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Development And Validation Of Sensitive LC-MS/MS Method For Determination Of Doravirine In Human Plasma Samples

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Abstract: Doravirine is a non-nucleoside reverse transcriptase inhibitor for use in the treatment of HIV/AIDS. A simple, rapid and sensitive liquid chromatography with tandem mass spectrometry (LC-MS/MS) assay method has been proposed for the determination of Doravirine in human plasma samples using Delavirdine as internal standard (IS). Analyte and the IS were extracted from the 100 μL of K2 EDTA human plasma by Solid Phase extraction (SPE). The chromatographic separation was achieved on a Zodiac C18 column by using a mixture of Methanol and 0.1% formic acid buffer (85:15, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The calibration curve obtained was linear (r^2-0.99) over the concentration range of 0.15 – 40.4 ng/mL. The Mass detection of Doravirine involves m/z-426.5 (parent) and 112.5 (product) and Delavirdine involves m/z - 457.2 (parent) and 362.1 (product) as internal standard in Positive ion mode. Method validation was performed as per FDA guidelines and the results met the acceptance criteria. The intra-day and inter-day precision (%CV) and accuracy results in three validation batches across six concentration levels were well within the acceptance limits. A run time of 2.00 min for each sample made it possible to analyze more number of samples in short time, thus increasing the productivity. The assay was found to be sensitive, selective and reproducible.

Keywords: Doravirine, Solid-Phase extraction, human plasma, Validation, LC-MS/MS.

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1.   INTRODUCTION

Doravirine has been used in trials studying the treatment of HIV-1, HIV-1 Infection, Renal Impairment, and Human Immunodeficiency Virus (HIV) Infection. [1-5] Doravirine does not inhibit the human cellular DNA polymerases α, ß, and mitochondrial DNA polymerase γ. The chemical name for doravirine is 3-chloro-5-[[1-[(4,5-dihydro-4-methyl-5-oxo-1H-1,2,4-triazol-3-yl)methyl]-1,2-dihydro-2-oxo-4-(trifluoromethyl)-3-pyridinyl]oxy]benzonitrile. It has a molecular formula of $C_{17}H_{11}ClF_3N_5O_3$ and a molecular weight of 425.75 (Figure.1)[6-8, 11-15].
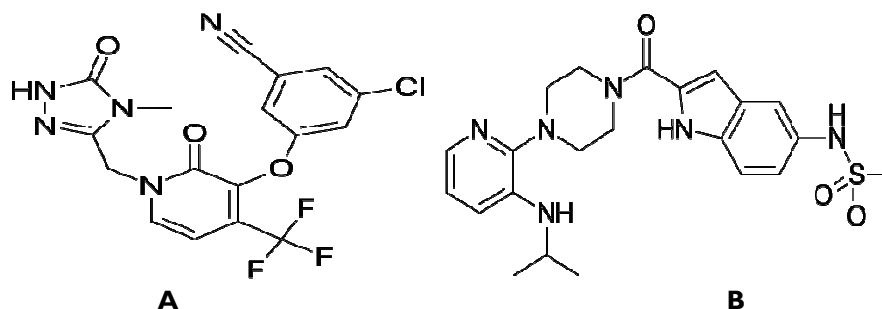
Fig 1. Chemical structures of A) Doravirine (DV) D) Delavirdine(DVIS)

From the literature, it is evident that there is only one LC-MS/MS [9] method available for the quantitative determination of Doravirine (DV) in human plasma samples using Delavirdine(DVIS) as internal standard. From literature survey it reveals that the requirement for change of analytical method due to their higher runtime and sample preparation process. An analytical method must be rugged, simple, reproducible and cost effective in quantifying analyte concentrations in plasma. A simple SPE method was developed on LC-MS/MS employing a low volume of 100 µL plasma without usage of high volumes of chemicals with a shorter chromatographic runtime of 2.0 minutes using Delavirdine(DVIS) as internal standard (Fig.1). Among the reported literature methods, the present method involves less retention time and Solid Phase extraction which is modern and less reported method. The analytical method should satisfy the scientists in terms of simplicity, sensitivity, runtime, time consumption, sample volume and efficient extraction procedure. The developed method was validated as per FDA guidelines[10]

2.   MATERIALS AND METHODS

A Reference sample of Doravirine was obtained from Clearsynth Labs Ltd. (Mumbai, India), while Delavirdine was form Clearsynth Labs Ltd (Mumbai, India).

2.1   LC–MS/MS instrumentation

An HPLC system consisting of a MDS Sciex API–4500 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface . The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 90, 38, 10, 11 V for Doravirine and 90, 38, 10, 11V for the IS. Detection of the ions was carried out in the multiple reaction monitoring mode (MRM), by monitoring the transition pairs of m/z 426.5 parent ion to the m/z 112.5 for product ion of Doravirine and m/z 457.2 parent ion to the m/z 362.1 product ion for the Delavirdine(IS) were shown in Fig.2. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst Software™ (version 1.6.3). The MRM technique provided intrinsic selectivity and sensitivity, hence chosen for the study.
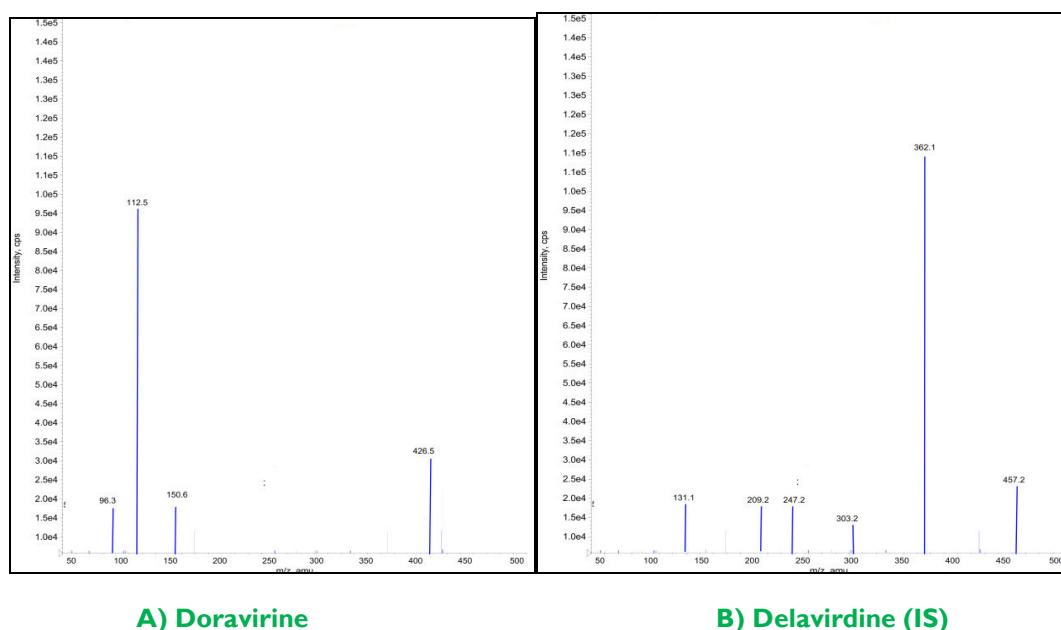
A) Doravirine

B) Delavirdine (IS)

Fig 2. Product ion mass spectra of [M+H]+ of Doravirine and Delavirdine (IS).

2.2 Preparation of plasma standards and quality controls

Standard stock solution of Doravirine and IS (1mg/mL) were prepared in Methanol. Working solutions for calibration and controls were prepared by appropriate dilution in Methanol and water (70:30, v/v; diluent). The IS working solution (200 ng/mL) was prepared by diluting its stock solution with diluent. Stock solutions of Doravirine and IS were found to be stable for 3 days at 2-8 C. Calibration samples were prepared by spiking 950 µL of control K2 EDTA human plasma with the 50 µL working standard solution of the analyte as a bulk, to obtain Doravirine concentration levels of 0.15, 0.30, 0.65, 1.62, 4.06, 8.12, 16.2, 24.2, 32.3 and 40.4 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.15 (LLOQ), 0.43 (LQC), 5.19 (MQC1), 20.7 (MQC2) and 31.2 ng/mL (HQC) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in microcentrifuge tubes (Tarson, 2 mL) and stored in the freezer at -70 ± 10° C until analyses.

2.3 Sample processing

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. One set of calibration curve standards, one or more sets of quality control samples and subject plasma samples were withdrawn from the deep freezer and allowed thawing at room temperature. The thawed samples were then vortexed to ensure complete mixing of the contents. Pipette out 100 µL

of the sample into pre-labelled RIA vial tubes. 10 µL of internal standards dilution (200 ng/mL of Delavirdine) were added except in blank and subject pre-dose sample wherein 10 µL of diluent were added and vortexed. About 100 µL of each pre-dose sample was processed with the addition of 10 µL of internal standard dilution and vortexed. To this, 500 µL of Milli Q/HPLC grade water was added and vortex. The sample mixture was late loaded onto Starata TM-X, 33µm, polymeric sorbent cartridges (30mg/1mL) that were pre-conditioned with 1.0 mL of HPLC grade methanol followed by 1.0 mL Milli Q/HPLC grade water. After applying the maximum pressure the extraction cartridge was washed with 3.0 mL of Milli Q/HPLC grade water (each time 1.0 mL). Analyte and IS was eluted with 1.0 mL of mobile phase. Aliquot of 20 µL of the extract was injected into the LC–MS/MS system. The typical chromatograms of Doravirine were shown in Fig.3.

2.4 Method development

The objective of the present work was to develop and fully validate an LC–MS/MS method for the determination of Doravirine in human plasma with high sensitivity to monitor the concentration of Doravirine for pharmacokinetic/bioequivalence studies. Once Chromatographic column, mobile phase pH and organic solvent are set then flow rate, column temperature and buffer type and concentration can be manipulated for optimal response. The internal standard should have a similar structure, same physicochemical properties or can be a labeled compound (Isotopic drugs).
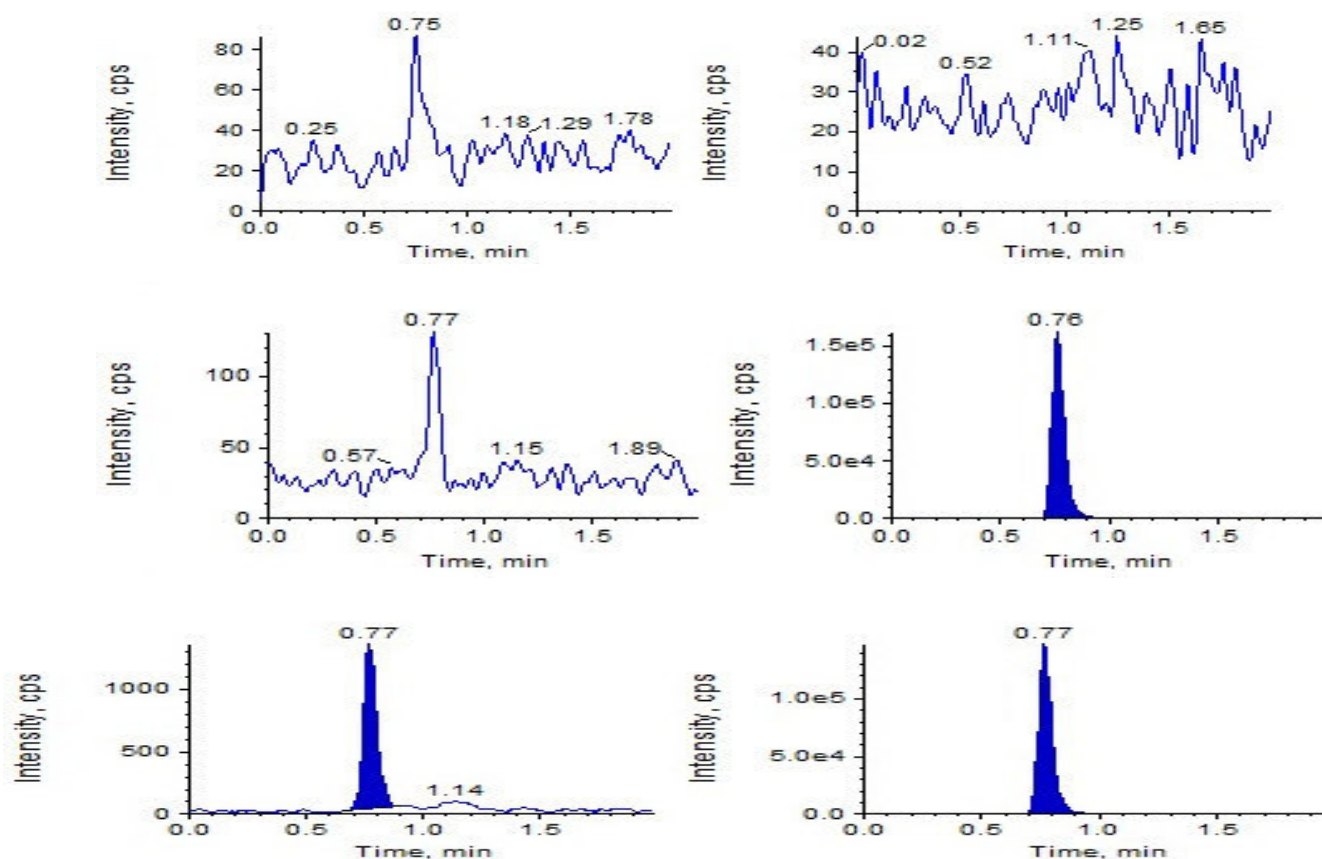

Fig 3. Typical MRM chromatograms of Doravirine (left panel) and IS (right panel) in human blank plasma and human plasma spiked with IS and LLOQ sample along with IS.

2.5   Method validation

A thorough and complete method validation of Doravirine in human plasma was carried out as per USFDA guidelines. The parameters determined were carryover test, selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability. Carry over experiment was performed to verify any carryover of analyte and IS which may reflect in subsequent runs. The design of the study comprised of the following sequence of injections i.e. blank plasma sample → six samples of LLOQ → blank plasma sample → ULOQ sample → blank plasma samples to check for any interference due to carry over. Matrix interference was identified by selectivity parameter usingsix different plasma lots and fortified LLOQ.s. Matrix effect was checked with six different lots of K2 EDTA plasma. Three replicate samples each of LQC and HQC were prepared from different lots of plasma (18 QC samples in total). The linearity of the method was determined by analysis of standard plots associated with a ten–point (non–zero standards) standard calibration curve. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. intra-day and Inter–day accuracy and precision were assessed by analyzing three batches of samples on two consecutive days. Recoveries of analyte and IS were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Recovery of Doravirine was determined at a concentration of 0.43 (LQC), 20.7 (MQC2) and 31.2 (HQC) ng/mL whereas for IS was determined at concentration of 200 ng/mL. The mean overall recovery of analyte was 80.62%. The mean recovery of IS was 78.31%. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8°C) was performed by comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (10 h), processed samples stability (autosampler stability for 54 h, wet extract stability for 50 h and reinjection stability for 44 h.), freeze-thaw stability (4 cycles), long-term stability (50 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and precision (15% RSD).

3.   STATISTICAL ANALYSIS

The statistical analysis of the validated method was performed using the MS excel Software (Microsoft Office Excel 97-2003 Worksheet), and for all statistical tests the level of statistical significance was $P < 0.05$.

4.   RESULTS AND DISCUSSION

4.1   System suitability

During method validation, the precision (% CV) of a system suitability test was found to be in the range 0.00-0.74% for retention time and 0.14–1.32% for the area response of Doravirine and IS.

4.2   Carryover effect

Carryover evaluation was performed to ensure that it does not affect the accuracy and precision of the proposed method. No significant carryover was observed in blank sample after injection highest concentration of analyte (ULOQ) which indicates no carryover of the analyte in subsequent samples.

4.3   Selectivity and chromatography

As shown in Fig. 3, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte and the IS.

4.4   Matrix effect

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The results found were well within the acceptable limits as shown in Table.1. The extraction method was rugged enough and gave accurate and consistent results when applied to real subject samples.

Table 1. Matrix effect of Doravirine in human plasma (n = 6).			
Analyte		**Doravirine**	
		LQC (0.43 ng/mL)	**HQC (31.2 ng/mL)**
Plasma lot	**QC ID**	**Mean Concentration found (ng/mL)**	**Mean Concentration found (ng/mL)**
Lot 1	1,2,3	0.40	31.9
Lot 2	1,2,3	0.41	32.2
Lot 3	1,2,3	0.43	33.0
Lot 4	1,2,3	0.43	32.5
Lot 5	1,2,3	0.44	32.5
Lot 6	1,2,3	0.43	32.3
Mean		**0.42**	**32.4**
S.D		**0.01**	**0.36**
%C.V		**3.38**	**1.12**
% Nominal		**99.4**	**104**
N		**6**	**6**
S.D-Standard Deviation; C.V- Coefficient of Variation			

4.5 Linearity, precision and accuracy

The ten point calibration curve was found to be linear over the concentration range of 0.15-40.4 ng/mL for Doravirine. After comparing the two weighting models (1/x and 1/x2), a regression equation with a weighting factor of 1/x2 of the

drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99. The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Tab.2.

Table 2. Precision and accuracy data for Doravirine.				
Quality control	Run	Concentration found (mean SD; ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (Six replicates at each concentration)				
LLOQ QC	1	0.13 0.01	4.24	83.0
	2	0.15 0.01	5.04	96.8
	3	0.14 0.01	5.57	93.6
	4	0.14 0.01	4.00	94.6
	5	0.15 001	4.93	98.7
LQC	1	0.39 0.01	3.01	90.7
	2	0.400.01	2.83	93.7
	3	0.38 0.02	4.23	89.8
	4	0.39 0.00	0.75	91.3
	5	0.40 0.01	1.82	94.5
MQC1	1	5.06 0.04	0.85	97.5
	2	5.27 0.06	1.16	102
	3	5.24 0.05	0.90	101
	4	5.20 0.04	0.85	100
	5	5.210.05	0.95	101
MQC2	1	20.2 0.09	0.42	97.2
	2	20.9 0.14	0.65	101
	3	20.8 0.19	0.89	100
	4	20.7 0.16	0.75	99.7
	5	20.60.16	0.76	99.5
HQC	1	31.3 0.20	0.64	100
	2	31.5 0.18	0.58	101
	3	31.7 0.16	0.49	102
	4	31.3 0.24	0.76	100
	5	31.5 0.19	0.59	101
Inter–day variations (Thirty replicates at each concentration)				
LLOQ QC		0.14 0.01	7.44	93.3
LQC		0.39 0.01	3.25	92.0
MQC1		5.19 0.09	1.66	100
MQC2		20.6 0.29	1.42	99.5
HQC		31.5 0.24	0.76	101
Spiked concentrations of LLOQ QC, LQC, MQC1, MQC2 and HQC are 0.15, 0.43, 5.19, 20.7 and 31.2 ng/mL, respectively.				

4.6 Stability studies and dilution integrity

In the different stability experiments carried out viz. bench top stability (10 h), autosampler stability (54 h), wet extract stability (50 h), repeated freeze–thaw cycles (4cycles), reinjection stability (44 h) and long term stability at –70 °C

for 50 days the mean % nominal values of the analyte were found to be within 15% of the predicted concentrations for the analyte at their LQC and HQC levels shown Tab.3. Thus, the results were found to be within the acceptable limits during the entire validation. The MRM chromatograms of Doravirine were shown in Fig.3.

Table 3. Stability data for Doravirine in plasma (n=6).				
Stability Parameter	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process (54 h at 10 °C)	0.43	0.40 ± 0.00	0.91	95.0
	31.2	31.3 ± 0.16	0.52	100
Wet extract (50 h at room temperature	0.43	0.41 ± 0.00	0.99	96.9
	31.2	31.4 ± 0.31	1.00	101

Bench top	0.43	0.40 ± 0.02	3.77	95.2
10 h at room temperature	31.2	31.2 ± 0.23	0.73	100
Freeze and	0.43	0.41 ± 0.00	1.12	96.1
thaw	31.2	31.3 ± 0.12	0.40	100
Re-Injection	0.43	0.40 ± 001	2.51	99.7
(44 h)	31.2	31.5 ± 0.17	0.54	99.7
Long–term	0.43	0.42 ± 0.03	7.55	110
(–70°C for 50 days).	31.2	30.0 ± 0.18	0.61	96.0

The described method offers several advantages such as a rapid and simple extraction scheme, and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from study of amlodipine and atorvastatin. The method exhibited acceptable recovery from spiked plasma samples. Also, the method was proved to be sensitive, demonstrating excellent LOD and LOQ levels, as mentioned.

5. CONCLUSION

The present research work involves successful method development and validation of a simple, sensitive and rapid LC-MS/MS method for the determination of Doravirine in human plasma samples according to commonly acceptable FDA guidelines. The SPE minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

6. AUTHORS CONTRIBUTION STATEMENT

Dr. Venkata Rao.V conceived and designed the study; Mr. Bonthu badru performed the experiment and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

7. ACKNOWLEDGEMENTS

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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