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Research Paper

A Liquid Chromatography Tandem Mass Spectrometry Regulatory Compliant Method for the Simultaneous Determination of Valsartan and Hydrochlorothiazide in Human Plasma

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The aim of current study was to develop and validate a rapid and specific assay based on solid phase extraction and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS-MS) for the simultaneous quantitative analysis of Valsartan (VAL) and Hydrochlorothiazide(HYD) in human plasma using Valsartan D3(IS1) and Hydrochlorothiazide^{13}C D2 (IS2) as internal standards. The precursor to product ion transitions of m/z 434.1/349.9 and m/z 296.1/204.6were used to measure the VAL and HYD respectively. The method was validated over a concentration range of 20.0 to 12008.8ng mL^{-1} for VAL and 0.50to 252.51ng mL^{-1} for HYD. The methodwas validated over the parameters like selectivity, matrix effect, sensitivity, specificity, linearity, precision and accuracy, various stabilities (bench top stability, standard stock solution stability, auto sampler stability, freeze thaw stability, long term stability - 65 °C ± 10°C & long term stability - 22 °C ± 5°C, dry extract stability, blood stability), recovery and reinjection reproducibility. The application of this assay was demonstrated in a bioequivalence study after an oral administration of a tablet containing 320 mg VAL and 25 mg HYD in healthy volunteers.

Keywords: Valsartan, Hydrochlorothiazide, electrospray ionization, tandem mass spectrometry, human plasma.

1. INTRODUCTION

Hypertension is emerging as one of the most significant health complications in recent years. Hence, antihypertensive therapy requires to reduce cardiovascular complications that cause a high mortality rate in the patients with hypertension. HYD, 6-chloro-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothia-diazine-1,1-dioxide is most popular thiazide diuretic.[1] HYD is one of the drug often prescribed in combination with other antihypertensive drugs such as beta blockers, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers.[2] VAL, N-(1-oxopentyl)-N-[2-(1H-terazole-5-yl) (1,1-biphenyl) -4-yl) methyl]-L-valine, is a angiotensin II receptor antagonists.[3,4]. The new drugs and their combination with another drug are being introduced in market as they have more patient compliance than a single drug. Combinations of two or more drugs in the pharmaceutical dosage forms are very much useful in Multiple therapies.[5]The combination therapies with VAL/HYDare associated with significantly greater blood pressure reductions compared with either monotherapy, are well tolerated, and are associated less hypokalemia than HYD alone.[6]

The US FDA approved fixed dose combination of HYD and VAL for 12.5 mg /80 mg, 12.5 mg /160 mg, 12.5 mg /320 mg, 25 mg /160 mg and 25 mg /320 mg respectively for patients with hypertension who do not respond properly to monotherapy of either drug.[7] The US FDA accept waiver request of in-vivo testing all other lower dose proportional similarity of the

formulations across all strengths if bioequivalence of higher strength are acceptable. Hence, method development for large range of linearity has challenge for their analysis with the help of number of analytical techniques that are available for the estimation of the individual drugs and their combination.

As per literature survey, several analytical methods have already been developed for the determination of HYD and VAL either individually or in combination with other drugs in plasma including liquid chromatography tandem mass spectrometry (LC–MS– MS) and HPLC.[8-16]The reported methods, which has time consuming and low sensitivity.[15]Further reported method, which has high sensitivity but linearity range of HYD and VAL did not cover the bioequivalence tablet for 25 mg HYD and 320 mg VAL strength.[16]The method also bears limitation for its use in pharmacokinetic and bioequivalence studies of HYD and VAL as it requires an additional demonstration of specificity of HYD in the presence of VAL and vice versa . The purpose of the current study was to develop and validate a sensitive, robust and a rapid LC–ESI-MS/MS method for simultaneous determination of HYD and VAL in human plasma over a wide range, which could make it applicable for use in a bioequivalence study of all strength.

2. EXPERIMENTAL

2.1 Materials and Method

VAL(>98.00% w/w on as is basis), IS 1(>98.00% w/w on as is basis),HYD(>99.10% w/w on as is basis) and IS 2(>98.10% w/w on as is basis) (IS2) were obtained from Clearsynth Labs Ltd, India. HPLC-grade acetonitrile and methanol were purchased from SD Fine Chem. Ltd. (Mumbai, India). Formic acid was purchased from Merck (Fluka Chemie, GmbH, Germany). Milli-Q water 18.2mΩ (milliohm) and TOC ≤ 50 ppb (parts per billion)] obtained from Milli-Q system (Millipore SAS, Molsheim, France). Discovery ® HS C18 15cm x 4.6 mm, 5µmHPLC column was purchased from Sigma-Aldrich Co., USA. All other reagents and chemicals used for these studies were of HPLC grade unless specified. The HLB cartridges (30 mg/1cc) used for the extraction of analytes along with internal standards were procured from Waters Corporation, Milford, MA, USA.

2.2 Instrumentation

LC MS-MS analysis was performed using API 3000 triple quadrupole instrument (Applied Biosystems MDS SCIEX, Toronto, Canada) coupled with Shimadzu HPLC system (Shimadzu SIL HTC, USA) in multiple reaction monitoring (MRM) mode. A turbo electrospray interface in negative ionization mode was used for ionization. Data processing was performed on Analyst software version 1.4.2 (Applied Biosystems MDS SCIEX, Toronto, Canada).

2.3 Standard and Quality Control Sample Preparation

Primary stock solution of VAL and HYD, for preparation of calibration standard and quality control (QC) samples were prepared separately. The primary stock solution of VAL (2mg mL^{-1}), HYD (1mg mL^{-1}), IS1(100 µg mL-1) and IS2(100 µg mL-1) were prepared in methanol.The stock solution of internal standards were diluted to concentration

(approximately 6000ng mL^{-1} and2000ng mL^{-1} for IS1and IS2respectively) with diluents solution (Methanol: Milli-Q/HPLC Grade Water:: 50:50, v/v).Aqueous dilutions for spiking were prepared by serially diluting the primary stock solution of VAL and HYD with diluent solution (Methanol: Milli-Q/HPLC Grade Water:: 50:50, v/v). Spiking of aqueous dilutions in human plasma was done to give eight-point calibration curve, (20.0 to 12008.8ng mL^{-1}) for VAL and (0.50ng $mL^{-1}$to 252.41ng mL^{-1}) for HYD. In a similar way spiking of aqueous quality control dilutions were done in human plasma to prepare the quality control samples consisting of VAL concentrations of 20.0ng mL^{-1} (LLOQ QC), 58.2ng mL^{-1} (LQC), 6010.7ng mL^{-1} (MQC) and 9941.8ng mL^{-1} (HQC) and for HYD 0.50ng mL^{-1} (LLOQ QC), 1.41ng mL^{-1} (LQC), 126.42ng mL^{-1} (MQC) and 202.28ng mL^{-1} (HQC).Primary stock solutions were kept at 2-8°C when not in use. Spiked calibration standards and QC samples were stored at –65°C(temp. range: -55ºC to -75°C) and few sets of LQC and HQC which were transferred for storage in cell frost deep freezer –22°C (temp. range: -17ºC to -27ºC) for the generation of long term stability at –22°C.

2.4 Preparation of mobile phase and Sample Preparation

Buffer solution (0.2 % Formic Acid in Milli-Q water) was prepared by 0.2 ml of formic acid in 100 mL reagent bottle followed by the addition of 100 mL milli-Q water. Organic mixture was prepared as a mixture of methanol: acetonitrile in the ratio of 60:30 v/v (Solution A). Mobile phase was prepared by adding solution Aand buffer solution in the ratio of 90:10 v/v. Mixed well, sonicated and degassed in an ultrasonicator bath. The solutions were used within 3 days from the date of preparation. A set of calibration curve standards and quality control samples were withdrawn from the deep freezer and allowed to thaw at room temperature in water bath. 300 µL of plasma was aliquoted into labeled polypropylene tubes and 100 µL of internal standard dilution (IS1= 6000 ng/mL and IS2=2000 ng/mL) was added and vortexed. The samples were loaded on conditioned HLB cartridge (30 mg/1cc) with 1.0 ml methanol followed by 1.0 ml of Milli-Q water. This was followed by washing the cartridges with 1 ml Milli-Q water and then cartridges were dried for approximately2 minutes. The samples were eluted from dried cartridges by 1.0 ml of Methanol into elution tubes. The eluate was evaporated to dryness at 50 °C & at constant pressure in nitrogen evaporator followed by reconstitution of the dried samples in 300 µl of mobile phase, Vortex and transfer to HPLC vials for analysis.

2.5 Chromatographic and Mass Spectrometric Conditions

The analytes were chromatographically separated using reversed-phase high-performance liquid chromatography (HPLC) with isocratic elution. The mobile phase was used at a flow rate of 1.0 mL min^{-1}. The chromatographic separation was performed using Discovery HS C18 150*4.6, 5µmcolumn. For all analyses 10 µL of extracted sample was injected. The total run time of 3.0 minute was found suitable for retaining and separating the analytes from each other and associated interference. The mass spectrometer was operated in the electro spray ionization mode

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with negative ion detection to monitor the ions with m/z 434.1/349.9for VAL, m/z 296.1/204.6 for HYD, m/z 437.2/350.1for IS1and m/z 298.8/206.7IS2refer Fig.1.

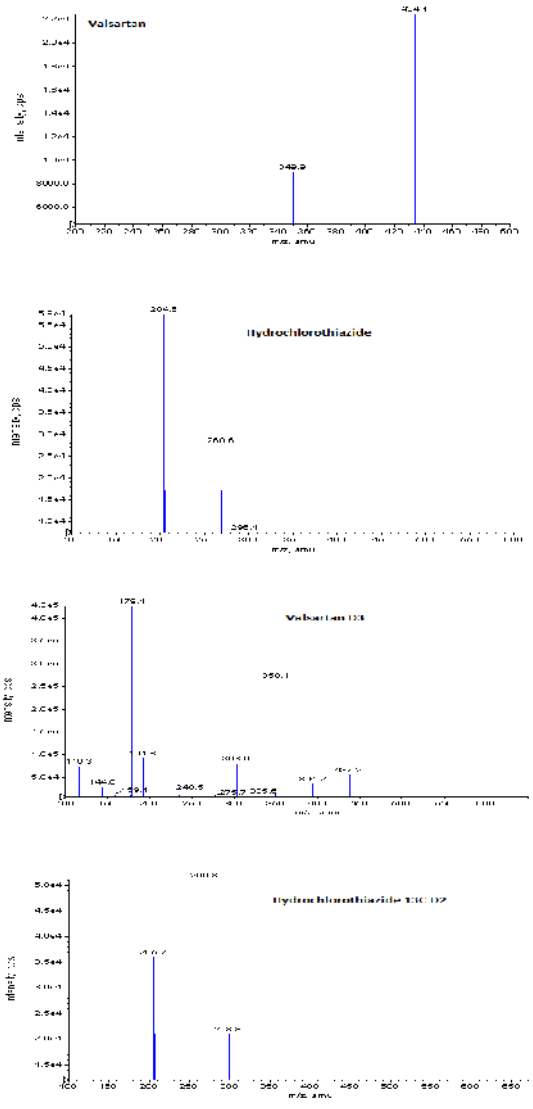

Fig. 1 Product ion spectra of VAL,HYD, IS1 and IS2

The source parameters for VAL,HYD,IS1 and IS2 optimized were Curtain gas (CUR): 6 psi, ion spray voltage (IS): -3500 V, source temperature (TEM): 450°C, collision gas (CAD): 7 psi, nebulizer gas (NEB): 12 psi, while the declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) applied were -40, -152, -35and-10 V for the VAL and IS1. The declustering potential (DP), focusing potential (FP), collision energy (CE) and cell exit potential (CXP) applied were -45, -160, -30 and -10 V for the HYD and IS2.

2.6 Data processing and Regression

The MRM chromatographic peaks were integrated using Analyst software version 1.4.2 after which peak area ratios of VAL to IS1and HYD to IS2 were plotted versus concentration with a linear curve fit, weighted by $1/x$ *and* $1/x^2$ (where x = concentration) was used to produce the regression line.

2.7 Bioanalytical method validation

As a part of method validations following parameters were evaluated as per the USFDA guidelines. [17]

2.7.1 Selectivity

Selectivity is the ability of the analytical method to differentiate and quantify the analytes in the presence of endogenous components in the sample. Selectivity was performed by evaluating at least six different blank matrices for interference at the retention time of analytes and internal standards.

2.7.2 Matrix Effect

Matrix effect was evaluated to asses any undesirable effect from the matrix that can bring about ion Matrix effect was estimated quantitatively through calculation of matrix factor, which is the ratio of peak suppression/enhancement, decease/increase in sensitivity, increased baseline, imprecision of results, retention time drift and chromatographic peak tailing

response in the presence of matrix ions to the peak response in the absence of matrix ions. Matrix effect can further be evaluated from matrix factor as follows.

$$\%\,\text{Matrix Effect} = \text{1- mean of the matrix factor for Analyte/IS} \times 100$$

2.7.3 Sensitivity

Sensitivity of the method was determined by the estimation of the lowest concentration that can be measured with an acceptable limit of accuracy and precision. For the estimation of sensitivity six lower limit of quantitation (LLOQ) samples were processed and analysed against a calibration curve and accuracy and precision were determined.

2.7.4 Specificity

The Specificity was performed to check the interference at the retention time of VAL in presence of HYD and vice- versa. The HQC concentration of VAL was spiked in six blank plasma lots to check the interference at the retention time of HYD and in the same way HQC concentration of HYD was spiked in six blank plasma lots to check the interference at the retention time of VAL.

2.7.5 Goodness of Fit

The data of three precision & accuracy batches were used for the estimation for goodness of fit. The back-calculated concentrations of Calibration Curve standards using 1/x and $1/x^2$weighing were considered for finding the best fit for regression

2.7.6 Linearity

A regression equation generated after processing the three precision and accuracy batches with the best-fit weighing factor was used to found out the linearity of the method. A correlation coefficient (r^2) was used as a benchmark to prove the linearity of the calibration curve.

2.7.7 Precision and Accuracy

Precision and accuracy for VAL and HYD was assessed by analyzing three batches comprising of standard blank (blank without IS), standard zero (blank with IS), calibration standards and six replicates of quality control samples (LOQQC, LQC, MQC and HQC) including both intra and inter day runs. The precision of the assay was measured by the calculation of percentage co-efficient of variation over the concentration range of LOQQC, LQC, MQC and HQC samples that were run within a day (intraday) or on different days (inter-day). The accuracy was expressed in percentage and it was calculated as the ratio of the calculated mean values of the LLOQ QC, LQC, MQC and HQC samples to their respective nominal values.

2.7.8 Stock Solution Stability at room temp. / in refrigerator

The stock solution stability was evaluated at room temperature and at 2-8°C for analytes (VAL and HYD) and internal standards (IS1 and IS2) by the preparation of two aqueous mixtures one from the stability standard stock solutions (kept on the bench at room temperature/in the refrigerator at 2-8°C)and the other from fresh standard stock solution (comparison stocks). An analysis of six replicates of aqueous mixture samples from stability stock and comparison stock was carried out to evaluate the stability. The percentage change of the mean response of the stability to the comparison stock

aqueous mixtures gives an estimate of the stability.

2.7.9 Auto sampler stability

To assess the auto sampler stability of VAL and HYD in processed samples, six quality control samples (at LQC and HQC level) were processed and stored into the auto sampler at 5 °C for the stability period of 49 hours. These pre-processed samples were than quantified against freshly spiked calibration curve standards and quality control samples.

2.7.10 Bench-top stability

The stability of analytes in human plasma stored at room temperature (bench-top stability) was determined by kept six quality control samples (at LQC and HQC level) idle on bench at room temperature then processing bench top stability quality control samples and freshly spiked quality control samples, quantifying them against the freshly spiked calibration curve standards.

2.7.11 Freeze-thaw stability

The freeze-thaw stability was conducted by analyzing the six quality control samples (at LQC and HQC level) that had been frozen and thawed five times. The processed freeze-thaw quality control samples were quantified against a freshly spiked calibration Curve along with freshly spiked quality control samples.

2.7.12 Long-term stability

The long-term stability was conducted by analyzing low and high quality control samples stored at -65°C and -22°C for 97 days and freshly spiked quality control samples with freshly prepared calibration standards.

2.7.13 Dry extract Stability

Dry extract Stability of VAL and HYD was determined by processing six set of low and high quality control samples, keeping them in refrigerator (-17 to -27 °C) for the stability period and finally quantifying them against freshly spiked calibration curve standards after reconstitution along with freshly spiked set of quality control samples.

2.7.14 Blood stability

Blood stability for spiked samples is carried out to assess the stability of the analyte(s) in blood. Blood stability was performed by the preparation of six sets of quality control samples [Medium Quality Control (MQC) and High Quality Control samples (HQC)] by spiking 2 % of MQC and HQC aqueous dilution in fresh blood and keeping them on ice bath for approximately 1 hour. Similarly, freshly spiked QC samples (MQC and HQC) were prepared and samples were then centrifuged at 4000 rpm at 4°C for 15 minutes to separate the plasma, then plasma samples were processed.

2.7.15 Recovery

Recovery of VAL and HYD from the extraction procedure was determined by preparing aqueous recovery comparison samples at LQC, MQC and HQC levels (representing 100 % extraction). Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by using 30 µL aqueous dilution of each VAL and HYD of respective quality control, 1000 µL of internal standard dilution (approximately6000.0 ng/ml for IS1 and 2000.00ng/mL for IS2) and 1940 µL of mobile phase. The following recovery dilution

represents100% unextracted samples. The aqueous samples (LQC, MQC and HQC) of VAL and HYD were compared against 6 sets of processed plasma samples of LQC, MQC and HQC. Recovery of internal standards was also compared at LQC, MQC and HQC level. The recovery was calculated in the following way

$$\% \text{Recovery} = \frac{\text{Mean Peak Area Response of Extractedsample}}{\text{Mean Peak Area Response of Unextracted sample}} \times 100$$

2.7.16 Reinjection reproducibility

Reinjection Reproducibility was performed to establish that the reinjection of the samples kept in the auto sampler at controlled temperature has no effect on the result reproducibility. Reinjection Reproducibility was performed by reinjection of a complete precision and accuracy batch after storage in the auto sampler for 47 hours at 5 °C from the last injection of original batch.

2.7.17 Pharmacokinetic study

A pharmacokinetic study to evaluate the bioequivalence of a test VAL and HYD formulation against innovator formulation at320 mg and 25 mg strength respectively was carried out using the validated method defined in the following manuscript. The study was carried using a open-label, balanced, randomized, two-treatment, two-sequence, four period, single-dose, replicate crossover design. The study was conducted as per the ICH-GCP guidelines after getting approval of the study protocol from the independent ethics committee.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

The scanning and acquisition of the parent and the product ions for VAL, HYD and internal standards were carried out by continuous infusion of the dilution of analytes and internal standards at appropriate concentration through a pump and sorting out appropriate polarity and ions. Afterwards optimization of mass spectrometric condition for each compound was carried out by continuous infusion and adjustment of the compound dependent parameters as declustering potential (DP), focusing potential (FP) and entrance potential (EP). Parameters such as DP, FP and EP were ramped to provide best signal to noise level for the parent ions. Optimization was carried out for the product ions to trace out the best combination of parameters as collision energy (CE), collision associated dissociation (CAD), cell exit potential (CXP). Afterwards source parameters as curtain gas, nebulizer gas, temperature, ion spray voltage and collision gas were optimized by flow injection analysis was using a union in place of column. Then chromatographic conditions were optimized to look for sensitivity, peak shape, separation of peaks and chromatographic run time. The selection of mobile phase was done taking into account symmetric separate peaks with no charge competition in source so that analytes and internal standards response not decrease with huge linear range of analytes. Results derived from several combinations showed that [Organic Mixture (Methanol: Acetonitrile:Buffer Solution (2 % formic acid in Milli-Q Water) :: 60:30:10 v/v/v serves the desired purpose with utmost effectiveness. During the early phase of method development attempts were

made to choose the right column. Waters Sunfire C18, PhenomenexGemeni C18, YMC pack C8 and Waters X bridge RP18 columns were tested. However, Discovery ® HS C18 15cm*4.6mm, 5µm column was found to be most suitable for analysis as VAL and HYD retention time were different and shorter run time that further leads to low consumption of mobile phase altogether making the method cost effective.The liquid-liquid extraction method were tested with different solvent alone or in different compositions, but analytes show matrix effect in optimized chromatographic condition. Hence, solid-phase extraction was tested to improve the recovery with no matrix effect.The use of proper internal standards was done to eliminate the quantitative bias caused by instrumental variation. IS1and IS2 were selected which has similar ionization condition, appropriate retention time and recovery compared to VAL and HYD leading to better tracking of analytes during the course of experiment.

3.2 Assay Performance

3.2.1 Selectivity and Sensitivity

The selectivity was carried out in six normal plasma lots. No interference of endogenous matrix/impurities was found at the retention time of the analytes and internal standards in normal plasma. Representative chromatograms of extracted blank human plasma (Fig. 2) and blank human plasma fortified with IS (Fig. 3), demonstrated the selectivity of the method. Sensitivity was determined by analyzing six replicates of blank human plasma spiked with the analytes at the lowest limit of the quantification (20.0ng mL-1 for VAL and 0.50ng mL-1 for HYD).The solid phase extraction procedure provide advance sample cleanup and gave very good sensitivity for the analysis of VAL and HYD in human plasma. The precision and accuracy for VAL at LLOQ was 1.49 % and 101.53 % and for HYD4.16% and 96.33 % respectively.The representative chromatogram for the LLOQ showing sensitivity was depicted in Fig. 4.

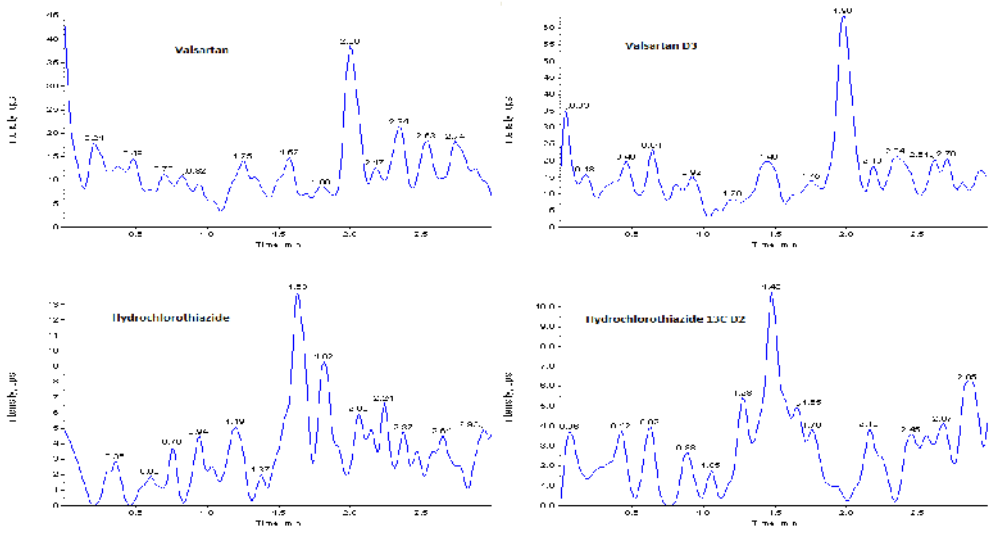

Fig. 2 Representative Chromatograms of extracted blank human plasma

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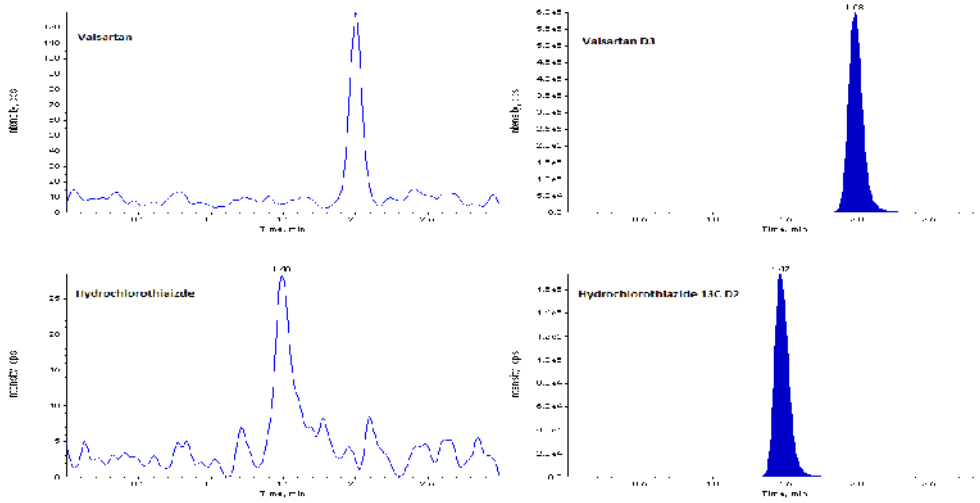

Fig. 3 Representative Chromatograms of extracted blank human plasma fortified with Internal Standard

3.2.2 Specificity

The Specificity was carried out in six plasma lots. There was no significant interference observed at the retention time of VAL in presence of HQC concentration of HYD and vice- versa.

3.2.3 Matrix effect assessment

The matrix effect on the ionization of VAL and HYD in the LC-MS/MS method was determined by processing of six different set of plasma lots in duplicate. The neat (non matrix based) samples were prepared using mobile phase, aqueous dilutions of VAL and HYDat the LQC and HQC levels and internal standards dilution to give concentration within the level of extracted value. The processed matrix effect blanks samples were reconstituted with neat samples. The matrix effect on the estimation of VAL and HYDwas determined as the % RSD (relative standard deviation) of the matrix factor or the variability of the matrix factor. Matrix factor is estimated by the comparison of the area response of the blank samples with the neat samples. The variability of matrix factor (reported as % CV of matrix factor) was 0.19 % (HQC) and 1.78 % (LQC) for VAL, -1.02 % (HQC) and 1.00 % (LQC) for HYD, -0.34 % (HQC) and 0.43 % (LQC) for IS1and-1.04 % (HQC) and 0.91 % (LQC) for IS2.The matrix effect was less than 15 % for VAL, HYD, IS1 and IS2.

3.2.4 Goodness of Fit and Linearity

The goodness of fit results showed 1/x2 to be the best fit for regression.Calibration curves were linear over the concentration range 20.0 to12008.8ng mL-1 for VAL and 0.50 to252.41ng mL-1 for HYD.The precision and accuracy batches evaluated gives a mean linear equation for the calibration curve y = (0.000200± 0.000019) x + (0.000189± 0.000059) for VAL and y = (0.006883± 0.000100) x + (-0.001897± 0.000361) for HYD, where y was the peak area ratio.of analyte to the IS and x was the concentration of analyte. The correlation coefficient (r2) for VAL and HYD was above 0.99

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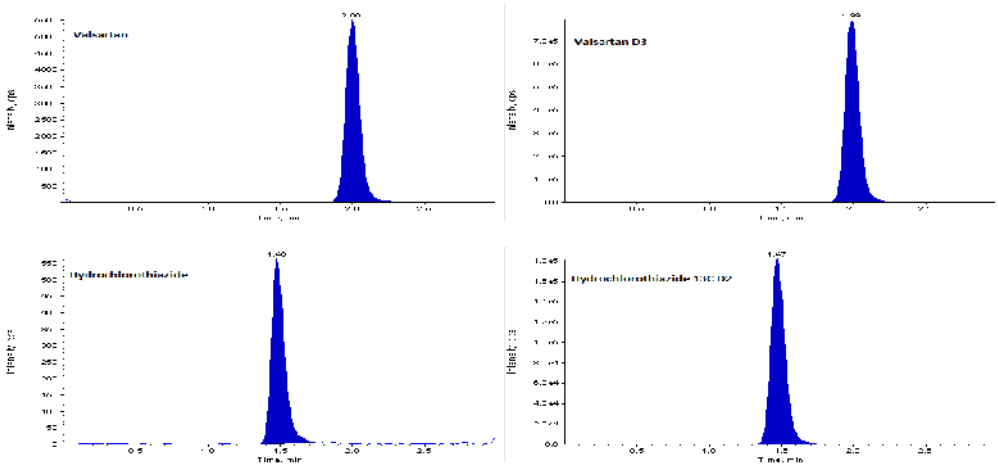

Fig. 4 Representative Chromatograms of LLOQ

3.2.5 Precision and Accuracy

The intraday precision and accuracy were calculated after repeated analysis in three analytical runs. The intra and inter-batch accuracy was determined by calculating percentage nominal of quality control sample from the theoretical concentration. The within- and between-day precision was determined in terms of relative standard deviation (% RSD).

Precision of the assay was measured by the percent coefficient of variation over different concentration levels. The acceptance criteria for within and between batch precision were 20% or better for LOQQC and 15% or better for other non-zero concentrations. Table 1 summarizes back calculated concentrations of calibration curve standards for VAL and HYD

Table 1 Back calculated concentration of calibration curve standards for VAL and HYD (*n* = 3)

Analyte	Standard concentration (ng/mL)	Mean (ng/mL)	SD	CV (%)	Nominal (%)	Slope	Intercept	r^2
VAL	20.0	19.5	0.8	4.10	97.50	0.000200	0.000189	0.9986
	40.0	40.8	1.5	3.68	102.00			
	200.1	206.8	1.8	0.87	103.35			
	1332.1	1262.1	40.9	3.24	94.75			
	2664.3	2730.8	48.8	1.79	102.50			
	5328.6	5304.9	51.6	0.97	99.56			
	10657.1	10666.6	106.4	1.00	100.09			
	12008.8	12014.8	316.1	2.63	100.05			
HYD	0.50	10.47	0.02	4.26	94.00	0.006883	0.001897	0.9979
	1.00	1.01	0.06	5.94	101.00			
	8.00	8.32	0.16	1.92	104.00			
	25.24	25.80	0.77	2.98	102.22			
	50.48	52.33	0.97	1.85	103.66			
	100.96	102.54	2.41	2.35	101.56			
	201.93	194.27	2.64	1.36	96.21			
	252.41	235.94	4.03	1.71	93.47			

whereas Table II represents the intraday and inter days precision and accuracy data. The intraday precision for VAL was ≤5.14% and accuracy was ≥98.35%, an intraday precision for HYD was ≤8.66% and accuracy was ≥94.00%.Where as the inter days precision for VAL was ≤3.92% and accuracy was≥99.41%, an inter days precision for HYD was ≤7.69% and accuracy was ≥98.00%.

3.2.6 Stability study

Stability studies were performed to evaluate the VAL and HYD stability in across different parameters. Stock solution of VAL, HYD and internal standards

Table 2 Inter day and intraday precision and accuracy of the method for VAL and HYD

Analyte	Level	Concentration added (ng/mL)	Inter-day (n=6)			Intra-day (n=18)		
			Mean conc. Found (ng/mL)	(%) Nominal	CV (%)	Mean conc. Found (ng/mL)	(%) Nominal	CV (%)
VAL	LLOQQC	20.0	20.5	102.50	5.14	20.2	101.00	3.92
	LQC	58.2	59.8	102.75	3.63	59.2	101.72	2.89
	MQC	6010.7	5911.7	98.35	2.12	5975.1	99.41	1.65
	HQC	9941.8	9794.4	98.52	1.74	9905.7	99.64	2.12
HYD	LLOQQC	0.50	0.47	94.00	8.66	0.49	98.00	7.69
	LQC	1.41	1.35	95.74	4.95	1.40	99.29	4.36
	MQC	126.42	128.55	101.68	2.31	130.12	102.93	2.29
	HQC	202.28	215.69	106.63	2.48	219.44	108.48	2.92

Table 3 Stability data of VAL and HYD in processed QC samples for different stability activities at different conditions (n= 6)

Stability	Analyte	Concentration (ng/mL)	Mean concentration found in stability samples (ng/mL)	Nominal (%)	CV (%)	Mean concentration found in comparison samples (ng/mL)	Nominal (%)	CV (%)	Change (%)
Bench Top Stability (14 h)	VAL	58.2	56.6	97.25	1.52	60.3	103.61	2.14	6.14
		9941.8	9962.8	100.21	1.27	9738.9	97.96	5.46	-2.30
	HYD	1.41	1.36	96.45	2.71	1.38	97.87	2.69	1.45
		202.28	210.21	103.92	5.73	209.57	103.60	4.9	-0.31
Auto sampler Stability (50 h)	VAL	58.2	59.0	101.37	6.4	60.3	103.61	2.14	2.16
		9941.8	9717.7	97.75	1.33	9738.9	97.96	5.46	0.22
	HYD	1.41	1.34	95.04	7.15	1.38	97.87	2.69	2.90
		202.28	213.79	105.69	1.42	209.57	103.60	4.9	-2.01
Freeze-Thaw Stability (5-cycles)	VAL	58.2	61.8	106.19	1.69	60.3	103.61	2.14	-2.49
		9941.8	9947.6	100.06	1.86	9738.9	97.96	5.46	-2.14
	HYD	1.41	1.40	99.29	3.61	1.38	97.87	2.69	-1.45
		202.28	209.98	103.81	5.99	209.57	103.60	4.9	-0.20
Dry Extract Stability (31 h)	VAL	58.2	61.7	106.01	2.34	60.3	103.61	2.14	-2.32
		9941.8	9994.1	100.53	1.86	9738.9	97.96	5.46	-2.62
	HYD	1.41	1.39	98.58	3.77	1.38	97.87	2.69	-0.72
		202.28	208.78	103.21	5.59	209.57	103.60	4.9	0.38
Long term stability at -65°C±10°C (97 days)	VAL	58.2	58.2	100.00	4.34	59.2	101.72	4.71	1.69
		9941.8	9973.0	100.31	1.58	9953.2	100.11	1.58	-0.20
	HYD	1.41	1.40	99.29	3.54	1.40	99.29	2.37	0.00
		202.28	201.30	99.52	1.9	204.57	101.13	3.63	1.60
Long term stability at -22°C±5°C (97 Days)	VAL	58.2	57.6	98.97	3.94	59.2	101.72	4.71	2.70
		9941.8	9963.3	100.22	1.53	9953.2	100.11	1.58	-0.10
	HYD	1.41	1.42	100.71	2.04	1.40	99.29	2.37	-1.43
		202.28	200.95	99.34	1.3	204.57	101.13	3.63	1.77

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(IS1 and IS2) were found stable at room temperature for 32 hours with a percentage change of 3.48 % to 5.95 %. Whereas the stock solutions were stable in refrigerator at 2-8°C for 7 days with a percentage change of -0.41 % to 0.16 %.Blood stability was expressed as the percentage change between the fresh and the stability samples. The % change for VALwas -0.27 % (HQC) to 0.57 % (MQC) andHYD

3.2.7 Reinjection Reproducibility and Recovery

The % change ranged from -5.25 % (LLOQ QC) to 0.07 % (LQC) for VAL and -3.50 % (LQC) to 1.12 % (MQC) for HYD in reinjection reproducibility of the samples at 5 °C for 47 hours.The mean % recovery of VAL, HYD, IS1 and IS2 were 86.34 %, 84.49 %, 89.91 % and 84.32 % with a precision of 3.71 %, 1.43 %, 3.29 % and 4.77 %respectively.

3.2.8 Pharmacokinetic evaluation

mean plasma concentration profile curve of test and reference given in Fig.5 and 6.

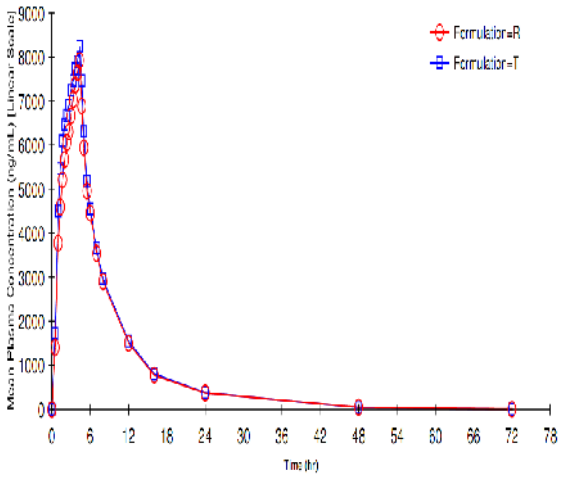

Fig. 5 Mean plasma concentration-time curves for VAL (320 mg) following single-dose administration of test and reference tablets

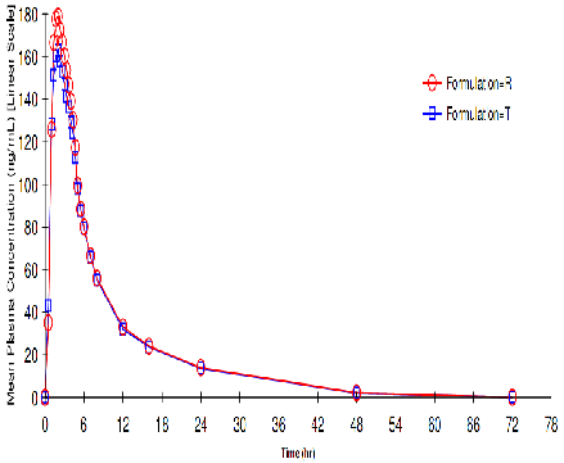

Fig. 6 Mean plasma concentration-time curves for HYD (25 mg) following single-dose administration of test and reference tablets

4. CONCLUSION

A highly specific, selective, and rapid LC-ESI MS-MS method for the simultaneous determination of VAL and HYD in human plasma has been developed and validated with a lower limit of quantification of 20.0ng mL-1and 0.50 ng mL-1 respectively. The validated method provides an easy way of estimation of VAL and HYD with deuterated internal standards in plasma with highly efficient sample cleanup associated with good recovery and negligible matrix effect. The validated method has been successfully applied for performing pharmacokinetic evaluation to adjudge the bioequivalence of two formulations of VAL and HYD with 320mg and 25 mg dose respectively in healthy volunteers.

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