RESEARCH ARTICLE

Quantization of Didanosine in Human Plasma using High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Present study reports the development and validation of Didanosine in human plasma by LCMS/MS using electron spray ionization technique. Zidovudine was used as an internal standard. The chromatographic separation of analyte and internal standard was achieved by using ACE 5µ, C18 50*4.6mm column as stationary phase and 5mM Ammonium Acetate : methanol (5:95) as mobile phase at a flow-rate of 0.8 ml/min. MS detection was performed at transitions of *m/z* 235.000/135.100 and 266.000/193.000 in multiple reaction monitoring for didanosine and zidovudine at negative mode. Didanosine was extracted from the plasma by solid phase extraction using Orochem-30mg cartridges. The present method was found to be linear over the concentration range of 10.022 - 3003.561 ng/ml (r^2- 0.9983). The limit of quantification of Didanosine in plasma was found to be 10.022ng/ml. The retention times of Didanosine and Zidovudine were found to be 0.77 min and 0.79 min respectively. The analyte was found to be stable under various stability tests such as freeze-thaw, bench top, wet extract, dry extract, auto sampler and interim studies. This simple, rapid and specific validated method was successfully applied for the faster analysis of Didanosine in human plasma in bioavailability and bioequivalence studies.

Keywords: LC-MS/MS, human plasma, Didanosine, Zidovudine, solid-phase extraction, validation.

INTRODUCTION

Didanosine, 9-[(2R, 5S)-5-(hydroxymethyl)oxolan-2-yl]-6,9-dihydro-3H-purin-6-one is an antiretroviral belonging to the class of Nucleoside Reverse Transcriptase Inhibitors (NRTIs). Didanosine is metabolized intracellularly by a series of cellular enzymes to its active moiety, dideoxyadenosine triphosphate (ddATP), which inhibits the HIV reverse transcriptase enzyme competitively by competing with natural dATP. It also acts as a chain terminator by its incorporation into viral DNA as the lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated.

Various analytical methods, such as voltammetric[1], spectrophotometry[2], liquid chromatography with UV detection[3-5], RP-HPLC[6], MALDI-TOF/TOF technology to quantify abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine in the plasma of HIV-infected patients by standard additions analysis[7] and various LC-MS/MS methods have been previously reported for the assay of Didanosine in human plasma[8-12]. Quantification of Didanosine in human plasma using HPLC was developed by few authors which involves longer run time and are more expensive. A method for quantification of Didanosine in human plasma using hyphenated LC-MS/MS method where, the linearity ranged between 25 to 2500 ng/ml and in which the samples were pretreated with solid phase extraction (SPE) was developed[11]. Another LCMS/MS method in which lamivudine was used as an internal standard with run time of 5 mins was developed[9]. The present study describes development and validation of a simple, specific, rapid and sensitive liquid chromatography - tandem mass spectrometry (LC-MS/MS) method for the determination of Didanosine in human plasma with a limit of quantification (LOQ) of 10.022 ng/ml

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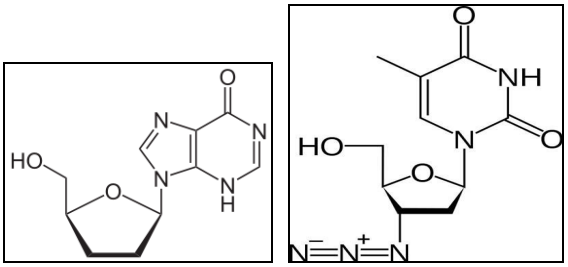
during a 1.8 min run time using Zidovudine as internal standard. The structures of Didanosine and Zidovudine are displayed in Figure 1.

Fig.1: Structure of Didanosine and Zidovudine



Didanosine Zidovudine

MATERIALS AND METHODS

Reagents and chemicals:

Didanosine (99.75% purity), Zidovudine (98.90% purity) were obtained from Vivan Life Sciences Pvt. Ltd., Thane, Maharashtra, India. HPLC grade methanol, ammonia, formic acid and ammonium acetate were purchased from Merck, Mumbai. Orochem SPE Cartridges (30mg) of grade DVB-LP were used. High purity water was prepared through a Milli-Q water purification system (Synergy UV Millipore, USA).

Instrumentation:

API 3200 triple quadrupole instrument (Applied Biosystems SCIEX, Toronto, Canada) was used for mass spectrometry. Shimadzu HPLC system (Shimadzu SIL HTC, USA) was used for chromatographic separation. Electro-spray ionization technique was used. Data was collected and processed using Analyst software version 1.5.1 (Applied Biosystems MDS SCIEX, Toronto, Canada). Ultra microbalance SE2 and Semi Microbalance CPA225D of Sartorius was used for weighing. A high speed desk centrifuge Sorvall Legend XTR of Thermo Scientific was used for centrifugation

MS/MS conditions:

Detection of the ion was performed in MRM mode with negative polarity. Concentrations of 100 ng/ml and 500 ng/ml solutions of Didanosine and Zidovudine were prepared in 80% methanol respectively for tuning the mass conditions. The precursor to product ion transitions for Didanosine and Zidovudine were found to be m/z 235.000/135.100 and 266.000/193.000 respectively. The tuned conditions of Declustering Potential (DP) were -58V, -25V; Collision energy (CE) were -32V, -19V; Collision Cell Entrance Potential (CEP) were -29.22V, -30.06V; Collision Exit Potential (CXP) -13V, -18V for analyte and internal standard respectively. Entrance Potential (EP) -10V, Heater temperature 500ºC, Curtain gas 25 psi, Collision associated dissociation (CAD) 5psi, Nebulizer gas (GS1) 40psi, Heater gas (GS2) 45psi and Ion spray voltage (ISV) -4500V were optimized for both analyte and internal standard. Mass spectrums of parent and product ions of analyte and internal standard are represented in Figure 2 (A, B) and Figure 3(A, B).

Fig. 2A: Parent ion scan of Didanosine

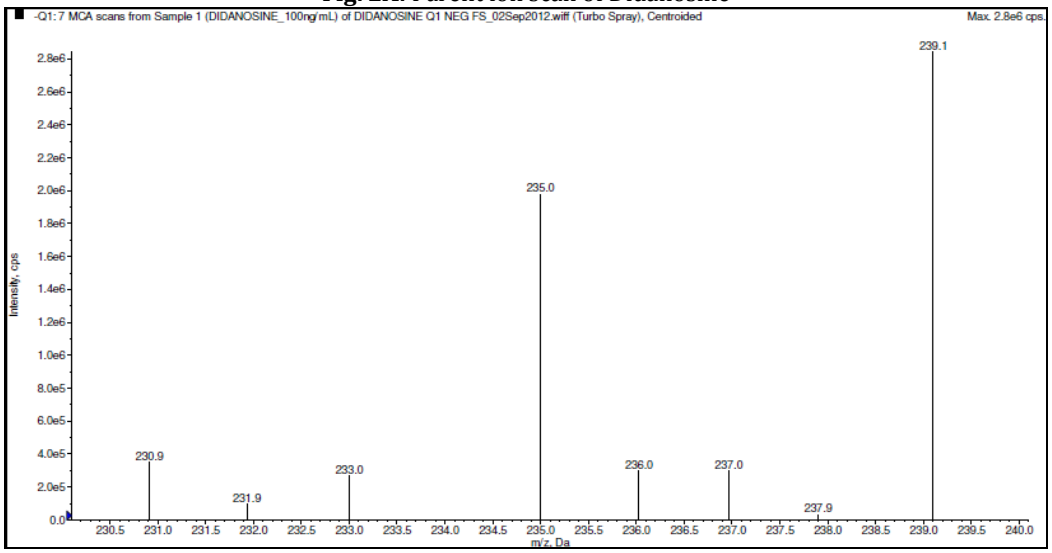


Fig. 2B: Product ion scan of Didanosine

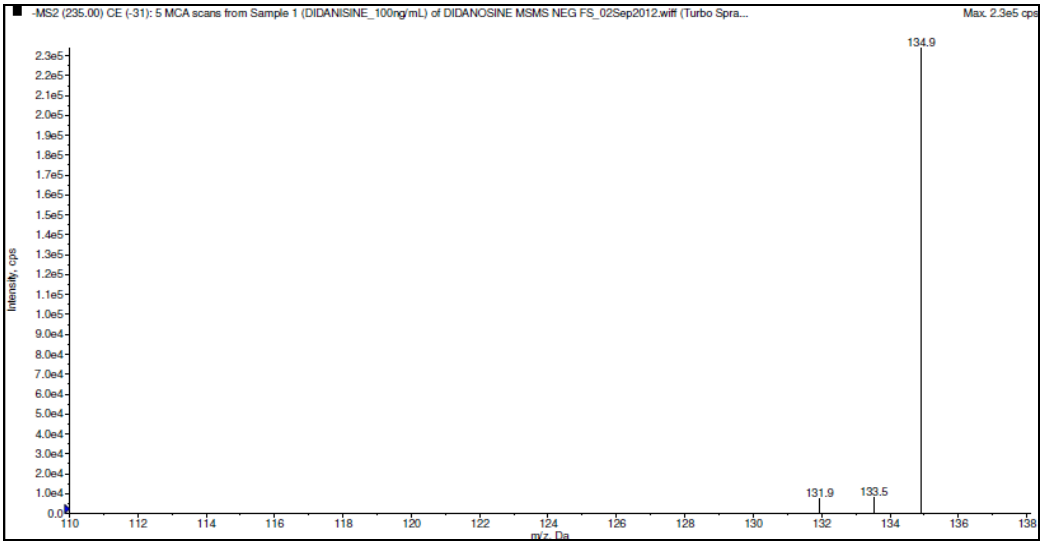


Fig. 3A: Parent ion scan of Zidovudine

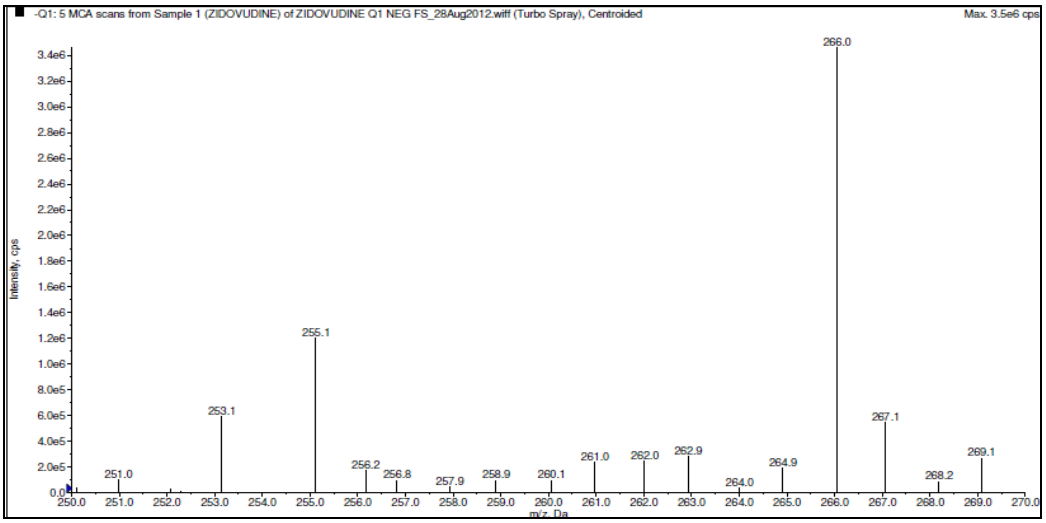
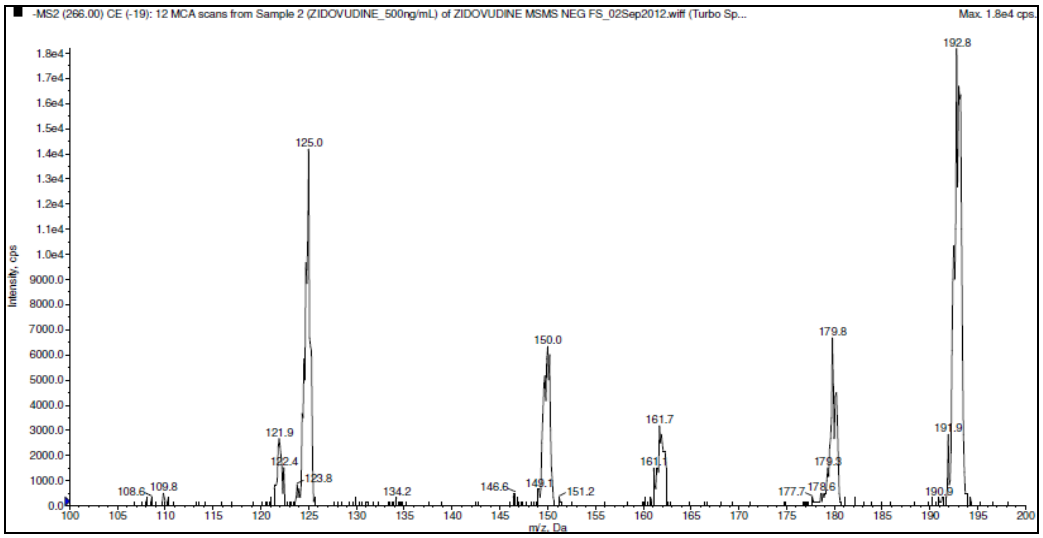


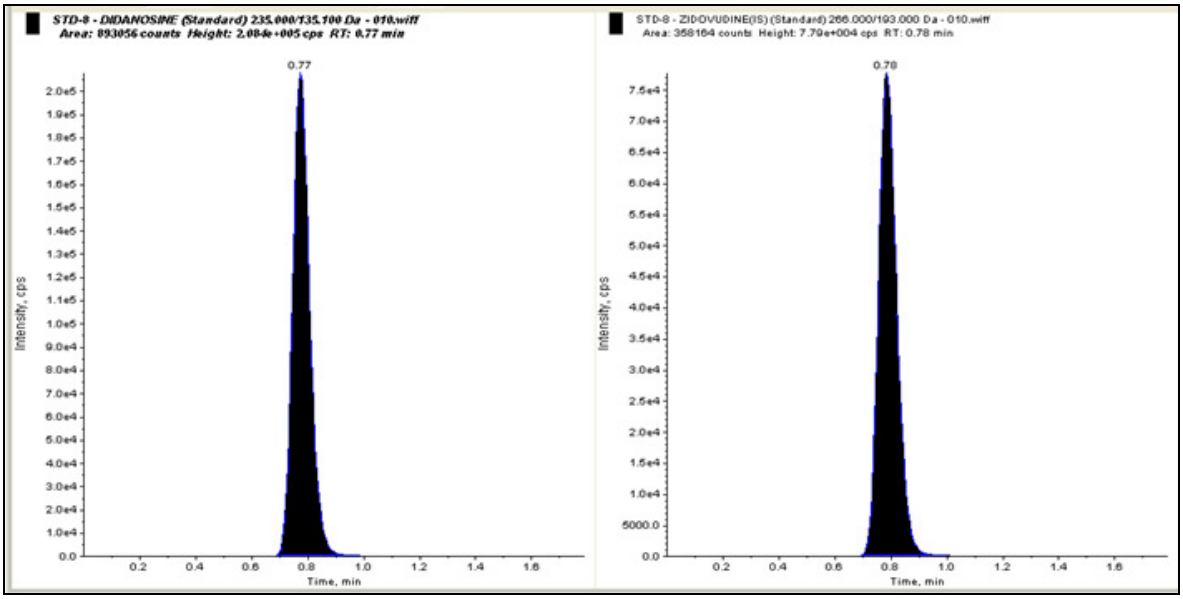
Fig. 3B: Product ion scan of Zidovudine



Chromatographic conditions:

The liquid chromatographic separation was carried out using ACE 5µ, C18 50*4.6mm analytical column and auto-injection volume of 15 µl. Mobile phase was composed of 5mM Ammonium Acetate:Methanol (5:95) and was delivered at a flow rate of 0.8ml/min with run time of 1.8 min. Retention times of Didanosine and Zidovudine were found to be 0.77 min and 0.79 min respectively (Figure 4).

Fig.4: Retention time of Didanosine and IS



Preparation of standards and quality control samples:

Standard stock solutions of Didanosine and Zidovudine of concentration 1mg/ml were prepared in methanol. The internal standard solution was diluted to a concentration of 100µg/ml by using diluent 80% Methanol. Calibration curve standard solutions were prepared by spiking stock solution into drug free human plasma to obtain 8 concentrations of 10.022, 20.043, 301.858, 603.716, 1207.431, 1802.137, 2402.849 and 3003.561 ng/ml by using diluent 80% Methanol. Three quality control (QC) samples, lower quality control (LQC) of 30.579 ng/ml, medium quality control (MQC) of 1528.932 ng/ml and higher quality control (HQC) of 2288.821 ng/ml were prepared in an analogous manner to the calibration standards. Matrix based samples were stored at -70 ±15°C and stock solutions were stored at 2-8°C.

Sample Preparation:

50 µl of internal standard solution (100 µg/ml) was added into labeled ria vial tubes and spiked with 100 µl of plasma sample (respective concentration) into each tube and vortexed briefly. 200 µl of 0.5% (v/v) formic acid was added to the above ria vial tubes and vortexed for 30s. Cartridges were conditioned with 1ml methanol and later equilibrated with 1 ml water. Samples were loaded into SPE cartridges (Orochem-30 mg) and washed twice with 1 ml water. Then the samples were eluted with 1ml of methanol. Each sample was transferred into pre-labeled auto sampler vials and was evaporated until dryness under the nitrogen evaporator. Then the residue was reconstituted with 300 µl of mobile phase and analyzed.

RESULTS AND DISCUSSION

Method Validation:

The study samples, QC samples and calibration standards were processed in accordance with the validated analytical method to ensure the acceptability of the analytical run. The analytical method was validated according to the guidance of US

Department of Health and Human Services Food and Drug Administration[13]. Each analytical run consists of the blank sample (processed matrix sample without analyte and IS) and a zero blank sample (processed matrix with IS), calibration standards at a minimum of 6 - 8 concentration levels, at least six sets of 3 levels of QC samples (low, medium and high) and study samples.

Specificity:

The specificity of the method was determined by comparing the blank sample and a zero blank sample with that of plasma samples spiked with analyte to find out the interferences caused by endogenous substances. This method was found to be specific. Chromatograms of blank and zero blank were represented in Figure 5 and 6.

Fig 5: Blank of Analyte and IS

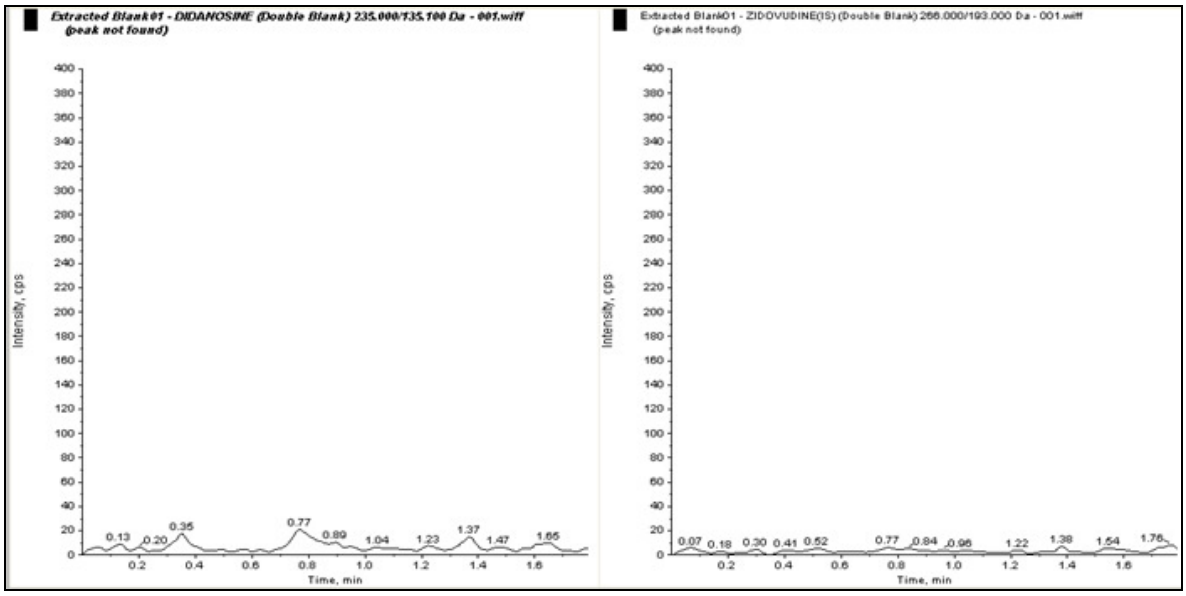
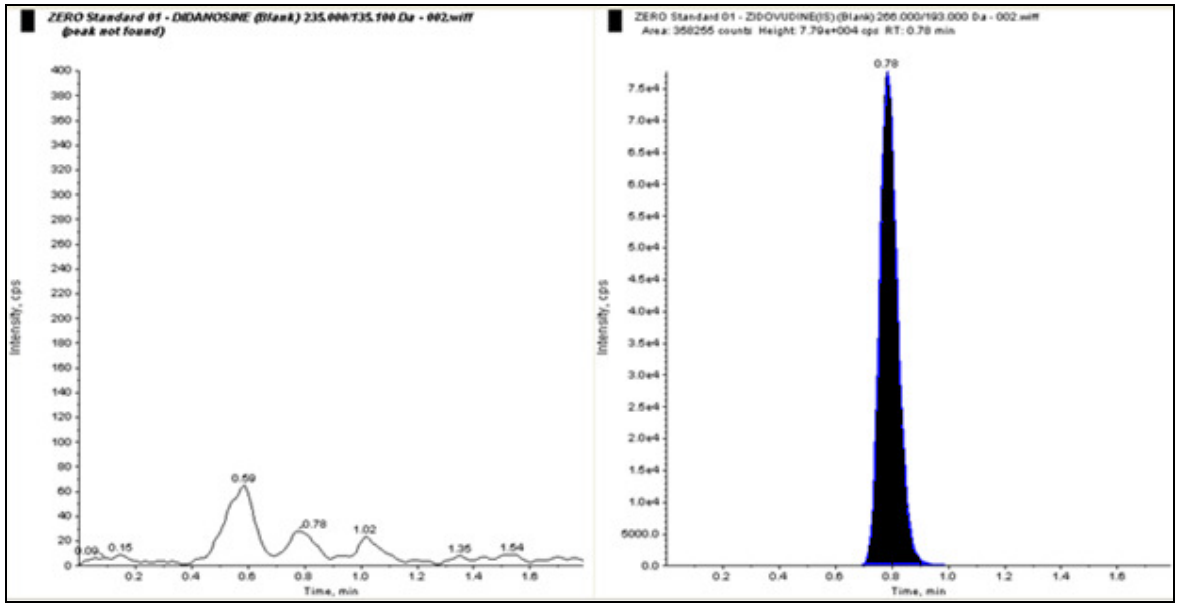


Fig 6: Zero Blank of Analyte and IS

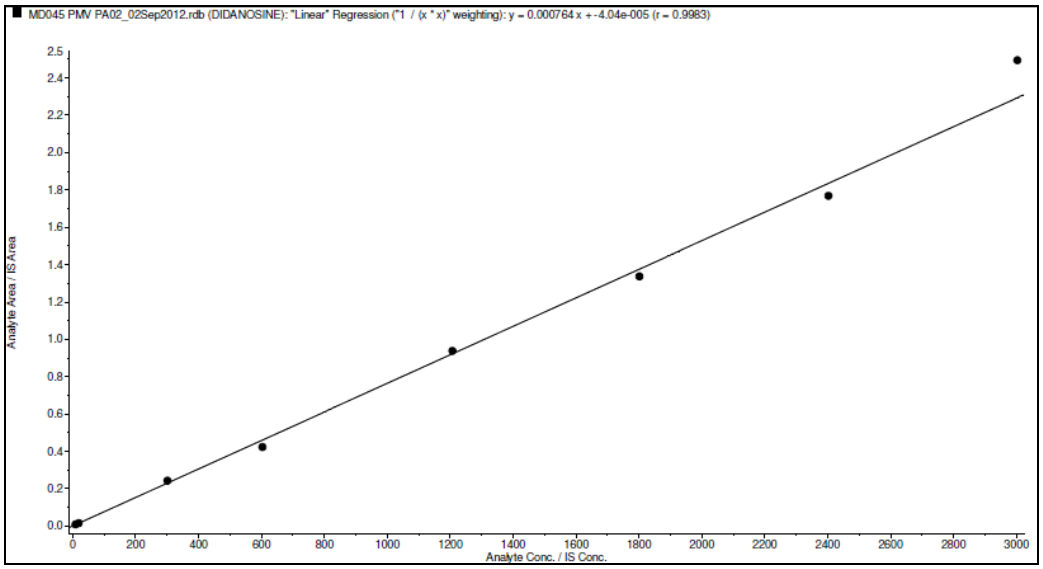


Linearity:

Calibration curve was linear over the concentration range of 10.022 - 3003.561 ng/ml for Didanosine with correlation coefficient (r) 0.9983(Figure 7). The best linear fit and least square residuals for the calibration curve were achieved with a $1/x^2$ weighing factor,

giving a mean linear equation for the calibration curve in the form of y = mx + c, where y was the peak area ratio of Didanosine to Zidovudine and x was the concentration of Didanosine. The lower limit of quantification was found to be 10.022 ng/ml.

Fig.7 Calibration Curve of Didanosine



Precision and Accuracy:

The method precision and accuracy of Didanosine in human plasma was evaluated by using three sets of QC's at three concentrations of HQC, MQC and LQC [Figure 8A, B, C]. Intra-day precision was done on set 1 and set 2, whereas inter-day precision was done on set 2 and set 3. The overall precision of the method expressed as relative standard deviation and accuracy of the method. Inter day batch accuracy ranged from 93.2 % to 99.9 %. Inter day batch precision ranged from 0.2 % to 8.1 %. Intraday batch accuracy ranged from 93.2 % to 99.9 %. Intraday batch precision ranged from 0.2 % to 8.8 %. The mean concentrations, standard deviation (SD), coefficient of variation (%CV) were evaluated and their results were tabulated in table 1. t-test was performed for interday and intraday precision concentrations and they were found to be statistically non-significant. Therefore, the method was found to be precise.

Table 1: Precision and Accuracy studies of Didanosine samples (ng/ml)

CONC. (ng/ml)	**LQC** (30.579)			**MQC** (1528.932)			**HQC** (2288.821)		
	Set 1	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**	**Set 1**	**Set 2**	**Set 3**
Mean	28.52	28.51	28.64	1523.61	1527.03	1525.59	2285.22	2282.95	2284.81
SD (±)	2.499	2.167	2.206	3.402	5.699	4.725	5.158	5.104	5.378
CV(%)	8.8	7.6	7.7	0.2	0.4	0.3	0.2	0.2	0.2
Accuracy (%)	93.3	93.2	93.7	99.7	99.9	99.8	99.8	99.7	99.8

Extraction recovery:

Recovery of Didanosine was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples (LQC, MQC and HQC) to mean peak areas of six unprocessed reference solutions. Recovery of internal standard Zidovudine was evaluated by comparing the mean peak areas of low, medium and high quality control samples to mean peak areas of unprocessed reference solutions of the same concentration. The results were represented in table 2.

Table 2: Extraction recovery data of analyte and internal standard

Drug	Nominal conc	% Recovery	Standard deviation	%CV
Didanosine				
LQC	30.579ng/ml	80.43	3.402	4.2
MQC	1528.932ng/ml	79.38	3.113	3.9
HQC	2288.821ng/ml	81.58	0.838	1.0
Zidovudine	100 µg/ml	82.02	0.0876	0.1

Re-injection Reproducibility:

The re-injection reproducibility was done by comparing the results of re-injected set of samples with that of the original set and results were represented in table 3.

Table 3: Results for reinjection reproducibility

Parameter	Observed Concentration(ng/ml)	
	HQC	LQC
Average Conc.	2285.600	28.213
Standard Deviation	4.1670	3.3824
CV (Precision %)	0.2	12.0
Nominal Conc.	2288.821	30.579
Accuracy (%)	99.9	92.3

Stability Studies:

As a part of method validation, stabilities such as bench top stability, auto-sampler stability, freeze thaw stability, dry extract stability, wet extract stability (in refrigerator and on bench top) were validated. Six replicates were analyzed for each of Low quality control (LQC) and high quality control (HQC) samples at each storage condition. The concentration of Didanosine after each storage period was compared to the initial concentration as determined for the samples that were freshly prepared and processed immediately. The precision and accuracy for the stability samples must be within ≤15 and ±15%, respectively, of their nominal concentrations. The results were represented in table 4.

Table 4: Results for Stability Studies

STABILITIES	TIME	HQC	LQC
Freeze-thaw	At 0 Cycles	2286.82±6.821	30.42±2.062
	After 5 cycles	2289.84±7.497	29.50±2.863
	% Stability	100.2	95.0
	t-Value	0.735	0.637
Bench top	At 0 h	2286.99±6.342	30.98±1.886
	After 24 h	2287.94±5.306	29.26±3.587
	% Stability	100.0	94.7
	t-Value	0.283	1.04
Wet extract at refrigerator	At 0 h	2286.03±6.714	29.93±2.967
	After 24 h	2286.95±6.459	28.82±2.258
	% Stability	100.1	100.4
	t-Value	0.243	0.725
Wet extract at bench top	At 0 h	2286.75±7.099	28.10±3.164
	After 24 h	2289.22±5.300	29.08±2.440
	% Stability	100.0	100.4
	t-Value	0.684	0.605
Dry extract	At 0 h	2286.14±6.323	28.35±3.087
	After 24 h	2288.07±7.504	29.51±3.117
	% Stability	100.1	99.4
	t-Value	0.482	0.651
Auto sampler	At 0 h	2283.13±6.019	30.12±2.650
	After 48 h	2286.90±4.105	28.52±2.508
	% Stability	100.1	96.3
	t-Value	1.27	1.07
Interim	At 0 h	2287.61±5.829	29.16±2.217
	After 48 h	2285.09±5.255	27.69±2.611
	% Stability	99.8	92.8
	t-Value	0.786	1.05

Bench Top Stability (BTS):

The stability of analyte in human plasma stored at room temperature (bench-top stability) was determined by processing bench top stability quality control samples after keeping them at room temperature approximately for 24h and quantifying them against the freshly spiked set of quality control samples.

Freeze-thaw Stability (FTS):

The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed five times against freshly spiked quality control samples.

Wet Extract Stability:

Wet extract bench top and wet extract refrigerator stability of Didanosine was determined by processing and reconstituting quality control samples, keeping them at room temperature and refrigerator (2-8°) approximately for 24h and quantifying them against freshly spiked set of quality control samples.

Dry Extract Stability:

The dry extract stability of Didanosine was determined by processing quality control samples, keeping them in refrigerator for 24h and quantifying against freshly spiked set of quality control samples.

Auto injector stability:

To assess the auto- injector stability of Didanosine, quality control samples were stored into the auto-sampler for the stability period of 48h. These samples were then quantified against freshly spiked quality control samples.

Interim stability:

Samples were initially stored in -25°C and later retrieved after 48h. The samples were then processed and quantified against freshly spiked quality control samples.

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CONCLUSION

A highly selective and rapid LC-MS/MS method employing solid-phase extraction for the determination of Didanosine in human plasma has been developed and validated with a lower limit of quantification of 10.022 ng/ml. The validation data also demonstrates good precision, accuracy and high extraction efficiency. The validated method allows quantification of Didanosine in the linear range of 10.022 - 3003.561 ng/ml. In conclusion, this paper describes a very simple and sensitive LCMS/MS method for the quantization of Didanosine suitable to monitor plasma concentrations during clinical pharmacokinetic and bioequivalence studies in humans.

REFERENCES

1. Percio Augusto Mardini Farias, Arnaldo Aguiar Castro and Ana Isa Perez Cordoves. Didanosine determination in diluted alkaline electrolyte by adsorptive stripping voltammetry at the mercury film electrode. Journal of Electrochemical Sciences. 2012; 2:133-142.
2. Jaiprakash N Sangshetti, Parag A Kulkarni and Devanand B Shinde. Spectrophotometric determination of didanosine in bulk and tablet formulation. Trends in Applied Sciences Research. 2007; 2:71-75.
3. Rosell-Rovira ML, Pou-Clave L, Lopez-Galera R and Pascual-Mostaza C. Determination of free serum didanosine by ultrafiltration and high-performance liquid chromatography. Journal of Chromatography B. 1996; 675 (1):89-92.
4. Naser L Rezk, Richard R Tidwell and Angela DM Kashuba. Simultaneous determination of six HIV nucleoside analogue reverse transcriptase inhibitors and nevirapine by liquid chromatography with ultraviolet absorbance detection. Journal of Chromatography B. 2003; 791 (1–2):137–147.
5. Antonia Maria Cavalcanti de Oliveira, Teresa Cristina Raposo Lowen, Lucio Mendes Cabral, Elizabeth Moreira dos Santos, Carlos Rangel Rodrigues, Helena C

Castro Tereza Cristina dos Santos. Development and validation of a HPLC-UV method for the determination in didanosine tablets. Journal of Pharmaceutical and Biomedical Analysis. 2005; 38 (4):751–756.

6. Verweij-van Wissen CPWGM, Aarnoutse RE and Burger DM. Simultaneous determination of the HIV nucleoside analogue reverse transcriptase inhibitors lamivudine, didanosine, stavudine, zidovudine and abacavir in human plasma by reversed phase high performance liquid chromatography. Journal of Chromatography B. 2005; 816 (1–2):121–129.
7. Stefania Notari, Carmine Mancone, Tonino Alonzi, Marco Tripodi, Pasquale Narciso and Paolo Ascenzi. Determination of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine concentration in human plasma by MALDI-TOF/TOF. Journal of Chromatography B. 2008; 863 (2):249–257.
8. Nicole Clark T, Catherine A White, Michael G Bartlett. Determination of Didanosine in maternal plasma, amniotic fluid, fetal and placental tissues by high-performance liquid chromatography–tandem mass spectrometry. Biomedical Chromatography. 2006; 20 (6-7):605–611.
9. Rita de Cassia E Estrela, Myriam C Salvadori, Renata S L Raices and Guilherme Suarez-Kurtz. Determination of didanosine in human serum by on-line solid-phase extraction coupled to high-performance liquid chromatography with electrospray ionization tandem mass spectrometric detection: application to a bioequivalence study. Journal of Mass Spectrometry. 2003; 38 (4):378–385.
10. Yong Huang, Elisabeth Zurlinden, Emil Lin, Xiaohua Li, Jason and Tokumoto. Liquid chromatographic–tandem mass spectrometric assay for the simultaneous determination of didanosine and stavudine in human plasma, bronchoalveolar lavage fluid, alveolar cells, peripheral blood mononuclear cells, seminal plasma, cerebrospinal fluid and tonsil tissue. Journal of Chromatography B. 2004; 799 (1):51–61.
11. Tan A, Pellerin B, Couture J and Vallee F. Analytical Method for the Determination of Didanosine in Human EDTA K2 Plasma by LC/MS/MS. Anapharm.
12. Zhongtian Yan, Jin Sun, Jinling Wang, Youjun Xu, Yannan Chang and Ping Meng. Simultaneous determination of didanosine and its amino acid prodrug, valdidanosine by hydrophilic interaction chromatography coupled with electrospray ionization tandem mass spectrometry: Application to a pharmacokinetic study in rats. Journal of Chromatography B. 2010; 878 (3–4):466–470.
13. US Department of Health and Human Services, Food and Drug Administration, Guidance for Industry Bioanalytical Method Validation. 2001; Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf [Last accessed on 2013 Jun 17].

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