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Method Development and Validation of Antihypertensive Drug Valsartan by Bioanalytical RP-HPLC Method in Human Plasma

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Keywords: Valsartan, Sofosbuvir, HPLC method, Human plasma, extraction, deprotonation, Matrix sample, Calibration curve

ABSTRACT

The article describes a high-performance liquid chromatography method for the determination of valsartan in human plasma. This method was systematically optimized, designed, developed, and validated as per USFDA guidelines. The solvent deprotonation technique opted for the extraction of valsartan from human plasma. The effect of the deproteinizing agent, volume of a deprotonating agent, speed of centrifugation was studied during the optimization studies which also have a great impact on the analysis of the drug. After deproteinization, the drug was subjected to Cromasil C18 column (5µm, 250×4.1mm) using Methanol: Water: OPA (75:22:1 v/v) as the mobile phase with a flow rate of 0.8 ml/min. The standard calibration curve was constructed in the concentration range of 0.35 µg/ml to 14 µg/ml and linearity was found to be 1. Sofosbuvir was used as the internal standard. The retention time of valsartan and the internal standard was found to be 7min and 4.9 min resp. There were no traces or interference of any peak undesired peak. From the accuracy results, the accuracy of the method was found to be between 97.68% and 99.7% and the relative standard deviation was between 0.26% and 2.31%. The % relative standard deviation (RSD) of intraday and interday precision was reported to be less than 15%. Limit of detection (LOD) and limit of quantitation (LOQ) was reported to be 0.05µg/ml and 0.14 µg/ml, individually. So, the proposed HPLC method was successfully demonstrated as rapid and sensitive which can be used as an alternative for the analysis of valsartan in any other matrix examples.

INTRODUCTION:-

Hypertension is the most commonly addressed disorder, which particularly shows its effect on past middle age. Hypertension and Cardiovascular (CV) diseases are the prime reason for morbidity and mortality over the globe. Blood pressure and the risk of Cardiovascular disease go hand in hand with continuing and independent of other risk factors. Therefore, the priority aims for treating hypertension is to lower the adverse CV and Chronic Kidney Failure (CKD). [1] Epidemiological studies have confirmed that the higher the pressure (Systolic or diastolic or both) greater is the risk of a cardiovascular set of symptoms. Thus, the clinical focus of treatment should be on lowering the Systolic Blood pressure (SBP) and Diastolic (DBP). The majority of cases are essential hypertension. The sympathetic and renin-angiotensin system (RAS) may or may not be overactive, but they do contribute to the tone of blood vessels and c.o. in hypertensive drugs, by chronically lowering BP, may reset the barostat to function at a lower level BP. [2] For the treatment of a hypertensive disorder, the drug of interest is Losartan, Valsartan, Irbesartan, Telmisartan, Candesartan which belongs to the class of Angiotensin (AT1 receptor) inhibitors (blockers) (ARBs). These are non-peptide orally active angiotensin receptors selectively antagonist of AT2 receptors that selectively block the binding of angiotensin II to the type I binding position. [3] Valsartan, is N-(1-oxopentyl)-N-[2-(1H-tetrazole-5-yl)[1,1-bi-phenyl]-4-yl]methyl]-L-valine **(Fig1)** has an empirical formula of $C_{24}H_{29}N_5O_3$ and molecular weight of 435.5 [4]. Valsartan is an orally active drug when administered orally, it is particularly selective as an angiotensin II receptor inhibitor, and therefore it is the choice of drug for the treatment of hypertension. Comparing Valsartan has less oral bioavailability (approx. 23%) which restricts its use as an antihypertensive agent. This is due to poor absorption and poor permeability of Valsartan in the gastrointestinal tract, within significant breakdown and large defecation (approx. 80%) as unchanged drug. [4,5,6,7] Valsartan has a long range of cardioprotective benefits which leads to the lowering the risk of various heart-related disorders such as CV morbidity which leads to Heart failure (HF) followed by Myocardial infarction (MI) thus the use of valsartan causes the maintenance of the Proper heart rate and Blood pressure leading to the reduction in hospitalization and fatal condition such as strokes. Valsartan has a more positive tolerability profile, with a significantly lower incidence of Angio-oedema. [8]

There are a number of bioanalytical methods which have been developed for the approximation of valsartan in plasma samples of humans. Sophisticated high-performance liquid chromatography (HPLC) methods combined with fluorimetric, PDA detector have

been there for the analysis of valsartan in human plasma [9, 10, 11, 12]. There are also the reported methods using HPLC- tandem mass spectroscopy (LC/MS) which is highly sensitive for the drug in plasma.[13,14,15] In a few years the most advanced and sensitive two liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has been developed for the estimation of drugs (fixed-dose combination) in rat and human plasma. [16,17] Using the most expensive detectors and instruments such as LC/MS/ MS, LC-ESI-MS/MS, and combining HPLCs with photodiode array fluorescence, ultraviolet-fluorescence, and fluorimetric detections result in the increase of expensiveness of the analysis which if compared with HPLCs attached to ultraviolet (UV) detection provide easy in handling and is affordable. Using simple and easy detectors such as photodiode array (PDA) and UV methods are been developed with good and precise results for its determination and a good amount of recovery of valsartan from human plasma by extraction techniques are achieved.[18, 19].

By the application of bioanalytical HPLC for the first time valsartan was isolated from plasma by opting solid-phase extraction which was performed on a Bond-Elute CH cartridge. Then the solvent was put for evaporation, and the filtrate was reconstituted in the solution of 0.1% trifluoroacetic acid (TFA) in acetonitrile Water (45/55 v/v) and the resulting drug solution was subjected for the analysis using HPLC with UV detector at 215 nm wavelength with the flow rate of 1.0 ml/min [18].

After a period of time the extraction was carried out using the organic solvent similarly, to that of solid-phase isolation using the bioanalytical HPLC method, the organic solvent was subjected to evaporation under a gentle stream of nitrogen at 40°. Further, the resultant residue was dissolved in a 0.1 ml mobile phase (ACN and 15 mm of potassium dihydrogen phosphate with the pH 2.0 in the ratio of 42:58 v/v) and then the resultant samples were subjected to analysis on a UV detector at a wavelength of 215 nm and the flow rate was 1.2 ml/min on Luna C_{18} (150×4.6 mm ID., particle size 5 µm) column (Phenomenex) [4]. There are also reported analyses using the PDA detectors which help the analysis of three components simultaneously which involves a multi-step extraction process and expensive gases (Nitrogen) for the evaporation of the extracted organic solvents.

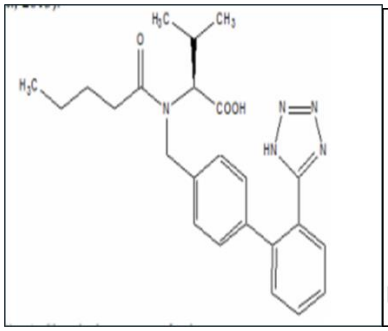
Therefore, there is the urge to design, improve and validate a simple, easy, faster, and economical, enviro-friendly bioanalytical method for the isolation and analysis of valsartan in plasma samples. A developed bioanalytical HPLC method should not only be rapid in

analysis but also the extraction process we use should be easy as possible and shouldn't be time-consuming which will ultimately lead to fewer mistakes and more accurate estimation 0f the desired drug. While optimizing the extraction process, the choice of deproteinizing agent used, the volume of the extracting solvent, simplicity of the extraction, and the protein separating process are also the most important factor which leads to a good and effective method established for estimation [4]. Further, in the process of drug estimation, sensitivity, robustness, short analysis time, resolution of peak, precision, accuracy, and other parameters also should be taken into the account. Selection of proper mobile phase with optimized concentration and PH range using the buffers to sink the condition for good retention time with suitable UV detector and flow rate using the proper column with respect to the pKa value of the drug, these are some major factors which lead to an excellent condition for analysis of the desired drug.[20,21]

The aim of the present study was to develop and validate a sensitive, simple, easy, fast, reproducible, precise, and economical bioanalytical HPLC method for the estimation of valsartan in human plasma. By using the internal standard the precession and robustness of the method were developed. Then the extract of the drug aliquot was put for analysis on the lately developed and validated HPLC bioanalytical method.

MATERIAL AND METHODS:-

The API Valsartan of potency (99.8%) and the internal standard Sofosbuvir (Fig 2) were procured as a gift sample from Macleod pharmaceutical and the human plasma was procured from the Curie Manavta, (HCG) Cancer center Nashik. No actual humans participated in this research work. The stored (Preserved) plasma was used as the biological matrix media treated as the representative biological matrix. For the preparation of mobile phase, (HPLC grade) Methanol of Merck life science was used, O-Phosphoric acid (AR grade) was used of Ranked. Ultra-purified water HPLC grade water was obtained from the Milli-Q purification system. Organic solvent Ethyl acetate was used as the deproteinizing agent for the extraction of drugs from plasma samples.



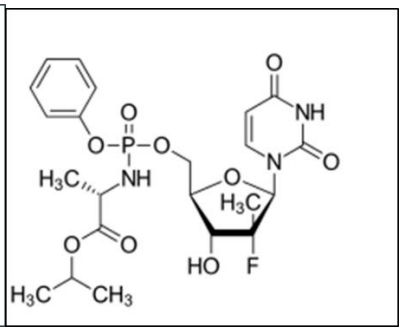


Fig. No. 1: Structure of Valsartan Fig. No. 2: Structure of Sofosbuvir

Estimation of valsartan in human plasma was carried out using an HPLC Binary Gradient System 3000 series, Analytical Technologies Ltd. connected to a dual-wavelength UV detector, column oven, and manual sample injector. HPLC workstation was used for the recording of HPLC chromatograms. Cromasil C18 column with (250mm×4.16ID, 5µ) was used to accomplish the separation and security guard column. Millipore glass filter (Millipore filter with cellulose nitrate gridded with 0.22 µ size and 47 mm diameter) assembly attached with vacuum pump was used for the filtration of the mobile phase. The mobile phase was sonicated for 15 min using an ultrasonicator to eliminate dissolved gases from the mobile phase. Vortex mixer and centrifuge were used for the extraction of the drug from human plasma. A pH Instrument was used to adjust the proper pH of the mobile phase.

Chromatographic Conditions:-

The mobile phase system was made by taking the desired concentration of Methanol: Water (75:25 v/v) which was adjusted to 3.0±0.02 with orthophosphoric acid (OPA). The Isocratic system of the mobile phase was kept with a 0.8ml/min flow rate. Cromasil C18 (5µm, 250mm × 4.1ID) column was connected with C_{18} security guard column was used as the stationary phase. The column temperature was kept at 25° which was thermo-statistically controlled by a column oven. The drug was quantified at 252. 21 nm wavelength with UV detector while keeping the 11 min run time for each chromatographic analysis.

Preparation of Stock solution:-

Accurately weighed 10mg of valsartan into 10ml of volumetric flask and was dissolved in 2 ml of methanol and was subjected for sonication after sonication volume was made up with the mobile phase up to 10ml the resultant solution prepared was 1000 µg/ml. Further, the Working standard solutions of 100 µg/ml were made with appropriate dilutions by using the above-prepared stock solution. Sofosbuvir is used as an internal standard in this study.

Accurately weighed 10mg of IS into 10 ml of volumetric flask and was dissolved in 2 ml of methanol and was subjected for sonication after sonication volume was made up with the mobile phase up to 10ml the resultant solution prepared was 1000 µg/ml, therefore the appropriate dilutions were made to produce working standards concentrations of Sofosbuvir.

Calibration standard plot from 0.35 to 14µg/ml was prepared in human plasma by spiking 20 µl drug solution of working stock solutions and 7µl of IS. The quality control (QCs) solutions of various concentrations such as lower limit of quantification (LLQC) 0.35 µg/ml, lower quality control (LQC) 1 µg/ml, medium quality control (MQC) 7µg/ml, and higher quality control (HQC) 14µg/ml were prepared by spiking 20 µl of each concentration and 7 µl of the internal standard.

Extraction techniques from human plasma:-

For extraction of valsartan from, solvent deproteinization technique was used. Two hundred microliters of human plasma, 20µl standard drug solutions, and 7µl IS were taken in a microcentrifuge tube and shaken for 5 mins to mix it properly. Further, the mixture was subjected to the vortex for thorough mixing and then after 5 min, the mixture was centrifuged at 7000–7500 rpm for 10 min at room temperature. After that supernatant was transferred into vials and filtered through a syringe filter of 0.2µm and further injected into the HPLC system.

METHOD DEVELOPMENT

Solubility and wavelength selection: -The selection of the solvents system is based on the solubility of the drug. Various solvents were used to perform solubility, the common solvents used are methanol, water, acetonitrile [22] (Table No. 1) Diluent: Methanol: water (75:25 v/v).

Table No. 1: Solvents and their solubility

Solubility studies		
Combination	Concentration	Observation
Combination 1	ACN: water (50:50)	Sparingly soluble
Combination 2	Methanol: ACN (30:70)	Sparingly soluble
Combination 3	100% methanol	Freely soluble
Combination 4	Water: methanol (25:75)	Freely soluble

Determination of adsorption maxima by UV-visible spectrophotometer the Spectrophotometer with Double beam UV-visible spectrophotometer with 1 cm; matched quartz cell was used. Valsartan and Sofosbuvir were subjected for the λ max. (Table no 2), λmax determination was executed by the isosbestic point method due to simultaneous analysis of valsartan and Sofosbuvir respectively. The UV Spectra are shown in fig no. 3.

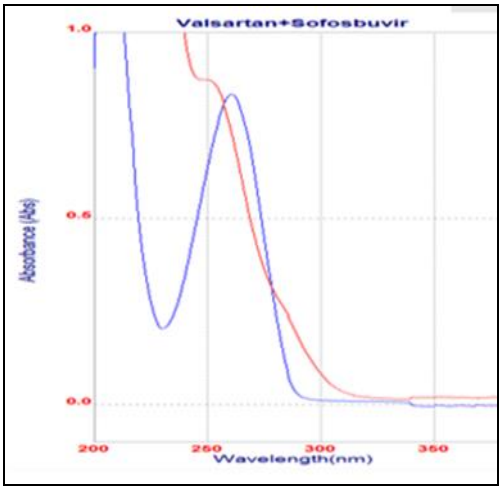


Fig. No. 3: Isobestic point determination UV-Absorbance

Table No. 2: Absorbance of the valsartan and IS

Wavelength selection	
Name of drug	Absorbance
Valsartan has the wavelength λmax	250.21 nm.
Sofosbuvir has the wavelength λmax	260 nm.
The isosbestic point was taken and the wavelength was finalized at 252.21 nm.	

Flow rate optimization:- The ultimate aim in any separation is to obtain resolution and optimum distance between peaks of your desired compounds. So, flow rate is very important for elution and, resolution. If we go into deeper fundamentals flow rate affects the separation performance can be calculated by Van Demeter plot. This plot tells us which flow rate gives the best performance for a given analyte. [23]

Optimized Chromatographic conditions:-

Column: Cromasil C18 (250mm×4.16ID, 5µ)

Mobile phase: Methanol: Water: OPA (75:25:1% v/v)

Diluent: Methanol

Flow rate: 0.8 mL/min

Column temperature: 25° C ± 2° C

Injection volume: 20 µL

Run time: 11 min

Detector: UV –detector

Detection wavelength: 252.21 nm

Elution: Isocratic

RT: Valsartan: 7 min

Sofosbuvir: 4.9 min

METHOD VALIDATION:-

System suitability test: -

System suitability parameters were performed via injecting the LLOQ solution test concentration, Six times the standard solutions are injected into the HPLC system. This parameter of system suitability was evaluated from the obtained report. [24].

Linearity:-

Preparation of working standard solutions: 0.35, 0.7, 0.7, 1.4, 4.2, 8.4, 11.2 and 14 µg/mL working standard solutions were prepared.

Procedure: Linearity was demonstrated over the range of 0.35-14 µg/ mL of test concentration. The solution at eight levels of concentration was prepared and 20 µL of each of the working standard solutions were injected into the HPLC system to obtain the chromatograms. The graph was plotted by peak areas against concentration, by using the least

square method the linearity curve was constructed and regression equations were calculated. Using the chromatograms we calculated the correlation coefficient (r^2), y-intercept, and slope of the regression line.

Accuracy:-

It is the degree of closeness of agreement between the test result (nominal values) and the true value.

Procedure: Accuracy was established by performing the recovery studies of valsartan. Recovery studies were performed by spiking sample solution with the pure authenticated standard drug at 3 different concentration levels i.e. HOC, MQC, and LQC each being injected five times for 3 different days. Mean %RSD was calculated.

Precision:-

It is the closeness of an agreement between the series of measurements obtained under the prescribed conditions.

The Inter-day and intra-day precision of the proposed method was shown by taking the test concentration. The test concentrations were five times injected into the HPLC system to get the desired chromatograms and the area of the peak was recorded from the obtained peaks. After that, the average and the standard deviation (SD) of the five peaks at each concentration level were calculated. Finally, % RSD was then calculated [25].

Specificity:-

The specificity of the method was proved by processing and analyzing blanks prepared from six independent lots of control plasma along with six extracted LLOQ samples. The method is selective if there is no interfering peak present at the retention time of the drug or IS. If there is an interfering peak present at the retention time of the drug then its response should be less than 20% of the mean response of six extracted LLOQ samples. [26]

Limit of Detection:-

The Limit of Detection (LOD) is the estimation of the lowest amount of analyte substance in a sample under analysis that can be detected but not certainly quantitated in an absolute value, by using a definite method under the required experimental conditions.

LOD=3σ/S Where, σ=Standard deviation of response, and S= Slope of the calibration curve.

Limit of Quantitation:-

The Limit of Quantitation (LOQ) is the smallest (minimum) concentration of drug analyte which produces a response that can be precisely computed. LOQ can be determined with Signal to Noise ratio of 10:1.

LOQ=10 σ/S Where, σ=Standard deviation, and S= Slope of the calibration curve.

Robustness:-

To check the robustness of the method, small deliberate changes were made in flow rate, mobile phase concentration, pH, Wavelength was made to demonstrate any impact of this variation on the proposed modus operandi.

Effect of variation of mobile phase composition: The effect of variation of mobile phase ratio was determined by changing the ratio of Methanol: Water. The Standard solutions of 100% test concentration were injected into the HPLC system and the obtained chromatogram was recorded. The retention time, tailing factor, and %RSD values were calculated using the obtained records.

Effect of variation of flow rate: The effect of variation of flow rate was determined by keeping flow rates at 0.7 mL/ min and 0.9 mL/min. A standard solution of 100% test concentration was injected into the HPLC system and the chromatogram was obtained. The retention time, tailing factor, and % RSD was calculated. [27,28]

RESULTS AND DISCUSSION:-

Various solvents were used to check the solubility of valsartan. The drug solubility was found in methanol and water and the IS Sofosbuvir was also soluble in methanol from the overlain UV-spectrum, it was demonstrated that maximum absorbance of valsartan and Sofosbuvir was found to at 252nm (Isobestic point) for simultaneous analysis. Therefore, the obtained value was been selected as the detection wavelength for this analysis. Chromatographic conditions were optimized by injecting the test concentration in several trials to get the optimized retention time (RT), peak symmetry (Tailing), plate count, and relative standard deviation (% RSD) within the desired limits. After the various trail runs, a final method using mobile phase which consisting of Methanol: Water: OPA in the ratio of 75:25:1 at a flow rate

of 0.8 mL/min, the run was performed on Cromasil C18 (250mm×4.16ID, 5µ) column at 252 nm, was established to be suitable and adequate. The optimized method resulted in a chromatogram with valsartan eluting at 6.8 min and Sofosbuvir at 4.9 min respectively, with a tailing factor of 1.2 and USP plate count of 7501.

System suitability:-

Tailing factor, % RSD, a number of theoretical plates found were calculated by performing the system suitability test. The retention time of 6.8 min was exhibited by the chromatograms. From the system suitability studies, it was observed that the % RSD of peak areas was to be 1% for standard preparation of valsartan. Theoretical plate count is > 2000 i.e. 7501 and the tailing factor <2 was found to be 0.021. (Table no 3)

Table No. 3: Results for the system suitability of Valsartan

Sr. No.	RT	Peak area	USP plates	Resolution	Peak tailing
1	6.93	132958	8064	3.4	1.22
2	6.87	133114	7051	3.48	1.24
3	6.82	134738	7130	3.52	1.23
4	6.74	134760	7655	2.48	1.28
5	6.74	134280	7436	2.53	1.23
6	6.72	131657	7670	2.5	1.28
mean	6.80333333	133584.5	7501	2.985	1.24666667
SD	0.08453796	1226.802	377.7862	0.529292	0.0265832
% RSD	1.24%	1%	0.05036478	0.17731725	0.02132342
Limits	RSD ≤ 2%	RSD ≤ 2%	N should be > 2000	T ≤ 2	NLT 2

Linearity:-

The standard calibration curve was constructed with concentration Vs peak area to establish the linearity of the drug. From the obtained calibration curve, it showed that the method was linear over the concentration range of the 0.35 µg/mL-14 µg/mL for valsartan and correlation

coefficient (r^2). Interday and intraday linearity was demonstrated and the r^2 was established as 1 for both inter day and intraday. (Figures 4{a, b} and Table 4,5). Chromatograms of the Standard are recorded (Fig no 5{a- h}).

Acceptance criteria: The range of correlation coefficient (r^2) should be NLT 0.99 and NMT 1. The straight-line equation that should be obtained for the calibration curve was found to be y= Correlation coefficient (r^2) was found to be 1. Linearity was observed within the range of 0.35-14 µg/mL for valsartan. Hence, thus the method was found to be linear.

Table No. 4: Result of inter-day linearity

Sr. No.	Concentration of STD	Concentration of IS	Area of STD	Area of IS	Slope	Intercept	Correlation coefficient
LLOQ	0.35	7	46849	607153	130367	1057	1
STD A	0.7	7	91769	604869			
STB B	0.7	7	92243	612378			
STD C	1.4	7	184471	612083			
STD D	4.2	7	547577	608992			
STD E	8.4	7	1097608	607600			
STD F	11.2	7	1459882	601506			
ULQC	14	7	1826585	606628			

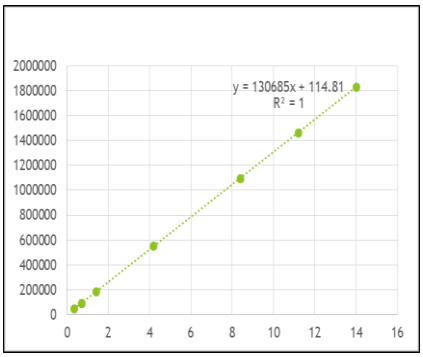


Fig. No. 4a: Calibration curve for inter-day Linearity

Table No. 5: Result for Intraday linearity is demonstrated

Sr. no	Concentration of STD	Concentration of IS	Area of STD	Area of IS	Slope	Intercept	Correlation coefficient
LLOQ	0.35	7	45049	607153	130696	114.81	1
STD A	0.7	7	92247	604869			
STB B	0.7	7	91243	612378			
STD C	1.4	7	183469	612083			
STD D	4.2	7	550214	608992			
STD E	8.4	7	1096234	607600			
STD F	11.2	7	1463483	601506			
ULQC	14	7	1830521	606628			

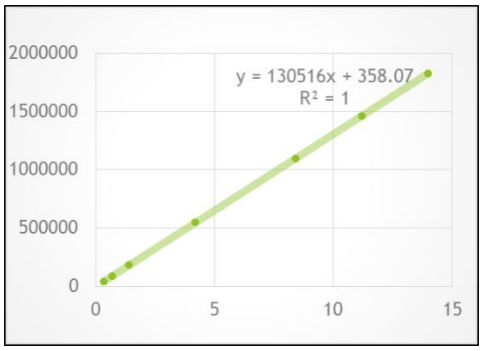


Fig. No. 4b: Calibration curve Inter day linearity

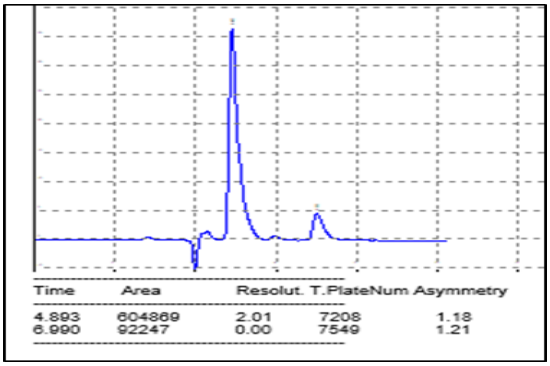


Fig. No. 5a: Chromatogram of STD A (0.7µg/ml)

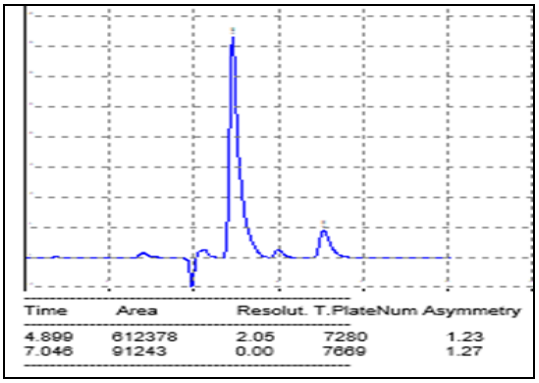


Fig. No. 5b: Chromatogram of STD B (0.7µg/ml)

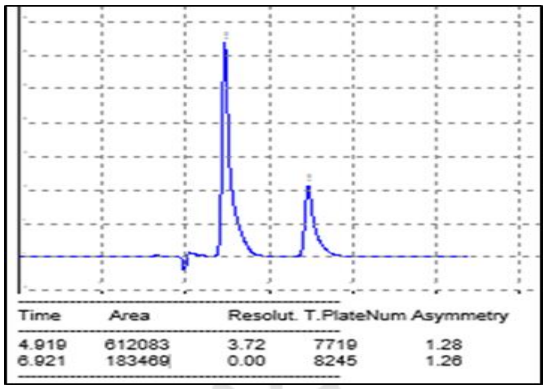


Fig. No. 5c: Chromatogram of STD C (1.4µg/ml)

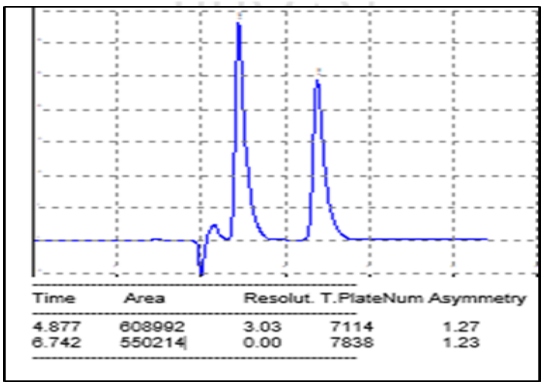


Fig. No. 5d: Chromatogram of Std D (4.2µg/ml)

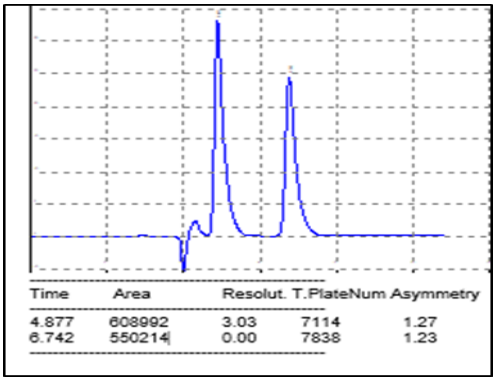


Fig. No. 5e: Chromatogram of Std E (8.4µg/ml)

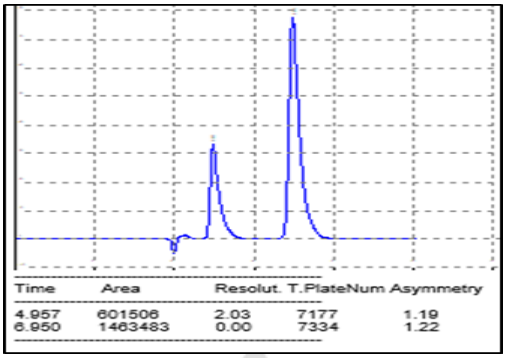


Fig. No. 5f: Chromatogram of Std F(11.2 µg/ml)

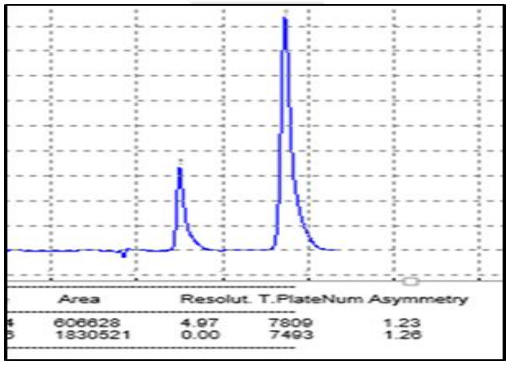


Fig. No. 5g: Chromatogram of ULOQ(14µg/ml)

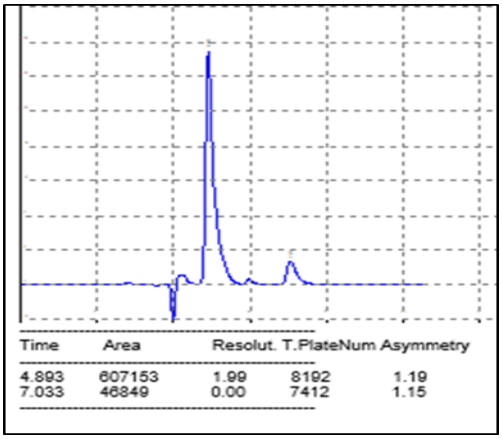


Fig. No. 5h: Chromatogram of LLOQ (0.35µg/ml)

Specificity/ Selectivity:-

The proposed method was evaluated to ensure that there was no interference of any plasma content and the IS. Specificity was demonstrated by analyzing the blank plasma samples and the 6 replications at the LLQC concentration level. Plasma samples were prepared as per the method described in the sample preparation above. The demonstrated method is shown in table no: 6. and the obtained chromatograms of STD and blank are shown in fig 6 (a, b).

Table No. 6: Sensitivity results are demonstrated in the table with % RSD 3.21%

Sr. No.	Concentration of STD	Actually taken concentration	Area of STD	Area of IS	Area of ratio	Accuracy	Limit	Precision (RSD)	Limit
1	0.35	0.36	46849	607153	0.077	102.1			
2	0.35	0.34	44442	604823	0.073	97.14			
3	0.35	0.33	45049	605030	0.074	94.3	80-120%	3.21%	<20%
4	0.35	0.34	44431	604820	0.073	97.14			
5	0.35	0.33	45444	605430	0.075	94.3			
6	0.35	0.351	46489	607853	0.076	100.2			

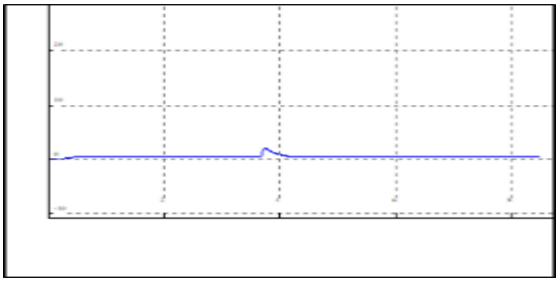


Fig. No. 6a: Chromatogram of Blank extracted from plasma

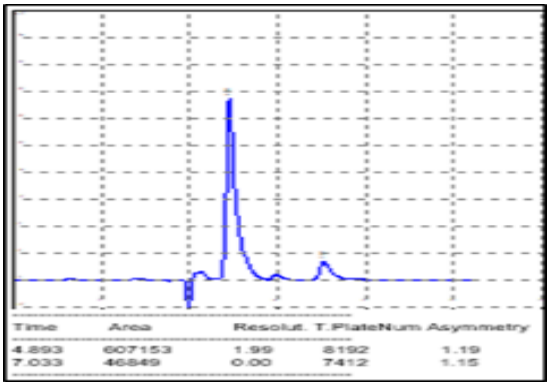


Fig. No. 6b: Chromatogram of STD for specificity of Valsartan and Sofosbuvir

Accuracy:-

The recovery studies were performed to check the accuracy of the method at LLOQ, LQC, MQC, and HQC. The mean recovery of the drug from the analyzed solution of the formulation is in the range of 97.68% to 99.85 %. Hence, the method is said to be accurate and the STD Concentration used chromatograms obtained are also shown below (Figure 7{a-d} and Table 7 -8).

Acceptance criteria: The mean % recovery of valsartan at each spiked level should be within the range of 97.68 %-102%. As per the recovery results, the test method has an acceptable level of accuracy shown in Table. The mean recovery of valsartan is in the range of 97.68 %-99.73 %.

Table No. 7: Accuracy levels at HQC, MQC, and LQC With the accuracy level range between 98.8% -99.7%

Sr. no (LQC)	Day 1	Day2	Day 3	Sr. no (MQC)	Day 1	Day2	Day 3	Sr. no (HQC)	Day 1	Day2	Day 3
Injection 1	132958	132228	138392	Injection 1	915777	918332	917540	Injection 1	1831597	1829925	1837809
Injection 2	133114	135098	135797	Injection 2	917263	916604	914026	Injection 2	1827012	1829369	1825655
Injection 3	134738	134280	135739	Injection 3	914863	911954	914695	Injection 3	1817122	1832369	1828980
Injection 4	132248	131657	131991	Injection 4	918506	913189	912290	Injection 4	1826710	1828442	1820444
Injection 5	134230	134760	135099	Injection 5	920883	914588	914981	Injection 5	1823335	1826928	1826700
mean	133457	133604.	135403	mean	917458	2736.2	2041.37	mean	1825155.2	1829406	1827917
SD	1008.3	1558.00	2287.8	SD	2367.84	0.3	0.22	SD	5366.733	2007.47	6352.65
%SD	0.75	1.16	1.68	%SD	0.25	0.30%	0.22%	%SD	0.29	0.1	0.34
%RSD	0.75%	1.16%	1.68%	%RSD	0.25%	99.70%	99.77%	%RSD	0.29%	0.10%	0.34%
Accuracy	99.25%	98.84%	98.32	Accuracy	99.74%	0.3	0.2	Accuracy	99.70%	99.90%	99.52%
%CV	0.75	1.16	1.68	%CV	0.25	0.3	0.2	%CV	0.29	0.1	0.34
LQC	98.8%			MQC	99.73%			HQC	99.7%		
	1.2%				0.26%				0.3%		

Table No. 8: Accuracy demonstrated for LLOQ

(LLOQ)	Day 1	Day 2	Day 3
Injection1	46489	44486	45369
Injection 2	44442	46985	44256
Injection 3	45049	46859	45239
Injection 4	45639	46583	45468
Injection 5	46856	45869	46981
Mean	45695	46156.4	45462.6
SD	996.094624	1028.961515	977.267773
%RSD	2.269	2.465	2.222
Accuracy	97.6%	97.7%	97.7%
%CV	2.31%		

Table No. 9: Interday and intraday Precision of the valsartan method is depicted

		LQC	MQC	HQC	LLOQ
	1	99.25	99.74	99.7	97.6
	2	98.84	99.7	99.9	97.7
Intraday	3	98.32	99.77	99.52	97.7
	Mean	98.80	99.73	99.70	97.7
	SD	0.46608	0.03511	0.19008	0.05773
	%RSD	0.47%	0.03%	0.2%	0.006%
	1	99.45	99.74	99.54	97.6
	2	98.55	98.69	99.86	97.7
Interday	3	99.88	99.64	99.46	97.7
	Mean	99.30	99.35	99.62	97.66
	SD	0.67869	0.57951	0.21166	0.05773

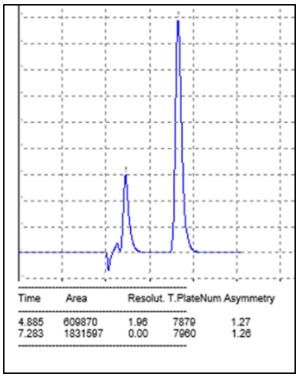


Fig. No. 7a: Chromatogram of HQC (14µg/ml)

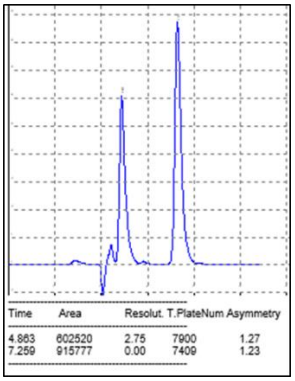


Fig. No. 7b: Chromatogram of MQC (7µg/ml)

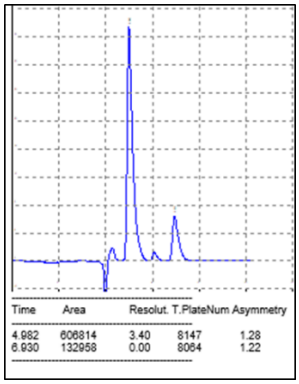


Fig. No. 7c: Chromatogram of LQC (1µg/ml)

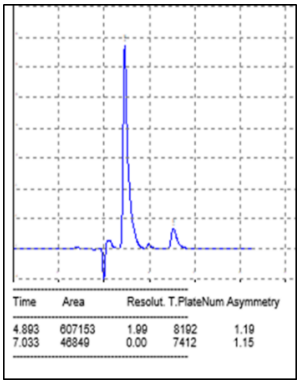


Fig. No. 7d: Chromatogram of LLOQ (0.35µg/ml)

Precision:-

The Precision was evaluated by using the working standard solutions and injecting triplicate for three consecutive days. The % RSD of peak area of chromatograms of valsartan is <2% for intraday and inter-day precision respectively (Table No. 9).

Acceptance criteria: % RSD of peak area of precision solution should not be more than 2.0%. The percentage RSD of peak areas of five injections of valsartan working standard solutions is in the range of 0.47 %-0.03 % and 0.22%, for intra-day and for inter-day precision was 0.7%, 0.6%, 0.2%, and for LLOQ it was 0.006% respectively. The % RSD value is <2%.

Sensitivity:-

The limit of detection is the least quantity of analyte which shows a peak area thrice that of the baseline of noise. The equation 2H/h is used to calculate the ratio of signal size to that of noise. The limit of quantification is the lowest concentration with a ratio of signal-to-noise.

Formula to calculate LOD:

$$LOD=3\sigma/S$$

Where, σ=Standard deviation of the response, and S=Slope of the calibration curve from the results, 3× 1951.811856/ 130516=0.05µg/ml, Therefore the LOD for valsartan is 0.05 µg/mL

Formula to calculate LOQ:

$$LOQ=10\ \sigma/S$$

Where σ=Standard deviation of the response; S = slope of the calibration curve LOQ=10×1951.811856/130516=0.15.

The LOD and LOQ are 0.05 µg/mL and 0.15 µg/mL for Valsartan respectively.

Robustness:-

The standard chromatograms of valsartan were within limits for variation in flow rate (± 0.1 mL), the flow rate within the range of 1.4 to 1.6 mL was allowable and variation in mobile phase composition (± 1%), pH (± 0.2), and Wavelength (± 2 nm) (Figures 8{a-d} and Tables 10). The % RSD values are <2.0%, hence the method is proved to be robust. There is not much difference when the small changes were made in the desired parameters.

Table No. 10: The robustness result of the method is represented in the table

Parameters	Change Levels	Area of valsartan	mean	SD	%RSD
Flow rate	0.7 ml/min	1822102	5517021	5225405	0.947142
(± 0.1 ml/min)	0.9 ml/min	9211940			
Wavelength	251	1837809	5887675	5727375	0.972774
(± 1 nm)	253	9937540			
Mobile phase	74:24:00	915593	748296	236593.7	0.316177
(± 1ml)	76:26:00	580999			
	Retention time				
PH	2	6.93	6.9	0.042426	0.006149
(± 1)	4	6.87			
Tailing factor		1.22	1.23	0.014142	0.011498
		1.24			

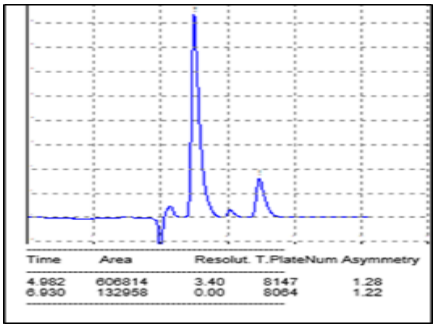


Fig. No. 8a: Chromatogram of Valsartan at PH 2

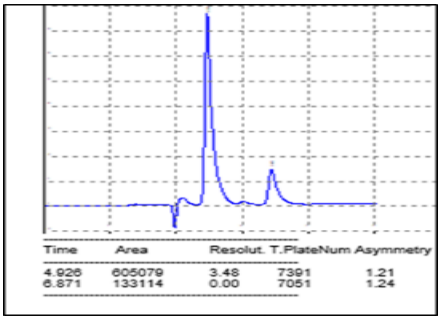


Fig. No. 8b: Chromatogram of Valsartan at PH 4

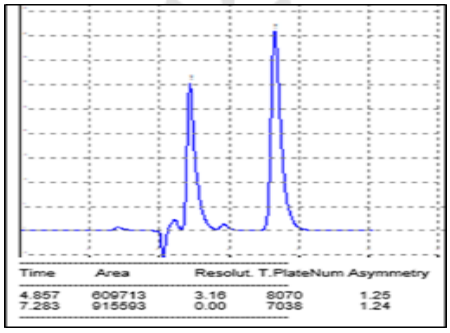


Fig. No. 8c: Chromatogram of valsartan with Mobile phase concentration 24:74:1 (water: methanol: OPA)

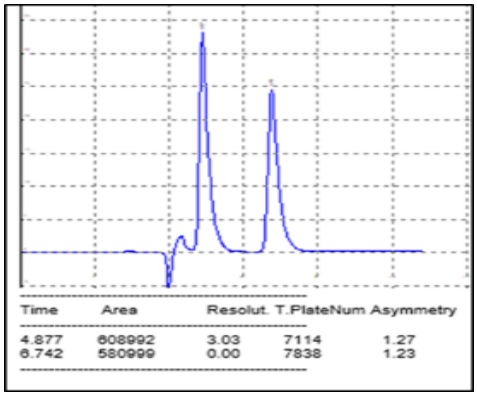


Fig. No. 8d: Chromatogram of Valsartan with Mobile phase concentration 26:76:3 (water: methanol: OPA)

Ruggedness:-

The ruggedness of the method was studied by determining the analyst to analyst variation by performing the inter-day analysis by two different analysts. % RSD between two analysts was not greater than 2.0 %. (Table No. 11)

Table No. 11: Ruggedness for the valsartan method

Sr. No.	Area of valsartan	
	Day 1 (Analyst 1)	**Day 2 (Analyst 2)**
Injection 1	132958	135098
Injection 2	133114	135428
Injection 3	134738	131657
Injection 4	132248	134760
Injection 5	134230	138392
Injection 6	132228	129254
Mean	133252.6667	134098.2
SD	1032.157869	3195.885
%RSD	0.00774587	0.023832
%RSD	0.015789149	

Recovery of Valsartan from Plasma:-

The recovery of an analyte in the method development process is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte insolvent. This parameter is considered to be important because the amount of drug.

Recovered should be maximum when we develop and should be consistent. The recovery for the 3 levels of QCs was taken and analyzed. And the chromatograms obtained were recorded. (Table 12, fig 9 {a-c})

Table No. 12: Recovery studies are shown in the table with a % recovery of 76.26% for valsartan from plasma.

Sr. No.	Concentration taken	Area of standard	Observed area	% Recovery	Mean Recovery
QC1 Low	0.35 ppm	46849	36660	78.20%	
QC2 medium	4.2 ppm	547577	400608	73.16%	76.26%
QC3 high	7 ppm	916604	709897	77.44%	

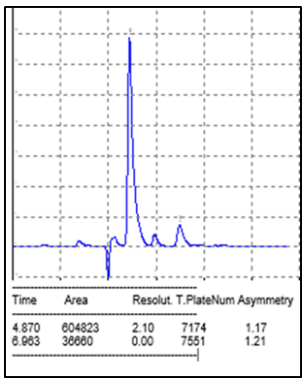


Fig. No. 9a: Chromatogram of valsartan recovery for QC1 concentration

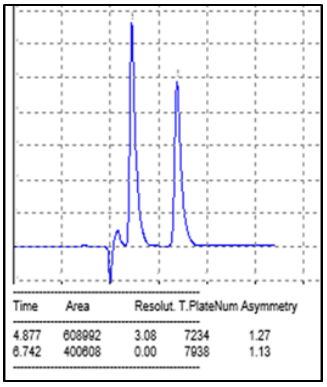


Fig. No. 9b: Chromatogram of Valsartan recovery for QC2 Concentration

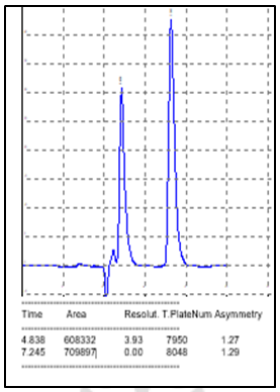


Fig. No. 9c: Chromatogram of valsartan recovery for QC3 concentration

CONCLUSION:-

In this bioanalytical Reverse Phase HPLC method, all the parameters were optimized so as to gain optimized conditions for the valsartan analysis using the IS method. The found optimum flow rate was found to be 0.8 mL/min using water and methanol and OPA (75:25:1 V/V) as mobile phase and the wavelength which was selected for detection was 252 nm at which good detector response for valsartan was achieved. The retention time is 6.8 min. To establish the efficiency of the system used, system suitability tests were done on new stock solutions and results met with acceptance criteria. The standard calibration was found to be linear in concentration range over 0.35-14µg/ mL with correlation coefficient 1 and no interference was seen due to mobile phase solvents and plasma content (blank) and IS (Sofosbuvir) at the retention times of valsartan which confirms that the method was specific. The LOD and LOQ for valsartan were demonstrated to be 0.05 µg/mL and 0.14 µg/mL respectively which postulate the method's sensitivity. The method is said to be precise when the indicated value of % RSD is below 2%. The mean recoveries are in the range of 73.16%-77.44 % indicating that this is an accurate method. The method also demonstrated its robustness as the % RSD

was below 2.0%. The proposed method was validated in accordance with USFDA guidelines and can be applied for analysis of the same in marketed bulk drugs. Finally, it can be concluded that this method is accurate, precise, robust, specific sensitive, has less retention time than previous methods, and can be successfully applied for the routine analysis of valsartan at the commercial level.

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