waters-Millipore, Bedford, MA, USA) with the particle size of 4 μm. Sodium citrate 0.02 *M* (pH 5)–isopropanol (97.5:2.5, v/v) was used as the mobile phase. The flow-rate was 1.0 ml/min. Didanosine was monitored with the detector set at 250 nm and 0.02 absorbance units full scale (AUFS).

2.4. Sample preparation

The samples were deproteinized by ultrafiltration of 250 μl of serum through Ultrafree-MC filters at 17 000 *g* for 1.5 h, then 50 μl of clear throughput was directly injected in duplicate into the column. The standards were assayed in the same manner.

2.5. Calibration graphs

Aqueous stock solutions containing 0.5, 1, 2.5 and 7.5 μg/ml of didanosine were prepared and stored at −20°C. Working standard solutions containing 50, 100, 250 and 750 ng/ml of didanosine were prepared in drug-free serum from healthy humans by 1/10 dilution of the stock solutions. Deproteinization was performed as described above. The peak area of the drug was plotted against its concentration.

2.6. Patients

Forty-one samples were obtained, under steady-state conditions, from six patients treated with didanosine orally at doses of 100 ($n = 1$), 150 ($n = 2$) and 200 ($n = 3$) mg twice a day. Serum samples were taken 0, 0.25, 0.50, 0.75, 1.25, 2 and 4 h after administration of the drug. Serum samples were stored at −20°C until assayed.

For each serum concentration versus time curve we compiled the maximum concentration found (C_{max}) and the time required to reach it (t_{max}). The area-under-the curve (AUC) was calculated with noncompartmental analysis by trapezoidal rule using a pharmacokinetics program (Abbot-base Pharmacokinetic Systems, version 1.00, Abbot Diagnostics, Irving, TX, USA), and the average steady-state concentration (C_{ssav}) was determined by dividing the AUC by the therapeutic interval.

2.7. Analytical recovery and precision

Analytical efficiency was determined by comparison of the peak area of didanosine from

deproteinized standard solution with aqueous calibration standards.

Within-day variation was determined by assaying serum samples of known concentrations, 50 and 250 ng/ml, 10 and 20 times, respectively, in the same run. Day-to-day variation was calculated by assaying serum samples of known concentrations, 50 and 230 ng/ml, once a day for 8 and 15 days, respectively.

2.8. Stability

Sample stability at 20°C, 4°C and −20°C was determined by assaying a serum sample spiked with 250 ng/ml of didanosine at 1, 2 and 7 days and 1, 2, 3 and 8.5 months.

2.9. Drug interferences

To ensure that other nucleoside analogues, such as zidovudine and zalcitabine, do not interfere in the HPLC separation of didanosine, we assayed a serum sample from healthy humans spiked with 250 ng/ml of didanosine, 2500 ng/ml of zidovudine and 500 ng/ml of zalcitabine.

3. Results and discussion

Fig. 1 presents a typical chromatogram obtained with this procedure. The retention time for didanosine was 5.32 min.

Linearity was verified from 25 to 3000 ng/ml. The limit of detection at a signal-to-noise ratio of 3 was 25 ng/ml. The regression equation and correlation coefficient between the peak area (y) and the concentration (x) of didanosine were $y = 0.05522x - 0.3608$; $r = 0.9983$ ($n = 14$). Mean recoveries from the extraction procedure were: 97.4 ± 13.3% at 50 ng/ml; 97.3 ± 6.2% at 100 ng/ml; 92.9 ± 1.3% at 250 ng/ml; and 95.4 ± 1.8% at 750 ng/ml. The within-day and day-to-day coefficients of variation (C.V.) are presented in Table 1.

Table 2 shows the median and interval values for didanosine C_{max}, t_{max}, AUC and C_{ssav} in the treated patients.

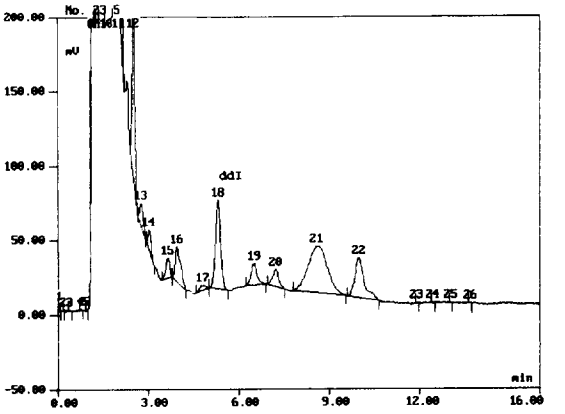

Fig. 1. Chromatogram of serum from a treated patient containing 243 ng/ml of didanosine. Retention time: 5.32 min.

Table 1
Precision of the HPLC assay of didanosine

Concentration added (ng/ml)	n	Concentration found (ng/ml)		C.V. (%)
		Mean ± S.D.	Range	
Within-day				
50	10	49.3 ± 1.8	45.3– 51.1	3.6
250	20	252.4 ± 4.3	240.0–259.1	1.7
Day-to-day				
50	8	47.0 ± 4.4	39.7– 52.1	9.3
230	15	233.6 ± 8.3	222.2–251.4	3.6

Fig. 2 shows the results of drug stability at 20°C, 4°C and −20°C. As may be seen, the didanosine concentration in the sample was maintained between 90% and 110% of the basal value, indicating that didanosine is stable in serum for at least 8.5 months under the storing conditions studied.

Table 2
Didanosine pharmacokinetics data from six treated patients

	Median	Interval
C_{max} (ng/ml)	504	375–1264
t_{max} (h)	0.5	0.25–0.75
AUC [(ng/ml)h]	1098	618–1704
C_{ssav} (ng/ml)	91.5	51.5–112

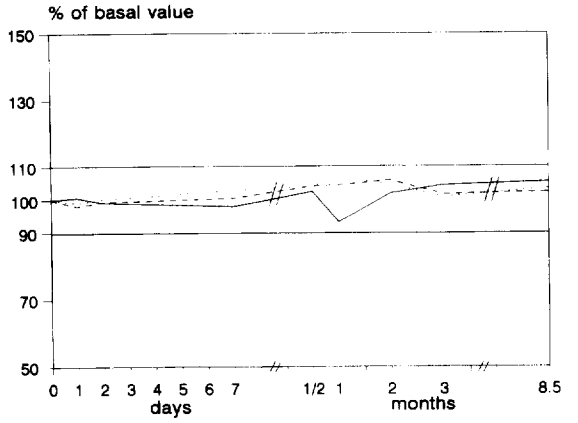

Fig. 2. Stability of a sample spiked with 250 ng/ml of didanosine stored at 20°C (solid line), 4°C (dotted line) and −20°C (dashed line). Results are expressed as percentage with respect to the basal value.

Fig. 3 presents the chromatogram of a serum sample containing didanosine, zidovudine and zalcitabine. The retention times for zidovudine and zalcitabine (22.4 and 3.0 min, respectively) did not affect the chromatographic separation of didanosine.

All the chromatographic methods previously described for the determination of didanosine in biological fluids have utilized solid-phase extraction or precipitation as sample preparation methods. Some impurity problems in the chromatogram were observed with these procedures, due to other co-extracted, unidentified components [9]. To overcome these problems, we applied an ultrafiltration technique, taking advantage of the low protein binding of didanosine and its low molecular mass. This simple and inexpensive procedure measures the concentration of free didanosine, the biologically active drug.

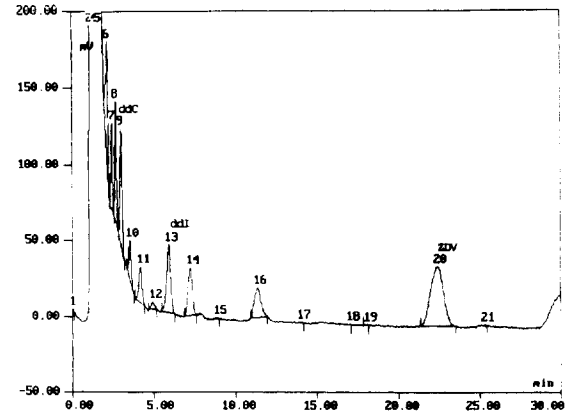

Fig. 3. Chromatogram of a normal serum sample spiked with 250 ng/ml of didanosine, 2500 ng/ml of zidovudine and 500 ng/ml of zalcitabine.

In conclusion, our results indicate that determination of free serum didanosine by ultrafiltration and HPLC is a simple, inexpensive and accurate method that may be used for therapeutic monitoring of this drug in clinical laboratories.

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