



ORIGINAL ARTICLE

# Analysis of *in vivo* absorption of didanosine tablets in male adult dogs by HPLC

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**Abstract** Didanosine is an effective antiviral drug in untreated and antiretroviral therapy-experienced patients with Human Immunodeficiency Virus (HIV). An automated system using on-line solid extraction and High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection was developed and validated for pharmacokinetic analysis of didanosine in dog plasma. Modifications were introduced on a previous methodology for simultaneous analysis of antiretroviral drugs in human plasma. Extraction was carried out on C18 cartridges, with high extraction yield as stationary phase, whereas mobile phase consisted of a mixture of 0.02 M potassium phosphate buffer, acetonitrile (KH<sub>2</sub>PO<sub>4</sub>: acetonitrile: 96:4, v/v) and 0.5% (w/v) of heptane sulphonic acid. The pH was adjusted to 6.5 with triethylamine. All samples and standard solutions were chromatographed at 28 °C. For an isocratic run, the flux was 1.0 mL/min, detection was at 250 nm and injected volume was 20 µL. The method was selective and linear for concentrations between 50 and 5000 ng/mL. Drug stability data ranged from 96% to 98%, and limit of quantification was 25 ng/mL. Extraction yield was up to 95%. Drug stability in dog plasma was kept frozen at –20 °C for one month after three freeze–thaw cycles, and for 24 h after processing in the auto sampler. Assay was successfully applied to measure didanosine concentrations in plasma dogs.

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## 1. Introduction

Didanosine is an important antiretroviral for Acquired Immune Deficiency Syndrome (AIDS) for producing beneficial effects on virological and immunological markers of Human Immunodeficiency Virus (HIV) disease, improving clinical outcome in adults or children with HIV infection [1,2]. It is a synthetic, intracellularly phosphorylated nucleoside analogous to the active metabolite, which inhibits the activity of HIV reverse transcriptase by competing with the natural substrate [3,4]. However, it exhibits a low oral bioavailability because of poor permeability across the intestinal epithelium [5,6]. Antiretroviral agents may be potential candidates for therapeutic drug monitoring to control efficacy and toxicity [7,8]. Our group has previously developed granules containing chitosan microspheres [9] encapsulating didanosine, which increased half life ( $t_{1/2}$ ), promoting the sustained delivery for 36 h, compared to the free buffer tablets and gastro-resistant granules, Videx<sup>®</sup> EC, following a pharmacokinetic profile. Since solid dosage forms cannot be administered in rats, pharmacokinetic analysis of didanosine was tested in male adult dogs. To quantify didanosine in dog plasma, a modified solid phase extraction (SPE) method for sample clean-up was carried out, followed by an on-line reversed phase high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The methodologies reported in the literature for analysis of didanosine in biological samples use SPE together with HPLC methods and UV detection for simultaneous analysis of groups of antiretrovirals in human plasma, such as abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine [10]. Plasma analysis using chromatographic methods always involves a sample preparation procedure using, e.g. liquid-liquid [11], solid-liquid extraction [12], or protein precipitation [13].

The application of SPE for analyses of biological matrices allows either higher recoveries or the elimination of some possible interference from co-administered drugs. It can be labor-intensive and prone to errors. A direct injection technique eliminates multiple sample pre-treatment steps and can increase the throughput of many bioanalytical methods. Automated sample processing reduces potentially dangerous sample handling [14]. In particular, SPE should be used for the analysis of didanosine, because this drug is poorly soluble in organic solvents.

Campbell et al. [15] developed and validated a high-performance liquid chromatographic assay for simultaneous determination of 2'-fluoro-2' 3'-dideoxyadenosine (FddA) and its metabolite, 2'-fluoro-2', 3'-dideoxyinosine (FddI), in dog plasma and urine. SPE was applied to extract FddA, FddI, and the internal standard (3',5'-anhydrothymidine) from the biological samples. The collected samples were chromatographed using a C<sub>8</sub> column coupled with a mobile phase consisting of monobasic phosphate, dibasic phosphate, ethylene glycol monomethyl ether, and water.

Some reports describe simultaneous determination of HIV drugs by HPLC with tandem mass spectrometry in plasma. This methodology can be used for pharmacological research and clinical purposes in rats [16,17] and humans [18–21]. In another study, Yan et al. [5] developed a rapid and efficient HPLC-tandem mass spectrometry coupled with SPE for determining didanosine concentrations in maternal rat plasma, amniotic fluid, placental and fetal tissue samples.

The method was developed and validated, and the results showed limits of detection of 1 ng/mL. Recoveries were 70% or greater for didanosine and valdidanosine compounds in the different matrices. Within and between run precision (%RSD) and accuracy (%error) were less than 15% for all matrices.

In this paper, we describe a modified method, which was validated for the analysis of didanosine in dog plasma, using the SPE C<sub>18</sub> cartridges (Bond Elut<sup>®</sup>), the HPLC stationary phase C<sub>18</sub> column. The mobile phase consisted of a mixture of potassium phosphate buffer, acetonitrile, and heptane sulphonic acid. The method was validated according to published FDA guidelines [22]. In general, analyses described in the literature, using HPLC methods and UV detection (240–260 nm), use mobile phase gradient elution; detection for drug concentrations ranges between 0.005 and 10 µg/mL, and the injected volume is 650 µL [10]. The advantages of our method include the smallest volume of sample (20 µL), which is needed for pharmacokinetic studies in animals, the adjustment of the mobile phase proportion for didanosine only, isocratic elution, and lower quantification levels (from 20 to 4500 ng/mL).

## 2. Experimental

### 2.1. Materials and reagents

Didanosine was provided by Labogen Química Fina e Biotecnologia S.A. (Indaiatuba, Brazil); Didanosine buffered tablets were received as a gift from the Brazilian Government Health Agency. Acetonitrile and methanol (HPLC grade) and triethylamine were purchased from Mtedia<sup>®</sup> (São Paulo, Brazil). Acyclovir (internal standard) was obtained from Henrifarma (São Paulo, Brazil). Heptane sulphonic acid (ion-exchange) was obtained from J.T. Baker<sup>®</sup> (Campinas, Brazil). Distilled water was purified by a Milli-Q system (Millipore<sup>®</sup>).

### 2.2. Preparation of sample solutions

Didanosine solution was prepared in 0.01 M potassium phosphate buffer, pH=7, with a final concentration of 0.2 µg/mL. The same procedure was followed using acyclovir as internal standard. These dilutions were used to sample plasma for the preparation of calibration curves. The standard stock solutions were prepared in triplicate for the calibration curve, the final concentrations of didanosine in dog plasma were 500, 1000, 2500, 5000, 10,000, 20,000, and 50,000 ng/mL with 0.2 µg/mL of acyclovir. Quality control (QC) samples were prepared at concentrations of 75, 1500, and 3500 ng/mL, and were used for validating the analytical method. The internal standard was added in all samples in a concentration of 0.2 µg/mL. Preliminary tests were carried out using a concentration range until the minimum and maximum values were obtained.

### 2.3. On-line SPE system and HPLC conditions

From the analytical methodology described by Notari et al. [10], the conditions were adjusted for the simultaneous determination of didanosine and acyclovir as an internal standard in dog

plasma. The method used in the on-line SPE was Prospekt 2<sup>TM</sup> (Spark Holland, Emmen, The Netherlands) on-line SPE system, consisting of the following modules: automated cartridge exchange (ACE) module for disposable cartridge exchange, high-pressure dispenser (HPD) module for handling of solvents and an auto sampler Triathlon. Prospekt 2<sup>TM</sup> was controlled using SparkLink<sup>TM</sup>. The SPE cartridges used consisted of BondElut C<sub>18</sub>, 10 mm × 2 mm i.d., 40–90 µm particles. HPLC apparatus consisted of a Shimadzu system equipped with LC-10AD VP pump with a low-pressure gradient flow control valve FCV-10AL VP, SCL-10A VP system controller, SIL-10AD VP auto-injector. Shimadzu CLASS-VP software (Version 6.12) was used for data acquisition and mathematical calculations. The chromatography was done by HPLC column C<sub>18</sub> 15 cm × 4 mm, V04-745, preceded by a guard column ACE<sup>®</sup> 5C18, V03-417, Analytica<sup>®</sup>. All samples and standard solutions were chromatographed at 28 °C (standardized lab temperature), using a mixture of potassium phosphate buffer (0.02 M) and acetonitrile (96:4, v/v), added with 0.5% (w/v) of heptane sulphonic acid as mobile phase. The pH was adjusted at 6.5 with triethylamine. The flux was 1.0 mL/min; injection samples were 20 µL, and UV detectors were set at 250 nm.

#### 2.4. Sample preparation by SPE

To prepare the calibration curves, 10 µL of didanosine and 10 µL of internal standard, acyclovir, were added to each 90 µL of dog plasma at different concentrations to obtain the final desired plasma concentrations, and the mixture was vortexed. Quality controls and plasma samples for the pharmacokinetic study followed the same procedures. The injection and the extraction were completely automated using the Prospekt<sup>2TM</sup> system; the cartridge was solvated with 1000 µL of methanol at 3000 µL/min and equilibrated with 1000 µL of water at the same flow rate. After the plasma sample was injected into the cartridge, it was eluted with 500 µL of water at 1000 µL/min to retain didanosine and acyclovir, and to eliminate plasma proteins. The cartridge was then washed with 1000 µL of water and 1000 µL of methanol/water (50:50) in flux 3000 µL/min. The cartridge was automatically changed for a new one, and a new cycle started.

#### 2.5. Calibration curve and validation

The analytical performance of the method was obtained by evaluation of the following parameters: calibration curve linearity, QC intra-day and inter-day precision and accuracy, lower limit of quantification (LLOQ), specificity and stability data [22]. To determine the linearity of the method, six calibration curves with seven calibration points ranging from 50 to 5000 ng/mL were prepared in duplicate and analyzed on two consecutive days. Linear regression analysis of the data from calibration curves gave slope, intercept and correlation coefficients, which were used to determine the concentration of each analyte in the QC control samples. The lowest concentration level giving a chromatographic response with acceptable coefficient of variation was defined as the LLOQ. To determine precision and accuracy, QC samples of didanosine were prepared in triplicate at four concentration levels (25, 75, 2500 and 4500 ng/mL) and analyzed using the procedure outlined above.

#### 2.6. Preliminary stability studies

The stability of didanosine and the internal standard in plasma was studied in three experimental conditions: during three freeze–thaw cycles and after storage at the autosampler carousel at room temperature, these samples were analyzed in zero time, 12 h and 24 h; and long-term stability in serum was assessed after 1 month storage at –20 °C. Stability was evaluated by comparing the obtained concentrations before and after storage. The analyses were carried out in triplicate.

#### 2.7. Pharmacokinetic study

The protocol used for didanosine pharmacokinetic evaluation was previously approved by the Ethics in Research Committee of Universidade Federal do Rio Grande do Sul-UFRGS (#2007760). Experiments with dogs were carried out in the Center of Research in Animal Health of the School of Agrarian Science and Veterinary of UNESP (CPPAR/Unesp-Jaboticabal-SP). A pilot study was carried out to demonstrate the applicability of the developed method. Adult male dogs (10 kg) were given vermifuges a week before the experiment, and were kept separately. The dogs fasted for 12 h before administration and received water and food 2 h after administering the formulation. Didanosine was available as didanosine tablets distributed by Brazilian Government's Health Agency administered orally as a single dose of 500 mg with 10 mL of water ( $n=9$ ). Blood samples (2 mL) were collected from the cephalic vase with heparinized VACUETTE<sup>®</sup> before drug administration (time 0) and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 18, 24, and 36 h after administration. Blood cells were removed by centrifugation for 10 min at 9000 rpm and the separated plasma was stored at –20 °C until assay. Pharmacokinetic parameters were determined by non-compartmental approach. The elimination rate constant ( $k_{el}$ ) was calculated by log-linear regression of the didanosine concentration during the elimination phase and the half-life ( $t_{1/2}$ ) was calculated as  $0.693/k_{el}$ . Didanosine peak plasma concentration ( $C_{max}$ ) and time of peak ( $t_{max}$ ) were obtained by visual inspection of the concentration–time profiles. The area under the plasma concentration versus time curve ( $AUC_{0-t}$ ) from time zero to the time of last concentration measured ( $C_{last}$ ) was calculated by the log-linear trapezoidal rule. The AUC zero to infinity ( $AUC_{0-\infty}$ ) was obtained by the addition of  $AUC_{0-t}$  and the extrapolated area was determined by  $C_{last}/k_{el}$ .

### 3. Results and discussion

The modified HPLC method provided a simple and fast procedure for the quantification of didanosine in dog plasma samples. Preliminary studies with different mobile phase combinations of phosphate buffer and acetonitrile were considered. Different pH values of the mixture were also evaluated. Chromatographic conditions were based on isocratic separation on a reverse phase column, preceded by a guard-column.

The LLOQ was 25 ng/mL, which was the lowest concentration of analyte in our modified method. This concentration presented an accuracy that does not deviate more than 20% of the actual value, and its precision does not exceed 20% of the

coefficient of variation under the experiment, according to FDA Guidelines.

Plasma samples were collected with different concentration levels of didanosine and were analyzed using the optimum SPE conditions. The linearity of the standard curve was checked in 6 different runs after calculating individual slopes and intercepts of each individual curve according to Table 1. The correlation coefficient was  $\geq 0.9994$  for all calibration curves in plasma.

Intra-assay, inter-assay precision and accuracy were performed. The results are reported in Table 2. Acceptable accuracy was achieved for all concentrations investigated and these values are considered adequate for biological samples.

When analyzing drugs from biological fluids using SPE, adequate selection of the convenient extraction conditions (sample pH, composition of the washing and elution solvents, and the nature of the sorbent material) provides the cleanest samples for the chromatographic analysis being an important factor for the selectivity of the entire analytical method. The correct selection of SPE washing and elution solvents provided very clean samples and an adequate selectivity for the analytical method developed. The optimum conditions were obtained using BondElut C<sub>18</sub> cartridges. As the chromatograms in Fig. 1 show, the retention time of acyclovir (internal

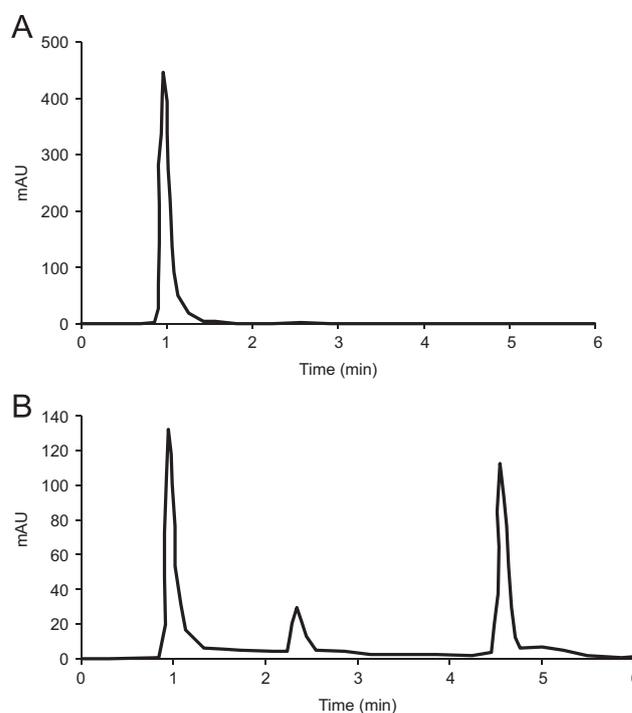
standard) and didanosine was approximately 2.2 and 4.5 min, respectively.

The concentrations of didanosine and acyclovir in plasma samples during three freeze–thaw cycles and in the auto sampler carousel for 24 h at room temperature did not reduce significantly in relation to time zero (Table 3). These results reveal the stability of both drugs in processed plasma samples during the time of analysis. The samples were stable in plasma at  $-20\text{ }^{\circ}\text{C}$  for at least 1 month, with stability data up to 95% in relation to zero time. Thus, the data show that didanosine concentrations remained unchanged under the conditions employed in the study. From these results didanosine stability in dog plasma was confirmed (Table 4).

**Table 1** Linearity of the standard curve in six runs.

Day	Run	Intercepts	Slope	Correlation coefficient
1	1	0.0162	0.0005	0.9972
	2	0.0302	0.0005	0.9981
	3	0.0058	0.0006	0.9964
2	1	0.0119	0.0005	0.9994
	2	0.0659	0.0005	0.9994
	3	0.0670	0.0006	0.9987
	Average	0.0328	0.0005	0.9994
	SD	0.0272	0.00005	
	RSD		9.43	

SD: Standard deviation; RSD: Relative standard deviation.



**Figure 1** HPLC chromatograms of blank dog plasma (A) and of dog plasma after didanosine and acyclovir intake (B).

**Table 2** Intra- and inter-day assay precision of the HPLC assay ( $n=6$ ).

Nominal concentration (ng/mL)	Measured concentration (ng/mL) <sup>a</sup>		Accuracy (%)		CV <sup>b</sup> (%)	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Intra-day						
25	26.0 ± 4.5	28.0 ± 2.6	104	112	17.6	9.4
75	83.0 ± 2.8	82.0 ± 7.0	110	109	3.4	8.5
2500	2526.0 ± 79.0	2637.0 ± 294.0	101	105	3.1	11.1
4500	4485.0 ± 409.0	4467.0 ± 48.0	99	99	9.1	1.0
Inter-day						
25		27.0 ± 1.4		108.0		5.2
75		82.5 ± 0.7		110.0		0.8
2500		2581.0 ± 78.4		103.5		3.0
4500		4476.0 ± 12.7		99.4		0.2

<sup>a</sup>Mean ± SD.

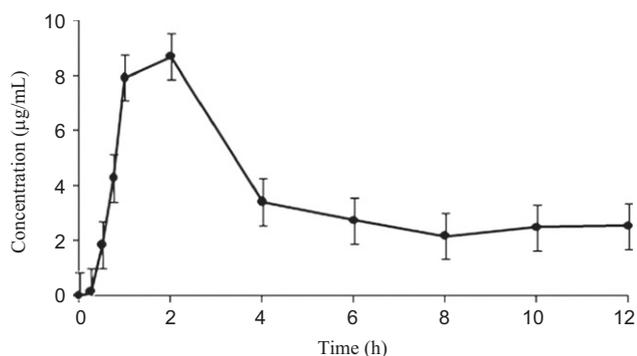
<sup>b</sup>CV: coefficient of variation.

**Table 3** Plasma samples in auto sampler carousel for 24 h at room temperature.

Concentration (ng/mL)	Concentration found (ng/mL)			Stability (%)
	Zero	12 h	24 h	
25	25.0±0.1	24.0±0.3	24.0±0.3	96
4500	4459.0±0.5	4450.0±0.4	4400.0±0.6	97

**Table 4** Plasma samples during three freeze–thaw cycles.

Concentration (ng/mL)	Concentration found (ng/mL)			Stability (%)
	Cycle 1	Cycle 2	Cycle 3	
25	24.0±0.4	24.0±0.2	24.0±0.4	96
4500	4450.0±10.0	4449.0±5.0	4439.0±10.0	98

**Figure 2** Mean plasma concentration–time profile after a single oral dose of 50 mg/kg of didanosine in dog ( $n=9$ ).

The developed SPE–HPLC method was employed to determine the pharmacokinetic profile of didanosine after a single oral dose of 500 mg was given to each dog. Didanosine average concentration versus time profile is shown in Fig. 2. The error bars were below 1%. The peak plasma concentration was  $7935 \pm 1.05$  ng/mL and it took place at 1 h. The half-life of the elimination phase was  $5.4 \pm 2.4$  h. The  $AUC_{0-\infty}$  was  $20 \pm 5$  µg h/mL and extrapolated AUC ( $AUC_{t-\infty}$ ) was  $2 \pm 1$  µg h/mL, around 10% of the total AUC, demonstrating that the sensitivity of the method developed is sufficient to allow quantifying the drug in dog plasma for a period of time long enough to adequately characterize its elimination phase.

#### 4. Conclusions

A bioanalytical modified method for didanosine quantification in dog plasma has been developed and validated, and didanosine showed to be stable in this process. The method reported the analysis of didanosine in plasma by combining a rapid and efficient on-line SPE with a specific and sensitive quantification by HPLC with UV detection. The validation parameters are in accordance with the FDA Guidelines.

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