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Determination of Didanosine in Human Plasma by Electro Spray Ionization LC-MS/MS Method

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A rapid and sensitive analytical method based on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed for the determination of didanosine in human plasma. Sample preparation involved solid phase extraction technique. Chromatography was carried out on an Inertisil ODS 3V column using a mixture of 10 mM ammonium acetate (pH 6.5 ± 0.1) and acetonitrile in a ratio of 10:90 v/v as the mobile phase at a flow rate of 0.6 mL/min. Detection by electro-spray negative ionization mass spectrometry and multiple-reaction monitoring of the transitions of didanosine at m/z 235.2 → 135.2 and of zidovudine at m/z 266.3 → 223.1 was linear over the concentration range 20 (LLOQ)-4000 (ULOQ) ng/mL. Intra-day and inter-day precisions (CV %) were ≤ 5.7 and ≤ 10.2 %, respectively. Accuracy (in terms of percentage relative error) was with in the range of -4.85 to 6.16. Recovery and matrix effect were examined and found to be within acceptable limit (percentage coefficient variance ≤ 15). The method was validated as per FDA and ICH guidelines.

Key Words: Determination, Didanosine, Human plasma, Zidovudine.

INTRODUCTION

Didanosine (9-[(2R,5S)-5-(hydroxymethyl)oxolan-2-yl]-3*H*-purin-6-one) is a cytidine nucleoside analogue. Didanosine[1,2] is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against human immunodeficiency virus type 1 (HIV-1). Didanosine differs from other nucleoside analogues, as it does not have any of the regular bases, instead it has hypoxanthine attached to the sugar ring. Didanosine is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. It is official in USP and BP. Its empirical formula is $C_{10}H_{12}N_4O_3$ with molecular weight 236.2. The structure of didanosine is shown in Fig. 1. A survey of literature reveals the report of a few methods of determination of didanosine in dosage forms[3] and simultaneous determinations in biological fluids based on HPLC[4-8] and LC-MS/MS[9-13]. The authors now propose a selective, accurate and precise LC-MS/MS method for the determination of didanosine in human plasma.

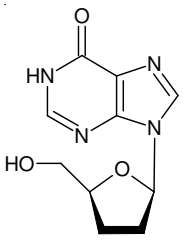

Fig. 1. Structure of didanosine

EXPERIMENTAL

HPLC grade acetonitrile and methanol used in the study were obtained from Qualigens, India. HPLC grade water (milli Q) was prepared from Millipore (USA) equipment. Buffer was prepared by dissolving 0.77 g of ammonium acetate in 1000 mL of milli-Q water. The pH of the buffer solution was adjusted to 6.5 ± 0.1 with acetic acid. The working standard samples of didanosine and zidovudine were obtained from Hetero Drugs Pvt. Ltd. (Hyderabad).

Chromatographic conditions: A Shimadzu HPLC equipment comprising of two LC-10AT VP pumps, VP CTO-10AS VP column oven, a Inertsil ODS, C_{18} (4.6 ID × 50 mm, 5 µ particle size) column and an API 2000 (MDS Sciex) mass detector was used for chromatographic separation. The detection of the compounds carried out by negative polarity mode ionization with multiple-reaction monitoring (MRM). The mass transitions of didanosine (mass spectra was shown in Fig. 2) were observed at m/z 235.2 → 135.2 and for zidovudine at m/z 266.3 → 223.1. A mobile phase consisting

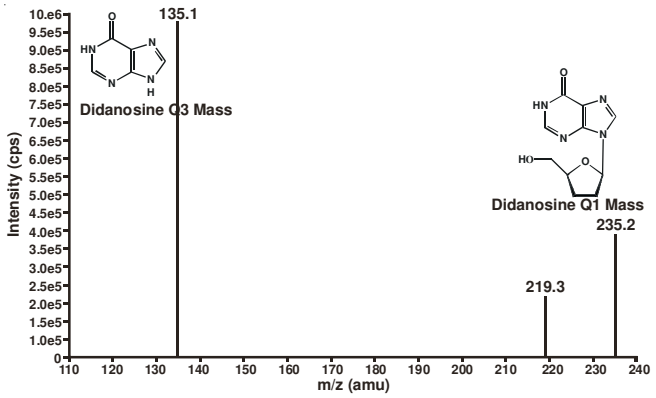

Fig. 2. Mass spectra of didanosine for parent and product masses

of 10 mM ammonium acetate buffer (pH 6.5 ± 0.1) and acetonitrile in a ratio of 10:90 v/v was used at a rate of 0.6 mL/min. Data was acquired and processed with analyst 1.4.2 Software.

Working standard solutions and the calibration curve: The stock solutions of the drug and the internal standard were prepared in a mixture of methanol and water (60:40 v/v) at a free base concentration of 1 mg/mL. The working standard serial dilutions were prepared from the stock solution by using the same diluent. These working standard serial dilutions were used to prepare the calibration curve standards and quality control samples. The solutions for obtaining the eight point standard calibration curve were prepared by spiking the screened blank plasma with appropriate amounts of didanosine working standard serial dilutions. The calibration curve was linear in the range of 20-4000 ng/mL ($r \geq 0.998$). The calibration plot was drawn with a weighing factor of $1/X^2$. The quality control samples were prepared at four concentration levels of 20.1 ng/mL (LLOQ QC), 56.4 ng/mL (LQC), 2015.3 ng/mL (MQC) and 3198.9 ng/mL (HQC).

Method development: For optimum detection up to nanogram level of didanosine and zidovudine (as internal standard) in human plasma, it was necessary to adjust not only the chromatographic conditions and the mass parameters but also to develop an efficient extraction method that gives consistent and reproducible recovery of the analyte from the plasma. The parent ion and the product ions areas were optimized by injecting 1000 ng/mL solutions of didanosine and zidovudine into mass spectrometer to observe 10-350 m/z range. The intensity was checked in positive as well negative polarity modes using electro spray ionization technique. But the best intensity for $[M-H]^+$ ions was found in negative mode for the analyte and internal standard as they have ability to donate protons. Most abundant product ions were obtained at m/z 135.2 and 223.1 for didanosine and zidovudine, respectively by applying sufficient collision assisted dissociation (CAD) gas and collision energy (CE). The optimization of source temperature and heater gas flow is important as they play a major role in minimizing ion suppression which alters the sensitivity. Increase in source temperature and heater gas above 375 ºC and 8000 cm^3/mL, respectively augmented the intensity for the drug. Minor changes in ion spray voltage (ISV) and the nebulizer gas did not have a marked effect on the signal intensity and were maintained at -4500 and 7 ψ, respectively. A dwell time of 200 milli seconds was found to be sufficient and no cross-talk was observed between the multi reaction monitoring cycles (MRM's) of the drug and the internal standard.

Chromatographic analysis of the drug and the internal standard was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Separation was attempted using various proportions of acetonitrile and the buffer on different columns of C_8-C_{18}. Among these, hypersil C_{18} advanced high purity column and inertsil ODS C_{18} (4.6 ID × 50 mm, 5μ) were proved to be ideal for separation of the components with high sensitivity and good peak shape. Usage of ammonium acetate buffer helped in achieving good response for mass detection operating in the negative polarity mode. To get a good chromatographic separation with desired reproducible response it was observed that mobile phase consisting of 10 mM ammonium acetate and acetonitrile (10:90 v/v) was found to be suitable as the analyte was deprotonated. High content of acetonitrile in the mobile phase helped in eluting didanosine and zidovudine within 2.5 min run time at a flow rate of 0.6 mL/min. Inertsil ODS C_{18} column gave good peak shape and response even at lower limit of quantification (LLOQ) for didanosine. Low injection volume (10 μL) reduces overloading of the column with the analytes, thereby ensuring more number of injections on the same column.

For the extraction of drug and the internal standard form plasma samples with reproducible recovery and negligible matrix effect, an efficient method was necessary. A simple and rapid solid phase extraction method was developed using a 30 mg/cc HLB cartridge. Elution was carried out with 1 mL of methanol. The eluate was subjected to drying and then reconstituted to increase sensitivity with improved response up to LLOQ level. No interference was observed from any component of plasma matrix at the retention times corresponding to the drug and the internal standard.

Of the several compounds tried for their suitability as internal standards in negative polarity mode zidovudine belonging to the same class of compounds was found to be more appropriate as there was no significant effect on the recovery, sensitivity and ion suppression of didanosine. The results of the method validation using zidovudine were acceptable in the entire study as per the guidelines.

Sample extraction procedure: Three hundred and fifty μL each of spiked plasma calibration curve standards and quality control samples were transferred to pre-labeled poly propylene tubes containing 50 μL of the internal standard (1500 ng/mL of zidovudine). The tubes were vortexed for 10 s. Each of the HLB 30 mg/1 CC cartridges was conditioned with 1 mL of methanol followed by equilibrating with 1 mL of water on the solid phase extraction chamber. The above samples were loaded on to the cartridges and the cartridges were washed with 1 mL of water followed by 1 mL of 5 % methanol in water. The cartridges were dried for *ca.* 1 min and eluted with 1 mL of methanol. The eluates were evaporated in a stream of nitrogen for 20 min at 50 ºC and the residues in the dried tubes were reconstituted with 0.3 mL of the mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed by injecting 10 μL of the sample into the column.

RESULTS AND DISCUSSION

Specificity: A representative chromatogram obtained from processing of blank plasma sample. The assay procedure is found to be specific as no interfering peaks were observed in the blank plasma at the retention times corresponding to the drug and the internal standard. All blank plasma lots used to prepare calibration standards and quality control samples were also free from interfering peaks.

Linearity: The peak area ratios of calibration curve standards were proportional with the concentration of analyte in each assay over the nominal concentration range of 20-4000 ng/mL. The calibration curves appeared linear and well described by least square method with correlation coefficient (r) ≥ 0.998. A weighing factor of $1/X^2$ (X = concentration) was chosen to achieve a homogeneity of variance. The observed mean back calculated concentrations with accuracy (percentage nominal concentration) and precision (percentage coefficient of variance) of six linearties are given in Tables 1 and 2.

TABLE-1
SUMMARY OF CALIBRATION CURVE PARAMETERS (FOR SIX CURVES/LINEARTIES)

Calibration curve	Slope	Intercept	Correlation coefficient
1	0.00021	-0.00081	0.9983
2	0.00020	0.00058	0.9984
3	0.00021	0.00000	1.0000
4	0.00020	0.00049	0.9996
5	0.00021	-0.00449	0.9981
6	0.00021	0.00250	0.9997

TABLE-2
PRECISION AND ACCURACY OF THE CALIBRATION CURVE STANDARDS (n = 6)

Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	Coefficient variation (%)	Relative error (%)
20.0	19.6	8.5	2.07
40.0	39.9	9.7	0.19
99.9	103.2	6.6	-3.27
249.7	257.4	9.6	-3.06
701.5	711.6	4.8	-1.44
1499.0	1500.2	7.1	-0.08
3202.9	3366.8	4.0	-5.12
4003.7	4084.0	3.5	-2.01

Precision and accuracy: Inter-day and intra-day precision and accuracy were calculated by replicate analysis of six quality control samples at each level of (LLOQ QC, LQC, MQC and HQC) and the results are presented in Table-3. The relative standard deviations for all quality control samples presented in the precision data and per cent nominal concentration values in the accuracy were within the acceptance criteria. The limit of detection and limit of quantification of didanosine were found to be 5 and 10 ng/mL, respectively.

Stability

Short term stability or bench top stability: Six replicates of QC samples at LQC and HQC levels were drawn from the deep freezer (stability samples). These samples were kept unprocessed at room temperature for a period of about 8 h (based on the expected duration of sample extraction). After that, calibration curve standards and six replicates of LQC and HQC samples (comparison samples) were freshly taken from deep freezer and processed along with the stability samples. The results (Table-4) are found to be within the acceptance limits.

Auto-sampler stability or wet extract stability: Six replicates of LQC and HQC samples were processed and loaded into the auto injector and kept for 22 h (stability samples). These stability samples were then injected and analyzed against freshly taken and processed calibration curve standards and 6 replicates of LQC, HQC samples (comparison samples). The results (Table-4) are found to be within the acceptance limits.

Freeze-thaw stability: Drug stability was determined after four freeze-thaw cycles for six aliquots of each of the LQC and HQC level quality control samples (stability samples). The stability samples stored at intended temperature (-20 ºC) were withdrawn after a minimum storage of 24 h and thawed unassisted at room temperature. When completely thawed, the samples were again frozen for 12-24 h under the same conditions. The same freeze thaw cycle was repeated thrice for the above samples, which were then analyzed along with freshly taken CC standards and 6 replicates of LQC and HQC samples (comparison samples). The results presented in Table-4 are found to be within the acceptance limits.

Dry extract stability: Six replicates of LQC and HQC samples were processed and kept for 27 h in refrigerator (stability samples). These stability samples were then injected and analyzed against freshly taken and processed calibration curve standards and 6 replicates of LQC, HQC samples (comparison samples). The results (Table-4) are found to be within the acceptance limits.

Dilution integrity: This was determined by diluting the ULOQ plasma concentration to 50 and 25 % individually (n = 6) with screened blank plasma. These samples were analyzed and estimated similarly like quality control samples. The results of dilution integrity were within acceptance and were presented in Table-4.

Long term stability: Six replicates of LQC and HQC samples were stored in the freezer at -20 ºC to perform long term stability of the drug in matrix for a period of 9 days. After completion of the appropriate duration, these QC samples

TABLE-3
PRECISION AND ACCURACY BATCH

QC ID	Nominal concentration (ng/mL)	Intra batch				Inter batch			
		n	Mean concentration observed (ng/mL)	CV (%)	RE (%)	n	Mean concentration observed (ng/mL)	CV (%)	RE (%)
HQC	3198.9	6	3002.0	5.0	6.16	36	3251.07	5.6	-4.85
MQC	2015.3	6	2065.5	4.0	-2.49	36	1999.69	7.5	-3.24
LQC	56.4	6	57.3	4.2	-1.54	36	53.58	6.4	4.35
LLOQ QC	20.1	6	20.9	5.7	-3.80	36	20.82	10.2	-3.51

TABLE-4
STABILITY EXPERIMENTS DATA

Details of stability experiment	n	Spiked concentration (ng mL^{-1})	Mean calculated comparison sample concentration	Mean calculated stability sample concentration	Mean percent change
Bench top stability HQC	6	3198.9	3212.40	3000.40	-6.60
Bench top stability LQC	6	20.1	20.50	20.70	0.98
Auto-sampler or Wet extract stability HQC	6	3198.9	2978.50	3142.40	5.50
Auto-sampler or Wet extract stability LQC	6	20.1	18.90	20.40	7.94
Freeze-thaw stability HQC	6	3198.9	3197.40	3000.40	-6.16
Freeze-thaw stability LQC	6	20.1	21.50	20.70	-3.72
Dry extract stability HQC	6	3198.9	2991.30	3142.40	5.05
Dry extract stability LQC	6	20.1	19.00	20.40	7.37
Long term stability HQC	6	3198.9	3111.80	2978.10	-4.30
Long term stability LQC	6	20.1	21.10	19.60	-7.11
Dilution integrity at 50 % of ULOQ (D/2)	6	2001.8	–	2042.98	-2.06
Dilution integrity at 25 % of ULOQ (D/4)	6	1000.9	–	1035.08	-3.42

were retrieved from the freezer (stability samples) and processed along with the fresh calibration standards and QC samples (comparison samples), which were prepared by using a new drug stock solution on the same day of the experiment. The results (Table-4) are found to be within the acceptance limits.

Acceptance limits: For all the experiments the coefficient variance (for precision) should be ≤ 15 % and the nominal concentration (for accuracy) should be 85-115 % in between.

Recovery: The absolute recovery was determined by comparing the mean (n = 6) peak area response of the extracted samples against un-extracted samples (same concentrations in the mobile phase). The per cent recovery at each level of LQC, MQC and HQC along with the overall mean recovery values for both the drug and the internal standard were calculated. The mean recovery of the drug at LQC, MQC and HQC level was 85 % and the recovery of the internal standard at MQC level was 90.

$$\text{Recovery of analyte (\%)} = \frac{\text{Mean analyte peak response in extracted samples}}{\text{Mean analyte peak response in unextracted samples}} \times 100$$

Matrix effect: Matrix effect is due to co elution of components present in biological samples. These components may not give a signal in MRM of target analyte but can certainly increase or decrease the analyte response dramatically to affect the sensitivity, accuracy and precision of the method. Thus effect of matrix plays an important and integral part of validation for quantitative LC-MS/MS method. The per cent matrix effect at each individual level of LQC, MQC and HQC was calculated by using the formula given below and were found to be 95, 98 and 105 %, respectively.

$$\text{Matrix effect (\%)} = \frac{\text{Mean peak area ratio in extracted samples}}{\text{Mean peak area ratio in unextracted samples}} \times 100$$

$$\text{Peak area ratio} = \frac{\text{Drug peak area counts}}{\text{Internal standard peak area counts}}$$

Conclusion

The developed and validated LC-MS/MS method for didanosine is selective, rugged and can also be applicable for bio-availability and bio-equivalence studies. This method has significant advantages in terms of clean and reproducible solid phase extraction procedure and a short chromatographic runtime of 2.5 min. The extraction method gave consistent and reproducible recoveries for the drug and the internal standard from plasma, with minimum matrix effect. The reconstituted residue after evaporation (10 µL) is directly submitted for LC-MS/MS analysis to give high throughput.

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