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Research Article

In Vitro Analytical Method Development And Validation Of Sacubitril And Valsartan In Rabbit Plasma Using RP-UPLC

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Abstract
A simple, Accurate, precise method was developed for the simultaneous estimation of Sacubitril and Valsartan in Rabbit plasma was developed and validated. By using solvent phase extraction [SPE] the sample preparation was prepared. Chromatogram was run through Std CHS (50mm x 2.1 mm, 1.8m). Mobile phase containing Buffer AmmoniumAceatate: Acetonitrile taken in the ratio 70:20 was pumped through column at a flow rate of 0.3ml/min. Buffer used is Disodium Phosphate buffer in this method was buffer. For the separation of Sacubitril and Valsartan Internal Standard [IS] used is Emtricitabine. The Temperature was maintained at 30°C. Optimized wavelength selected was 260nm. Retention time of Sacubitril and Valsartan was found to be 1.196min (IS) and 1.528min of Sacubitril and 1.799min of Valsartan. The standard curve was linear (R2 >0.995) over the concentration range of 0.4-8 µg/ml of Valsartan & 0.2-4 µg/ml of Sacubitril. All the analytical validation parameters were determined as per ICH guidelines The bioanalytical method developed approach was selective, robust, and reliable, as accuracy, precision, recovery, and other validation parameters were all within the recommendations' limitations. The peaks produced for the drug of interest and the internal standard were well separated from one another without any plasma interferences, and the peaks were symmetrical with an adequate tailing factor. The method has the potential to be very beneficial in therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biomedical investigations.

Key Words: Sacubitril and Valsartan, Internal Standard, RP- UPLC, Bioanalysis, Rabbit Plasma

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Introduction

Bioanalytical techniques, employed for the quantitative determination of drugs and their metabolites in biological fluids and creates a specific procedure to enable a coalesce of interest to be identified and at the same time to be quantified in a matrix. A coalesce is measured by several procedures. The choice of analytical procedures involve many considerations, such as: concentration levels, chemical properties of the analyte, specimen matrix, cost of the analysis, experimental speed, quantitative or qualitative measurement, required precision and necessary equipment[2]. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability.

DRUG ANALYSIS IN VARIOUS BIOLOGICAL MEDIA

Blood, urine, and faeces are the most commonly acquired samples for biopharmaceutical analysis, especially if the drug or metabolite is poorly absorbed or substantially eliminated in the bile.Saliva, breath, and tissue are examples of other media that can be used. The nature of the investigation heavily influences the selection of sampling media. In a clinical pharmacokinetic investigation, for example, medication levels necessitate the use of blood, urine, and saliva. A bioavailability study may necessitate drug level data in blood and/or urine, but a drug identification or drug addiction concern may only necessitate one type of biological sample.

The nature of the drug investigation heavily influences the selection of sample media. In a clinical pharmacokinetic study, for example, medication levels necessitate the use of blood, urine, and perhaps saliva. A bioavailability research may necessitate medication level measurements in blood or urine. The steps involved in estimating medicines in biological fluid are sample collection, sample treatment, separation of the compound of interest from the matrix, and analysis.

Bioanalysis can determine the therapeutic efficacy of a specific medicine. Bioanalysis is important in the pharmaceutical industry. The following steps are involved in bioanalysis.

- Biological fluid selection and collection
- Sample preparation -Analyte extraction from biological matrix.
- Analyte detection is accomplished through a variety of approaches.

The desired analyte should be extracted from the biological fluid after it has been selected. This phase in the bioanalytical approach is more crucial since sample preparation can be done using several extraction methods. The preparation of the sample takes time and should be done carefully due to its importance. If the biological matrix is liquid, such as blood, plasma, or urine, liquid-liquid extraction is employed; if it is solid, liquid-solid extraction is utilized.

The following are the most well-known and widely utilized extraction methods

1. Protein precipitation method.
2. Liquid-liquid extraction method.(LLE)
3. Solid-phase extraction method.(SPE)

Sacubitril is a prodrug neprilysin inhibitor used in combination with valsartan to reduce the risk of cardiovascular events in patients with chronic heart failure (NYHA Class II-IV) and reduced ejection fraction. It was approved by the FDA after being given the status of priority review for on July 7, 2015. Sacubitril's active metabolite, LBQ657 inhibits neprilysin, a neutral endopeptidase that would typically cleave natiuretic peptides such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and c-type natriuretic peptide (CNP). ANP and BNP are released under atrial and ventricle stress, which activate downstream receptors leading to vasodilation, natriuresis and diuresis. Under normal conditions, neprilysin breaks down other vasodilating peptides and also vasoconstrictors such as angiotensin I and II, endothelin-1 and peptide amyloid beta-protein. Inhibition of neprilysin therefore leads to reduced breakdown and increased concentration of endogenous natriuretic peptides in addition to increased levels of vasoconstricting hormones such as angiotensin II.

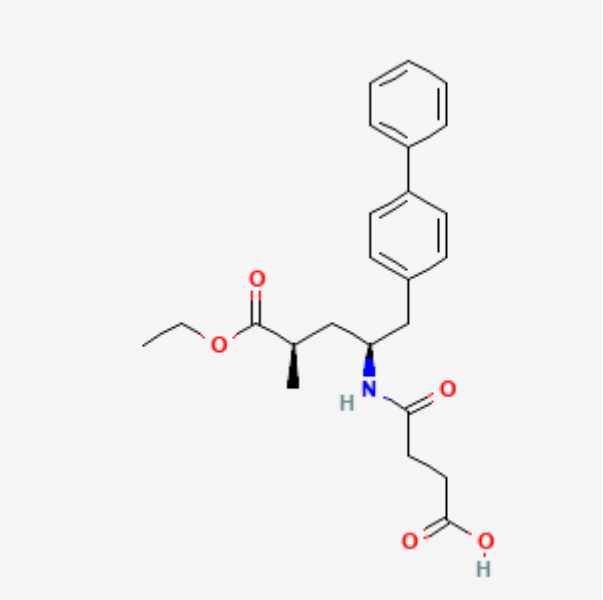

Figure 1 Chemical Structure of Sacubitril

Valsartan is a monocarboxylic acid amide consisting of L-valine in which the amino hydrogens have been replaced by a pentanoyl and a [2'-(1H-tetrazol-5-yl)biphenyl]-4-yl]methyl group. It exhibits antihypertensive activity. It has a role as an antihypertensive agent, an angiotensin receptor antagonist, a xenobiotic and an environmental contaminant. It is a biphenylyltetrazole, a monocarboxylic acid amide and a monocarboxylic acid**.**

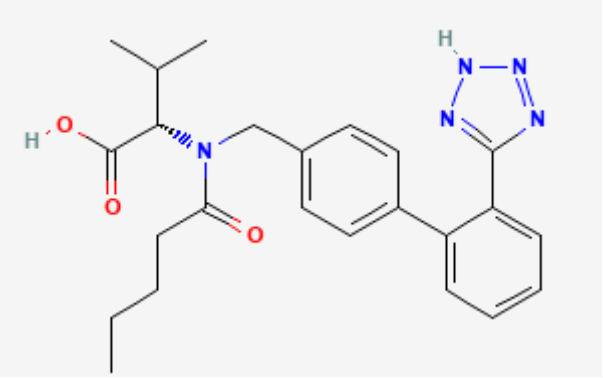

Figure 2: Chemical Structure of Valsartan

Experimental Work:

Materials and Chemical's Used: 1. API: Sacubitril and Valsartan API was obtained as a gift sample from Akirvis Pvt Limited , Kukatpally, Hyderabad, Internal Standard From Akrivis Pharma pvt Ltd. Rabbit plasma: The Plasma was supplied from Akrivis Pharma pvt Ltd., Hyderabad Chemicals used in the AR and HPLC grades are Used. All instrument used in the Work calibrated

Methodology:

Preparation of solutions: - All solutions performed sonication, were stored at room temperature, and were utilized within 24 hours after their production. The next section outlines the methodology for preparing buffers and possible solutions.

Preparation of diluent (v/v):

Based up on the solubility of the drugs, diluent was selected, Na_2HPO_4 and acetonitrile taken in the ratio of 70:20.

Preparation of Buffer (v/v):

0.01N Na2HPO4 Buffer: Accurately weighed 1.41gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.8 with dil. Orthophosphoric acid solution.

Preparation of stock solutions: -

Standard Preparation: Accurately Weighed and transferred 20mg of Sacubitril and 40 mg of Valsartan working Standards into a 100ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents, and filter the solution with Hplc nylon 0.5µm size filters (200 ppm/µg/ml of Sacubitril and 400 ppm/µg/ml of Valsartan).

Standard Working Solution: From the above stock solution 0.1ml, 0.2ml, 0.3ml, 0.8ml, 1.0ml, 1.2ml, 1.6ml and 2.0 ml was pipette and transferred to 8 individual of 10 ml volumetric flask and make up the volume up to the mark with diluent to produce 0.4 µg/ml, 0.8 µg/ml, 1.2µg/ml, 3.2 µg/ml, 4.0 µg/ml, 4.8 µg/ml, 6.4 µg/ml and 8.0µg/ml of Valsartan, 0.2 µg/ml, 0.4 µg/ml, 0.6µg/ml, 1.6 µg/ml, 2.0 µg/ml, 2.4 µg/ml, 3.2 µg/ml and 4.0µg/ml of Sacubitril.

Stock solution of internal standard (Emtricitabine): -

Standard Preparation: Accurately Weighed and transferred 50 mg of Emtricitabine working Standards into a 100ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents, and filter the solution with Hplc nylon 0.5µm size filters (500 ppm/µg/ml of Emtricitabine).

Final concentration: From the above solution, take 1ml of solution and spiking blank plasma with working stock dilutions of analyte to produce 50µg/ml ISD concentration.

Preparation of calibration curve (CC) standards and quality control (QC) samples

Quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.4 µg/ml (Standard-1/LLOQ), 0.8 µg/ml (Standard-2), 1.2 µg/ml (Standard-3/LQC), 3.2 µg/ml (Standard-4), 4.0 µg/ml (Standard-5/MQC), 4.8µg/ml (Standard-6), 6.4µg/ml (Standard-7/HQC) and 8.0µg/ml (Standard-8/ULOQ) of Valsartan and 0.2 µg/ml (Standard-1/LLOQ), 0.4 µg/ml (Standard-2), 0.6 µg/ml (Standard-3/LQC), 1.6 µg/ml (Standard-4), 2.0 µg/ml (Standard-5/MQC), 2.4µg/ml (Standard-6), 3.2µg/ml (Standard-7/HQC) and 4.0µg/ml (Standard-8/ULOQ) of Sacubitril.

Table 1 CC spiking solutions of Sacubitril.

Spiking solution	pippetout in ML	make up in ML	spiking in ML	make upon ML	final conc in ng/ml
Standard-1	0.1	10	0.25	2.5	200
Standard-2	0.2	10	0.25	2.5	400
Standard-3	0.3	10	0.25	2.5	600
Standard-4	0.8	10	0.25	2.5	1600
Standard-5	1.0	10	0.25	2.5	2000
Standard-6	1.2	10	0.25	2.5	2400
Standard-7	1.6	10	0.25	2.5	3200
Standard-8	2.0	10	0.25	2.5	4000

Table 2 CC spiking solutions of Valsartan.

Spiking solution	pippetout in ML	make up in ML	spiking in ML	make upon ML	final conc in ng/ml
Standard-1	0.1	10	0.25	2.5	400
Standard-2	0.2	10	0.25	2.5	800
Standard-3	0.3	10	0.25	2.5	1200
Standard-4	0.8	10	0.25	2.5	3200
Standard-5	1.0	10	0.25	2.5	4000
Standard-6	1.2	10	0.25	2.5	4800
Standard-7	1.6	10	0.25	2.5	6400
Standard-8	2.0	10	0.25	2.5	8000

Table 3 Preparation of QC spiking solutions

Spiking solution	pippetout in ML	make up in ML	spiking in ML	make upon ML	final conc in ng/ml of Sacubitril	Final conc in ng/ml of Valsartan.
LLOQ	0.1	10	0.25	2.5	200	400
LQC	0.3	10	0.25	2.5	600	1200
MQC	1.0	10	0.25	2.5	2000	4000
HQC	1.6	10	0.25	2.5	3200	6400
ULOQ	2.0	10	0.25	2.5	4000	8000

The solutions containing carbon compounds (CCs) and quality controls (QCs) were stored in a deepfreeze at a temperature of -20°C. A 0.25 mL amount of spiked samples was hermetically sealed and stored in several pre-labeled vials at a temperature of -20°C.

1. Common Core standards.
2. Quality control samples.
3. A blank sample including both spiking internal standard (IS) and analyte.
4. The standard zero sample involves adding an internal standard (IS) working solution to blank plasma during sample processing.
5. The aforementioned samples were subsequently utilized for conducting several validation experiments and assessing samples from animal studies.

Extraction procedure for Bio-Sample analysis.

The protein precipitation method was employed to extract Sacubitril and Valsartan from rat plasma, utilizing Emtricitabine as an internal standard (IS), in the subsequent procedure.

In this experiment, a total of 750µl of plasma was combined with 500µl of internal standard and an additional 250µl of Eluite. The mixture was subjected to a 15-second cyclomixing process. Following this, 1 ml of acetonitrile was added to the mixture, and the resulting solution was subjected to vertexing for a duration of 2 minutes. Subsequently, the solution was centrifuged at a speed of 3200 rpm for a period of 5 minutes, allowing for the collection of the supernatant sample. To ensure the removal of any impurities, the sample was then filtered using a polyvinylidene fluoride or polyvinylidene difluoride 0.45µ filter. Finally, 10 µL of the filtered sample was injected into the high-performance liquid chromatography (HPLC) system for further analysis.

Data analysis

The Analyst software version empower 2 was used to data acquisition and analysis, and additionally, a validated excel sheet was used to compute the statistics like mean, SD and %CV for analytical values generated during method validation.

Validation Methodology in bioanalytical method: - System Suitability Parameter

System Suitability test are performed that the test mixture is essential to check the specifications of a liquid chromatographic system. The System suitability testing limits are acceptance criteria that must be prior to sample analysis**.**

Methodology: The experiment involves the administration of six quality control samples of MQC from a single vial at the beginning of the study.

Acceptance criteria: The criteria acceptance accordingly as the % CV of the retention time (RT) should be ≤ 2.00 %., The % CV of the area ratio should be ≤ 5.00 %.

Auto Sampler Carryover

Carry-over is an alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument, during validation carry-over should be assessed by analyzing blank samples after the calibration standard at the ULOQ.

Methodology: The high-performance liquid chromatography (HPLC) technology was evaluated in order to investigate the potential occurrence of carry-over. The carryover was evaluated by injecting the following samples in a sequential manner.

- Blank refers to a solution that is used as a mobile phase and contains water as the solvent.
- Standard_QC (ULOQ).
- Blank
- Standard_QC (ULOQ)
- lower standard (AQ LLOQ)

Acceptance criteria: - The carryover area response in subsequent injections of RS or STD Bulk after aqueous or extracted ULOQ should be ≤ 20.00 % of the equivalent aqueous or extracted LLOQ standard area.

Specificity and Screening of Biological matrix

Specificity is the ability of a bioanalytical method to detect and differentiate the analyte from other substances, including its related substances (e.g., substances that are structurally similar to the analyte, metabolites, isomer, impurities, and degradation products formed during sample preparation or concomitant medications that are expected to be used in the treatment of patients with the intended indication).

Methodology: Specificity is determined by the injecting six samples of standard solution and the LLOQC sample solution and

Acceptance criteria: - check the % Interference Response of interfering peaks in STD Blk at the retention time of analyte should be ≤20.00 % of that in LLOQ and At least 80 % of the matrix lots (Biological

Sample) with intended anticoagulant should be within the acceptance criteria.

Sensitivity
Sensitivity is often interpreted as related to the detection/determination ability, LLOQ based on precision and accuracy (bias) data, this is probably the most practical approach and defines the LLOQ as the lowest concentration of a sample that can still be quantified with acceptable Limit.

Methodology: - the sensitivity is performed by injecting six injections of lower concentration of sample (LLOQ).
Acceptance criteria: -the acceptance criteria of sensitivity of LLOQ are At least 67 % (4 out of 6) of samples should be within 80.00-120.00 %.

Matrix Factor evaluation
A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation it is necessary to evaluate the matrix effect between different independent sources/lots.

Methodology: - The matrix effect should be evaluated by analyzing at least 3 replicates of **low and high QCs (LQC and HQC),** each prepared using matrix from at least 6 different sources/lots.
Acceptance criteria: - The accuracy should be within ±15% of the nominal concentration and the precision (per cent coefficient of variation (%CV)) should not be greater than 15% in all individual matrix sources/lots.

Linearity (Calibration Curve and Range)
the relationship between the nominal analyte concentration and the response of the analytical platform to the analyte, Calibration standards, prepared by spiking matrix with a known quantity of analyte, span the calibration range and comprise the calibration curve. Calibration standards should be prepared in the same biological matrix as the study samples.

Methodology: - The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method
Acceptance criteria: - The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %. The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Rugged Linearity
Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis,

Methodology: -The calibration range is obtained by injecting 6 concentrations of calibration standards not including blank and zero samples and establishing the concentration-response relationship by the sample regression model method and
Acceptance criteria: - The % accuracy for all CC standards except of LLOQ (STD 1) standard should be within 85.00-115.00 %. The % accuracy for LLOQ standard should be within 80.00-120.00 %.

Precision and Accuracy (Intra-day)
Accuracy and precision should be determined by analysing the QCs within each run (within-run) and in different runs (between-run). Accuracy and precision should be evaluated using the same runs and data.

Methodology: -
The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run.

Acceptance criteria: - The overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Rugged Precision and Accuracy (Inter-Day)
Accuracy and precision should be evaluated using the same runs and data.
Methodology: -The test is performed injecting the QC samples were injected 6 replicates at each qc concentration level in each analytical run
Acceptance criteria: the overall accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Recovery
Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Drug.

Methodology: -The recoveries for Sucubitril and Valsartan
at LQC, MQC and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the six samples of LQC, MQC and HQC with the main drug and check the interference with unextracted and extracted

Acceptance criteria:
The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Recovery of Internal Standard
The measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with Internal Standards containing the same area with known amount of Drug.

Methodology: -The recoveries for IS at 6 replicates the results demonstrated that the bioanalytical method had good extraction efficiency by injecting the six samples and check the interference with unextracted and extracted.

Acceptance criteria: The % CV of recovery at each QC level should be ≤ 15.00 %. The overall mean recovery % CV for all QC levels should be ≤ 20.00 %.

Reinjection Reproducibility

Reproducibility of the method is assessed by replicate measurements of the QCs and is usually included in the assessment of precision and accuracy. However, if samples could be reinjected (e.g., in the case of instrument interruptions or other reasons such as equipment failure), reinjection reproducibility should be evaluated and included in the Validation Report or provided in the Bioanalytical Report of the study where it was conducted.

Methodology: -The reproducibility was performed by injecting the qc samples in 6 replicates and check the acceptance limits.

Acceptance criteria: The % mean accuracy for LQC, MQC and HQC samples should be within 85.00-115.00 % and for the LLOQ QC sample it should be within 80.00-120.00 %.

Stabilities

Stability evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte.

Methodology: -The stability is assessed by long term stock solution stability and Matrix samples stability at -

Optimized method:

28±5 °C for 37 days & -80±5 ^{0}C, stability testing is performed by injecting the QC samples of high and low concentrations(HQC and LQC) with taken biological matrix

Acceptance criteria: The mean concentration at each QC level should be within ±15% of the nominal.

RESULTS AND DISCUSSIONS

METHOD DEVELOPMENT

Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Sacubitril and Valsartan as per current ICH guidelines.

Optimization of the chromatographic conditions

For developing the method for the assay of Sacubitril and Valsartan, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A hy_purity advance C18column was chosen as the stationary phase for this study.The mobile phase and the flow rate in order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C18 stationary phase. A binary mixture of acetonitrile and 0.01N Sodium dihyrogen ortho phosphate buffer in a ratio of 70:20 v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.3mL/min was found to be suitable.

Table4 : Chromatographic conditions

Mobile phase	: Acetonitrile: Na2HPo4 (20:70)
Flow rate	: 0.3ml/min
Column	: CHS (50mm x 2.1 mm, 1.8µ)
Detector wavelength	: 260nm
Column temperature	: 42^{0}C
Injection volume	: 1.0µL
Run time	: 4.0min

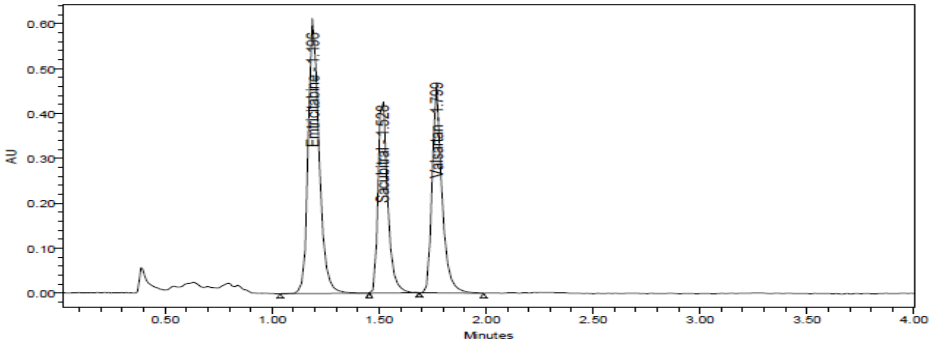

Fig no 15: Chromatogram of Optimized

Table5 : Observation of Optimized Chromatogram

	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing
1	Emtricitabine	1.196	97586	2465.5		1.2
2	Sacubitral	1.528	11465	4572.5	3.5	1.2
3	Valsartan	1.799	67765	6263.5	3.5	1.2

Observation: Sacubitril and Valsartan and Internal Standard were eluted at 1.528 min, 1.788min respectively and 1.196 min(IS) with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits

METHOD VALIDATION

System suitability of Sacubitril and Valsartan

This system suitability method is intended to guarantee that the UPLC system is working in such a way that correct and reproducible data may be submitted to regulatory agencies with confidence. This procedure includes signal stability, carryover, and instrument response tests.

Table 6 : System Suitability of Sacubitril and Valsartan

Sample Name	File Name	Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	Area Ratio
AQ MQC		34278	4.28	97580	2.565	0.3513
AQ MQC		34336	4.30	97623	2.565	0.3517
AQ MQC		34380	4.36	97593	2.566	0.3523
AQ MQC		34425	4.37	97720	2.571	0.3523
AQ MQC		34486	4.37	97815	2.594	0.3526
AQ MQC		34538	4.38	97872	2.597	0.3529
MEAN			4.346		2.576	0.35217
SD			0.0434		0.0150	0.000581
%CV			1.00		0.58	0.17

System Suitability						
Sample Name	File Name	Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	Area Ratio
AQ MQC		5823	3.11	97580	2.565	0.0597
AQ MQC		5890	3.13	97623	2.565	0.0603
AQ MQC		5763	3.13	97593	2.566	0.0591
AQ MQC		5823	3.13	97720	2.571	0.0596
AQ MQC		5862	3.18	97815	2.594	0.0599
AQ MQC		5792	3.19	97872	2.597	0.0592
MEAN			3.146		2.576	0.05963
SD			0.0316		0.0150	0.000475
%CV			1.00		0.58	0.80

Discussion: plate count, tailing factor, resolution of Sacubitril and Valsartan was According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The % CV of the retention time (RT) should be ≤ 2.00 %.

Auto sampler carryover of Sacubitril and Valsartan
The carryover was tracked back to the injection valve and eradicated by converting from a partial loop injection to a full loop injection, which allowed more effective cleansing of the sample flow channel. The UPLC system's susceptibility to carryover was shown to be dependent on the detection method's absolute sensitivity and the mass of analyte injected at the assay's lower limit of quantitation (LLOQ).

Table 7 : Auto sampler carryover of Sacubitril and Valsartan

Parameters	**Peak Area**		**% Carryover**	
	Drug	**ISTD**	**Drug**	**ISTD**
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	69523	98362	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	1792	97980	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	68449	97570	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	1709	97532	N/A	N/A

Parameters	**Peak Area**		**% Carryover**	
	Drug	**ISTD**	**Drug**	**ISTD**
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	22986	98623	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	594	98485	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	22720	97532	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	577	97539	N/A	N/A

Discussion: - The area obtained is less than 20 % of extracted LLOQ standard area to unextracted area by injected of replicate manner

Specificity and Screening of Biological Matrix
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present

Table 8 Specificity and Screening of Biological Matrix of Sacubitril and Valsartan

S.No.	**Parameters**	**Response**		**% Interference**		**Pass/Fail**
		Drug	**ISTD**	**Drug**	**ISTD**	
1	**STD Blk1**	**0**	**0**	**0.00**	**0.00**	**Pass**
2	**LLOQ1**	**1709**	**97532**			
3	**STD Blk2**	**0**	**0**	**0.00**	**0.00**	**Pass**
4	**LLOQ2**	**1725**	**97625**			
5	**STD Blk3**	**0**	**0**	**0.00**	**0.00**	**Pass**
6	**LLOQ3**	**1718**	**97680**			
7	**STD Blk4**	**0**	**0**	**0.00**	**0.00**	**Pass**
8	**LLOQ4**	**1735**	**97762**			
9	**STD Blk5**	**0**	**0**	**0.00**	**0.00**	**Pass**
10	**LLOQ5**	**1738**	**97846**			
11	**STD Blk6**	**0**	**0**	**0.00**	**0.00**	**Pass**
12	**LLOQ6**	**1715**	**97558**			
S.No.	**Parameters**	**Response**		**% Interference**		**Pass/Fail**
		Drug	**ISTD**	**Drug**	**ISTD**	
1	**STD Blk1**	**0**	**0**	**0.00**	**0.00**	**Pass**

2	**LLOQ1**	**577**	**97577**			
3	**STD Blk2**	**0**	**0**	**0.00**	**0.00**	**Pass**
4	**LLOQ2**	**583**	**97862**			
5	**STD Blk3**	**0**	**0**	**0.00**	**0.00**	**Pass**
6	**LLOQ3**	**592**	**97965**			
7	**STD Blk4**	**0**	**0**	**0.00**	**0.00**	**Pass**
8	**LLOQ4**	**586**	**98120**			
9	**STD Blk5**	**0**	**0**	**0.00**	**0.00**	**Pass**
10	**LLOQ5**	**568**	**97653**			
11	**STD Blk6**	**0**	**0**	**0.00**	**0.00**	**Pass**
12	**LLOQ6**	**572**	**97590**			

Observation: We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

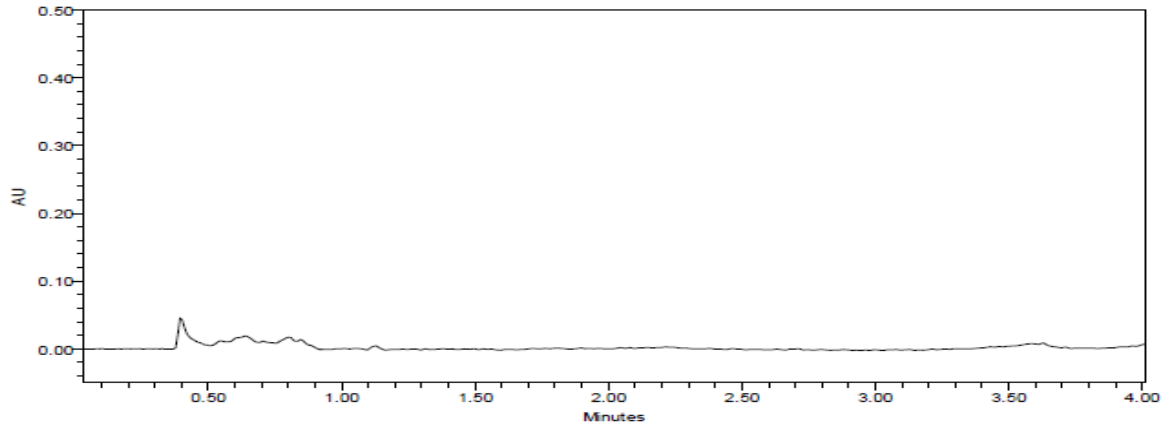

Fig.16 Representative Chromatogram of a Blank Plasma Sample

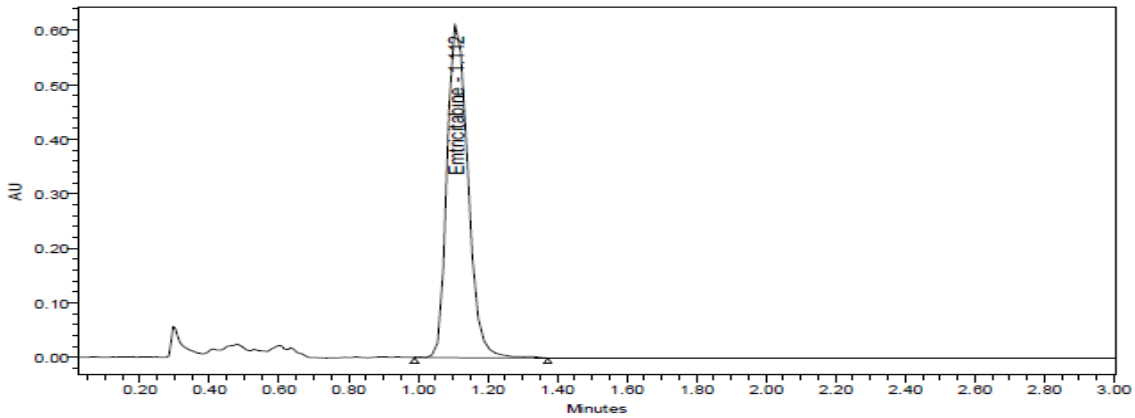

Fig. 17 Representative Chromatogram of Blank Plasma with Internal StandardSample

Discussion – The response areas obtained of analyte and internal standard are less than 20% and 5 % of LLoq Area. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific

Sensitivity

A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ

Table 9 Sensitivity of Sacubitril and Valsartan

Sensitivity of Sacubitril	
S No.	**LLOQ**
	Nominal Concentration (ng/mL)
	300.000
	Nominal Concentration Range (ng/mL)
	(240.000-360.000)
	Calculated Concentration (ng/mL)
1	**298.000**
2	**302.000**
3	**306.000**
4	**295.000**
5	**301.000**
6	**290.000**
n	**6**
Mean	**298.6667**
SD	**5.64506**
% CV	**1.89**
% Mean Accuracy	**99.56**
Sensitivity of Valsartan	
S No.	**LLOQ**
	Nominal Concentration (ng/mL)
	200.000
	Nominal Concentration Range (ng/mL)
	(160.000-240.000)
	Calculated Concentration (ng/mL)
1	**198.000**
2	**196.000**
3	**200.000**
4	**203.000**
5	**205.000**
6	**198.000**
n	**6**
Mean	**200.0000**
SD	**3.40588**
% CV	**1.70**
% Mean Accuracy	**100.00**

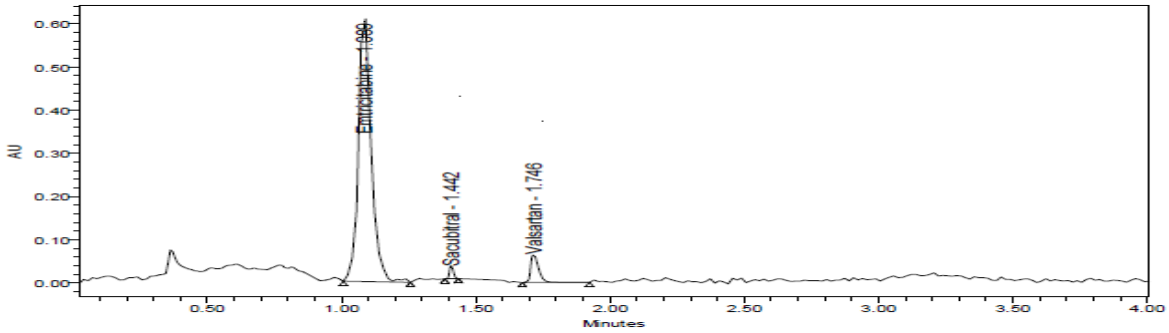

Figure 18: Sensitivity Chromatogram.

Discussion: - The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 1.87% and 1.70% of Valsartan and Sacubitril and Mean of 6 injections was found to be 99.56% & 100.00 % of Valsartan and Sacubitril within the acceptance limits. As the limit of Sensitivity % CV was less than "20%" the system Sensitivity was passed in this method.

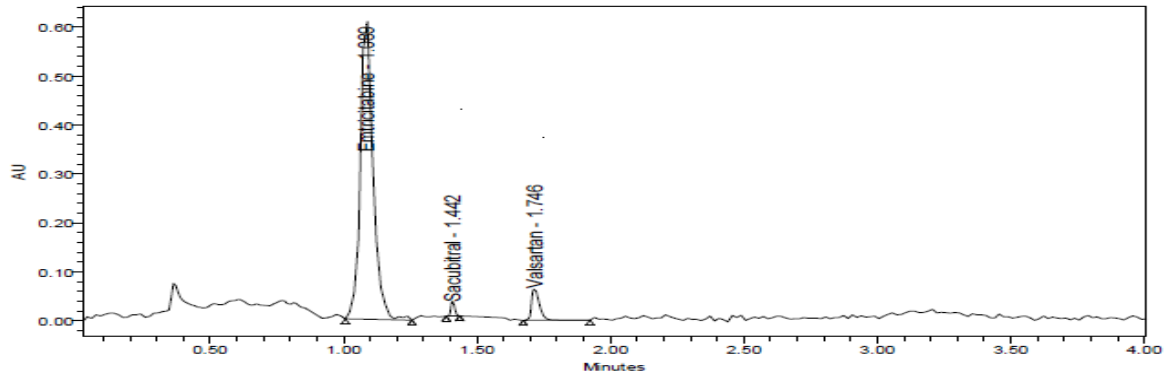

Figure 19: LLOQ Chromatogram

Matrix factor evaluation

Table 10 Matrix factor evaluation for Sacubitril (absence of matrix factor)

Matrix Effect			
S. No.	**Plasma Lot No.**	**HQC**	**LQC**
		Nominal Concentration (ng/mL)	
		4800.000	**600.000**
		Nominal Concentration Range (ng/mL)	
		(4,080.000-5,520.000)	**(510.000-690.000)**
		Calculated Concentration (ng/mL)	
1	**LOT1**	**4830.000**	**578.000**
		4750.000	**655.000**
		4871.000	**620.000**
2	**LOT2**	**4701.000**	**601.000**
		4890.000	**574.000**
		4819.000	**565.000**
3	**LOT3**	**4782.000**	**590.000**
		4825.000	**599.000**
		4875.000	**602.000**
4	**LOT4**	**4966.000**	**555.000**
		4875.000	**609.000**
		4850.000	**596.000**
5	**LOT5**	**4812.000**	**685.000**
		4810.000	**645.000**
		4789.000	**612.000**
6	**LOT6**	**4880.000**	**627.000**
		4865.000	**630.000**
		4820.000	**615.000**
n		**18**	**18**
Mean		**4833.8889**	**608.7778**
SD		**59.24812**	**32.44825**
% CV		**1.23**	**5.33**
% Mean Accuracy		**100.71**	**101.46**
No. of QC Failed		**0**	**0**

Table 11 : Matrix factor evaluation for Sacubitril

S. No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration (ng/mL)	
		3200.000	600.000
		Nominal Concentration Range (ng/mL)	
		(2,720.000-3,680.000)	(510.000-690.000)
		Calculated Concentration (ng/mL)	
1	LOT1	3198.00	599.00
		3210.00	605.00
		3171.00	601.00
2	LOT2	3205.00	566.00
		3201.00	617.00
		3183.00	591.00
3	LOT3	3211.00	613.00
		3218.00	621.00
		3233.00	632.00
4	LOT4	3189.00	628.00
		3185.00	616.00
		3285.00	598.00
5	LOT5	3175.00	652.00
		3089.00	618.00
		3101.00	605.00
6	LOT6	3088.00	599.00
		3199.00	618.00
		3179.00	617.00
n		18	18
Mean		3184.4444	610.8889
SD		49.55238	18.41320
% CV		1.56	3.01
% Mean Accuracy		99.51	101.81
No. of QC Failed		0	0

Discussion- The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 100.71% and 101.46 of HQC and LOQ of Valsartan & 99.51% and 101.81% of HQC and LOQ of Sacubitril and % CV obtained are 1.23% and 5.33% of HQC and LOQ of Valsartan & % CV obtained are 1.56% and 3.01% of HQC and LOQ of Sacubitril. As the limit of CV was, less than "20%" the system Matrix was passed in this method.

Linearity:

Table 12: Linearity of Sacubitril and Valsartan

	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	300.000	600.000	900.000	2400.000	3000.000	3600.000	4800.000	6000.000
	Nominal Concentration Range (ng/mL)							
	(240.000-360.000)	(510.000-690.000)	(765.000-1,035.000)	(2,040.000-2,760.000)	(2,550.000-3,450.000)	(3,060.000-4,140.000)	(4,080.000-5,520.000)	(5,100.000-6,900.000)
	Back Calculated Concentration (ng/mL)							
P&A1	290.000	590.000	916.000	2399.00	2999.00	3697.00	4790.00	6001.00
P&A2	295.000	595.000	905.000	2419.00	3010.00	3602.00	4894.00	5999.00
P&A3	301.000	605.000	909.000	2401.00	3000.00	3670.00	4808.00	6010.00
n	3	3	3	3	3	3	3	3
Mean	295.3333	596.6667	910.0000	2406.3333	3003.0000	3656.3333	4830.6667	6003.3333
SD	5.50757	7.63763	5.56776	11.01514	6.08276	48.95236	55.58177	5.85947
%CV	1.86	1.28	0.61	0.46	0.20	1.34	1.15	0.10
% Mean Accuracy	98.44	99.44	101.11	100.26	100.10	101.56	100.64	100.06

Table 13: Linearity of Valsartan

	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	200.000	400.000	600.000	1600.000	2000.000	2400.000	3200.000	4000.000
	Nominal Concentration Range (ng/mL)							
	(160.000-240.000)	(340.000-460.000)	(510.000-690.000)	(1,360.000-1,840.000)	(1,700.000-2,300.000)	(2,040.000-2,760.000)	(2,720.000-3,680.000)	(3,400.000-4,600.000)
	Back Calculated Concentration (ng/mL)							
P&A1	189.000	402.000	595.000	1589.000	1989.000	2382.000	3125.000	3989.000
P&A2	211.000	389.000	598.000	1625.000	2089.000	2474.000	3189.000	3992.000
P&A3	195.000	405.000	609.000	1601.000	1985.000	2401.000	3219.000	4018.000
n	3	3	3	3	3	3	3	3
Mean	198.3333	398.6667	600.6667	1605.0000	2021.0000	2419.0000	3177.6667	3999.6667
SD	11.37248	8.50490	7.37111	18.33030	58.92368	48.56954	48.01389	15.94783
%CV	5.73	2.13	1.23	1.14	2.92	2.01	1.51	0.40
% Mean Accuracy	99.17	99.67	100.11	100.31	101.05	100.79	99.30	99.99

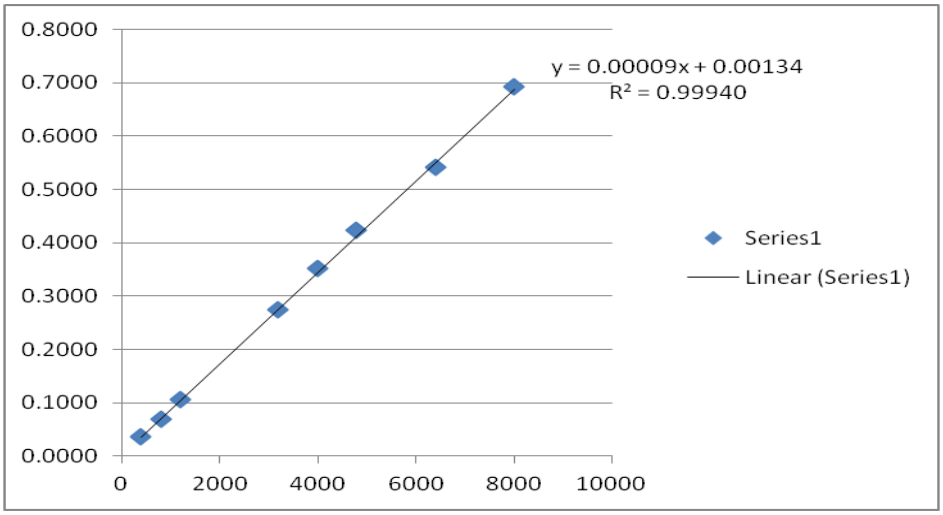

Figure Representative Calibration Curve for sacubitril

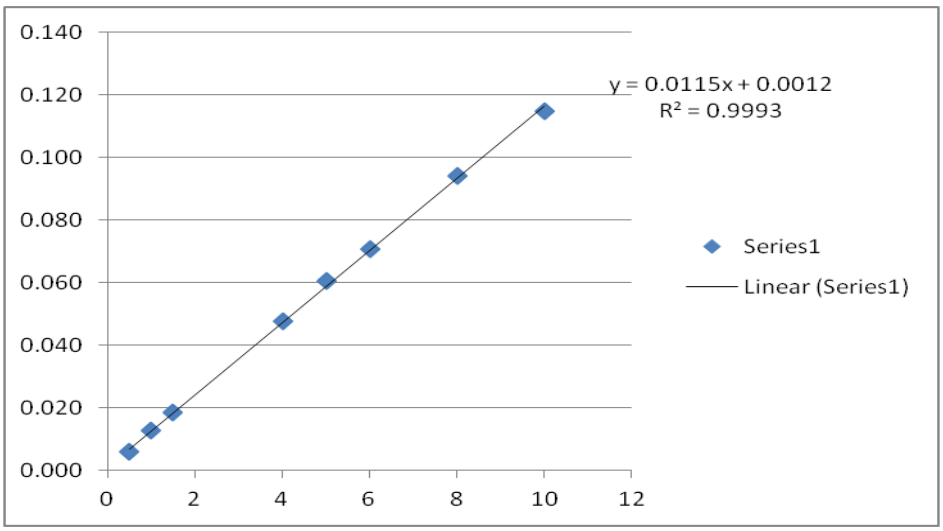

Fig. 20: Representative Calibration Curve for valsartan

Discussion :- Calibration was found to be linear over the concentration range of 0.15 to 6 µg /ml. The coefficient correlation (r^2) value was found consistently greater than 0.999 in all the cases. This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

Precision and accuracy (intra-day runs of Sacubitril and Valsartan)

Table 14 : precision data for intra-day runs of Sacubitril and Valsartan

Precision and Accuracy of Sacubitril				
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	4800.000	**3000.000**	**600.000**	**300.000**
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	**(2,550.000-3,450.000)**	**(510.000-690.000)**	**(240.000-360.000)**
	Back Calculated Concentration (ng/mL)			
	4799.00	**2995.00**	**599.000**	**295.000**
	4801.00	**3001.00**	**539.000**	**299.000**
	4820.00	**3010.00**	**589.000**	**300.000**
	4789.00	**2920.00**	**679.000**	**310.000**
	4800.00	**2990.00**	**607.000**	**298.000**
	4810.00	**2989.00**	**610.000**	**297.000**
n	**6**	**6**	**6**	**6**
Mean	**4803.1667**	**2984.1667**	**603.8333**	**299.8333**
SD	**10.60974**	**32.38158**	**45.04405**	**5.26941**
%CV	**0.22**	**1.09**	**7.46**	**1.76**
% Mean Accuracy	**100.07**	**99.47**	**100.64**	**99.94**
	4896.00	**2888.00**	**598.000**	**299.000**
	4900.00	**2999.00**	**602.000**	**292.000**
	4995.00	**3001.00**	**612.000**	**300.000**
	4940.00	**2889.00**	**590.000**	**301.000**
	4811.00	**3011.00**	**596.000**	**299.000**
	4985.00	**3000.00**	**601.000**	**310.000**
n	**6**	**6**	**6**	**6**
Mean	**4921.1667**	**2964.6667**	**599.8333**	**300.1667**
SD	**67.96887**	**59.15629**	**7.33258**	**5.77639**
%CV	**1.38**	**2.00**	**1.22**	**1.92**

% Mean Accuracy	**102.52**	**98.82**	**99.97**	**100.06**
	4800.00	**2892.00**	**591.000**	**289.000**
	4920.00	**2985.00**	**604.000**	**299.000**
	4899.00	**3001.00**	**589.000**	**307.000**
	4888.00	**3020.00**	**607.000**	**305.000**
	4874.00	**2986.00**	**579.000**	**300.000**
	4985.00	**3110.00**	**600.000**	**301.000**
n	**6**	**6**	**6**	**6**
Mean	**4894.3333**	**2999.0000**	**595.0000**	**300.1667**
SD	**60.42075**	**70.11419**	**10.56409**	**6.27429**
%CV	**1.23**	**2.34**	**1.78**	**2.09**
% Mean Accuracy	**101.97**	**99.97**	**99.17**	**100.06**
Between Batch Precision and Accuracy				
n	**18**	**18**	**18**	**18**
Mean	**4872.8889**	**2982.6111**	**599.5556**	**300.0556**
SD	**71.87644**	**54.70685**	**25.67494**	**5.43921**
%CV	**1.48**	**1.83**	**4.28**	**1.81**
% Mean Accuracy	**101.52**	**99.42**	**99.93**	**100.02**
Precision and Accuracy of Valsartan				
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	4800.000	**3000.000**	**600.000**	**300.000**
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	**(2,550.000-3,450.000)**	**(510.000-690.000)**	**(240.000-360.000)**
	Back Calculated Concentration (ng/mL)			
	4799.00	**2995.00**	**599.000**	**295.000**

	4801.00	**3001.00**	**539.000**	**299.000**
	4820.00	**3010.00**	**589.000**	**300.000**
	4789.00	**2920.00**	**679.000**	**310.000**
	4800.00	**2990.00**	**607.000**	**298.000**
	4810.00	**2989.00**	**610.000**	**297.000**
n	**6**	**6**	**6**	**6**
Mean	**4803.1667**	**2984.1667**	**603.8333**	**299.8333**
SD	**10.60974**	**32.38158**	**45.04405**	**5.26941**
%CV	**0.22**	**1.09**	**7.46**	**1.76**
% Mean Accuracy	**100.07**	**99.47**	**100.64**	**99.94**
	4896.00	**2888.00**	**598.000**	**299.000**
	4900.00	**2999.00**	**602.000**	**292.000**
	4995.00	**3001.00**	**612.000**	**300.000**
	4940.00	**2889.00**	**590.000**	**301.000**
	4811.00	**3011.00**	**596.000**	**299.000**
	4985.00	**3000.00**	**601.000**	**310.000**
n	**6**	**6**	**6**	**6**
Mean	**4921.1667**	**2964.6667**	**599.8333**	**300.1667**
SD	**67.96887**	**59.15629**	**7.33258**	**5.77639**
%CV	**1.38**	**2.00**	**1.22**	**1.92**
% Mean Accuracy	**102.52**	**98.82**	**99.97**	**100.06**
	4800.00	**2892.00**	**591.000**	**289.000**
	4920.00	**2985.00**	**604.000**	**299.000**
	4899.00	**3001.00**	**589.000**	**307.000**
	4888.00	**3020.00**	**607.000**	**305.000**
	4874.00	**2986.00**	**579.000**	**300.000**
	4985.00	**3110.00**	**600.000**	**301.000**
n	**6**	**6**	**6**	**6**
Mean	**4894.3333**	**2999.0000**	**595.0000**	**300.1667**
SD	**60.42075**	**70.11419**	**10.56409**	**6.27429**
%CV	**1.23**	**2.34**	**1.78**	**2.09**
% Mean Accuracy	**101.97**	**99.97**	**99.17**	**100.06**
Between Batch Precision and Accuracy				

n	**18**	**18**	**18**	**18**
Mean	**4872.8889**	**2982.6111**	**599.5556**	**300.0556**
SD	**71.87644**	**54.70685**	**25.67494**	**5.43921**
%CV	**1.48**	**1.83**	**4.28**	**1.81**
% Mean Accuracy	**101.52**	**99.42**	**99.93**	**100.02**

Rugged Precision and Accuracy (inter-day runs of Sacubitril and Valsartan)

Table 15 : precision data for inter-day runs of Sacubitril and Valsartan

Ruggedness Precision and Accuracy				
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	3200.000	**2000.000**	**600.000**	**200.000**
	Nominal Concentration Range (ng/mL)			
	(2,720.000-3,680.000)	**(1,700.000-2,300.000)**	**(510.000-690.000)**	**(160.000-240.000)**
	Calculated Concentration (ng/mL)			
Different Column	**3199.00**	**1968.00**	**592.00**	**199.00**
	3151.00	**2025.00**	**601.00**	**201.00**
	3191.00	**1915.00**	**595.00**	**193.00**
	3209.00	**1968.00**	**618.00**	**191.00**
	3221.00	**1989.00**	**619.00**	**221.00**
	3211.00	**1985.00**	**609.00**	**197.00**
n	**6**	**6**	**6**	**6**
Mean	**3197.0000**	**1975.0000**	**605.6667**	**200.3333**
SD	**24.78709**	**36.03887**	**11.51810**	**10.78270**
% CV	**0.78**	**1.82**	**1.90**	**5.38**
% Mean Accuracy	**99.91**	**98.75**	**100.94**	**100.17**
Different Analyst	**3188.00**	**2065.00**	**591.00**	**198.00**
	3114.00	**1951.00**	**599.00**	**218.00**
	3268.00	**1978.00**	**608.00**	**196.00**
	3211.00	**2011.00**	**601.00**	**204.00**
	3232.00	**2026.00**	**615.00**	**192.00**
	3189.00	**1971.00**	**617.00**	**198.00**
n	**6**		**6**	**6**
Mean	**3200.3333**	**2000.3333**	**605.1667**	**201.0000**
SD	**51.82535**	**41.82663**	**10.00833**	**9.18695**
% CV	**1.62**	**2.09**	**1.65**	**4.57**
% Mean Accuracy	**100.01**	**100.02**	**100.86**	**100.50**
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	4800.000	**3000.000**	**600.000**	**300.000**
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	**(2,550.000-3,450.000)**	**(510.000-690.000)**	**(240.000-360.000)**
	Calculated Concentration (ng/mL)			
Different Column	**4795.000**	**2995.000**	**595.000**	**289.000**
	4800.000	**2889.000**	**586.000**	**299.000**
	4770.000	**3000.000**	**595.000**	**300.000**
	4885.000	**3109.000**	**605.000**	**302.000**
	4798.000	**2997.000**	**601.000**	**292.000**
	4810.000	**2920.000**	**610.000**	**299.000**
n	**6**	**6**	**6**	**6**
Mean	**4809.6667**	**2985.0000**	**598.6667**	**296.8333**
SD	**39.22584**	**76.53235**	**8.50098**	**5.11534**
% CV	**0.82**	**2.56**	**1.42**	**1.72**
% Mean Accuracy	**100.20**	**99.50**	**99.78**	**98.94**
Different Analyst	**4820.000**	**2988.000**	**597.000**	**288.000**

	4787.000	**2992.000**	**589.000**	**291.000**
	4777.000	**2887.000**	**590.000**	**298.000**
	4808.000	**3085.000**	**610.000**	**310.000**
	4775.000	**3100.000**	**620.000**	**302.000**
	4790.000	**3108.000**	**590.000**	**308.000**
n	**6**	**6**	**6**	**6**
Mean	**4792.8333**	**3026.6667**	**599.3333**	**299.5000**
SD	**17.76982**	**86.71716**	**12.86338**	**8.89382**
% CV	**0.37**	**2.87**	**2.15**	**2.97**
% Mean Accuracy	**99.85**	**100.89**	**99.89**	**99.83**

Discussion:- The intraday and inter day accuracy and precision was assessed by analysing six replicates at five different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for Sacubitril and Valsartan at four concentration levels (LLOQ), (LQC), (MQC) and HQC The intra-day and inter day accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 99.96-100.35%, and 98.99%-99.93 % for intraday and 99.06%-100.02 and 98.91%-100.24 for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.38-11.54% and 0.76%-13.49% for intraday and 0.66%-14.23% and 0.77 %-13.16% for inter day respectively.

Recovery of Sacubitril and Valsartan-

Table 16 : Recovery of Sacubitril and Valsartan

Recovery – Analyte for Sacubitril						
S. No.	**HQC**		**MQC1**		**LQC**	
	Un extracted Response	**Extracted Response**	**Un extracted Response**	**Extracted Response**	**Un extracted Response**	**Extracted Response**
1	**18809**	**18237**	**11894**	**11482**	**1792**	**1740**
2	**18503**	**18051**	**11584**	**11470**	**1798**	**1760**
3	**18432**	**18650**	**11697**	**11499**	**1774**	**1756**
4	**18537**	**18121**	**11688**	**11475**	**1748**	**1750**
5	**18559**	**18331**	**11784**	**11486**	**1745**	**1749**
6	**18624**	**18237**	**11698**	**11466**	**1801**	**1753**
n	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**18577**	**18271**	**11724**	**11480**	**1776**	**1751**
SD	**129.97**	**209.98**	**104.69**	**12.01**	**24.95**	**6.86**
% CV	**0.70**	**1.15**	**0.89**	**0.10**	**1.40**	**0.39**
% Mean Recovery	**98.35**		**97.91**		**98.59**	
Overall % Mean Recovery	**98.286**					
Overall SD	**0.3437**					

Recovery – Analyte for valsartan						
S. No.	**HQC**		**MQC1**		**LQC**	
	Un extracted Response	**Extracted Response**	**Un extracted Response**	**Extracted Response**	**Un extracted Response**	**Extracted Response**
1	**55423**	**54832**	**35050**	**34273**	**5280**	**5209**
2	**55848**	**54453**	**35195**	**34366**	**5359**	**5211**
3	**56561**	**55036**	**35075**	**34123**	**5269**	**5203**
4	**55417**	**53821**	**34701**	**34247**	**5282**	**5210**
5	**55738**	**54117**	**35300**	**34911**	**5275**	**5209**
6	**55866**	**54353**	**34909**	**34688**	**5262**	**5215**
n	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**55809**	**54435**	**35038**	**34435**	**5288**	**5210**
SD	**419.04**	**448.10**	**212.14**	**301.42**	**35.63**	**3.89**
% CV	**0.75**	**0.82**	**0.61**	**0.88**	**0.67**	**0.07**
% Mean Recovery	**97.54**		**98.28**		**98.52**	
Overall % Mean Recovery	**98.112**					
Overall SD	**0.5104**					
Overall % CV	**0.52**					

Recovery - Internal standard

Table 17 : Recovery of Emtricitabine (IS)

Recovery - Internal standard for Sacubitril		
S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	98480	97532
2	98693	97585
3	98662	97638
4	98723	97480
5	98520	97570
6	98282	97630
n	6	6
Mean	98560.0	97572.5
SD	167.30	59.93
% CV	0.17	0.06
% Mean Recovery	99.00	
Recovery - Internal standard for Valsartan		
S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	98380	97532
2	98693	97585
3	98682	97638
4	98763	97480
5	98510	97570
6	98252	97630
n	6	6
Mean	98546.7	97572.5
SD	201.24	59.93
% CV	0.20	0.06
% Mean Recovery	99.01	

Discussion: Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Sacubitril and Valsartan

Rugged Linearity:

Table 18 : Rugged Linearity of Sacubitril and Valsartan

Ruggedness Linearity for Sacubitril								
	STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	200.000	**400.000**	**600.000**	**1600.000**	**2000.000**	**2400.000**	**3200.000**	**4000.000**
	Nominal Concentration Range (ng/mL)							
	(160.000-240.000)	**(340.000-460.000)**	**(510.000-690.000)**	**(1,360.000-1,840.000)**	**(1,700.000-2,300.000)**	**(2,040.000-2,760.000)**	**(2,720.000-3,680.000)**	**(3,400.000-4,600.000)**
	Calculated Concentration (ng/mL)							
Different Column								
	0.035	**0.075**	**595.000**	**1595.000**	**1998.000**	**2388.000**	**3197.000**	**4008.000**
Different Analyst								
	0.038	**0.073**	**601.000**	**6011.000**	**2011.000**	**2418.000**	**3299.000**	**4026.000**
Ruggedness Linearity fro Valsartan								
	STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	300.000	**600.000**	**900.000**	**2400.000**	**3000.000**	**3600.000**	**4800.000**	**6000.000**
	Nominal Concentration Range (ng/mL)							
	(240.000-360.000)	**(510.000-690.000)**	**(765.000-1,035.000)**	**(2,040.000-2,760.000)**	**(2,550.000-3,450.000)**	**(3,060.000-4,140.000)**	**(4,080.000-5,520.000)**	**(5,100.000-6,900.000)**
	Calculated Concentration (ng/mL)							
Different Column								
	299.000	**599.000**	**1089.000**	**2399.000**	**2998.000**	**3699.000**	**4820.000**	**6002.000**
Different Analyst								
	301.000	**606.000**	**990.000**	**2482.000**	**3013.000**	**3709.000**	**4885.000**	**6201.000**

Discussion: Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis,The calibration range is obtained by injecting 6 concentrations(0.15 ng/ml-6ng/ml) of calibration standards not including blank and zero samples and establishing, The calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Sacubitril and Valsartan.

Reinjection Reproducibility

Table 19 : Reinjection Reproducibility of Sacubitril and Valsartan

	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	3200.000	2000.000	600.000	200.000
	Nominal Concentration Range (ng/mL)			
	(2,720.000-3,680.000)	(1,700.000-2,300.000)	(510.000-690.000)	(160.000-240.000)
	Calculated Concentration (ng/mL)			
Different Column	3199.00	1968.00	592.00	199.00
	3151.00	2025.00	601.00	201.00
	3191.00	1915.00	595.00	193.00
	3209.00	1968.00	618.00	191.00
	3221.00	1989.00	619.00	221.00
	3211.00	1985.00	609.00	197.00
n	6	6	6	6
Mean	3197.0000	1975.0000	605.6667	200.3333
SD	24.78709	36.03887	11.51810	10.78270
% CV	0.78	1.82	1.90	5.38
% Mean Accuracy	99.91	98.75	100.94	100.17
Different Analyst	3188.00	2065.00	591.00	198.00
	3114.00	1951.00	599.00	218.00
	3268.00	1978.00	608.00	196.00
	3211.00	2011.00	601.00	204.00
	3232.00	2026.00	615.00	192.00
	3189.00	1971.00	617.00	198.00
n	6		6	6
Mean	3200.3333	2000.3333	605.1667	201.0000
SD	51.82535	41.82663	10.00833	9.18695
% CV	1.62	2.09	1.65	4.57
% Mean Accuracy	100.01	100.02	100.86	100.50

Reinjection Reproducibility				
	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	4800.000	3000.000	600.000	300.000
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	(2,550.000-3,450.000)	(510.000-690.000)	(240.000-360.000)
	Calculated Concentration (ng/mL)			
P&A01	4865.000	2965.000	586.000	288.000
	4884.000	2950.000	672.000	298.000
	4921.000	2989.000	620.000	309.000
	4879.000	3009.000	584.000	312.000
	4886.000	2889.000	610.000	302.000
	4908.000	3019.000	625.000	299.000
n	6	6	6	6
Mean	4890.5000	2970.1667	616.1667	301.3333
SD	20.40343	47.47385	32.22680	8.57127
% CV	0.42	1.60	5.23	2.84
% Mean Accuracy	101.89	99.01	102.69	100.44

Stabilities

Long term stock solution stability

Table no 20: stability of Sacubitril and Valsartan (zero days)

S. No.	HQC	LQC
	Nominal Concentration (ng/mL)	
	3200.000	600.000
	Nominal Concentration Range (ng/mL)	
	(2,720.000-3,680.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)	
1	3169.000	598.000
2	3189.000	609.000
3	3211.000	592.000
4	3222.000	601.000
5	3191.000	612.000
6	3181.000	603.000
n	6	6
Mean	3193.8333	602.5000
SD	19.49786	7.28697
% CV	0.61	1.21
% Mean Accuracy	99.81	100.42
S. No.	HQC	LQC
	Nominal Concentration (ng/mL)	
	4800.000	600.000
	Nominal Concentration Range (ng/mL)	
	(4,080.000-5,520.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)	
1	4965.000	589.000
2	4889.000	598.000
3	4895.000	610.000
4	4920.000	599.000
5	4810.000	625.000
6	4871.000	620.000
n	6	6
Mean	4891.6667	606.8333
SD	51.56614	13.93437
% CV	1.05	2.30
% Mean Accuracy	101.91	101.14

Matrix samples stability at -28±5 °C for 37 days

Table 21 : Matrix samples stability at -28±5 °C for 37 days

Long Term Analyte Stability in Matrix for Sacubitril				
S. No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	3200.000	3200.000	600.000	600.000
	Nominal Concentration Range (ng/mL)			
	(2,720.000-3,680.000)	(2,720.000-3,680.000)	(510.000-690.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	3213.00	3189.00	598.000	592.000
2	3212.00	3198.00	605.000	585.000
3	3188.00	3168.00	589.000	595.000
4	3178.00	3178.00	601.000	601.000
5	3206.00	3165.00	595.000	605.000
6	3219.00	3025.00	601.000	596.000
n	6	6	6	6
Mean	3202.6667	3153.8333	598.1667	595.6667
SD	16.09555	64.33480	5.60060	6.97615
% CV	0.50	2.04	0.94	1.17
%Mean Accuracy	100.08	98.56	99.69	99.28
% Mean Stability	98.48		99.58	

Long Term Analyte Stability in Matrix for Valsartan				
S. No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	4800.000	4800.000	600.000	600.000
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	(4,080.000-5,520.000)	(510.000-690.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	4825.000	4822.000	605.000	599.000
2	4801.000	4768.000	596.000	609.000
3	4795.000	4798.000	608.000	600.000
4	4859.000	4778.000	610.000	598.000
5	4810.000	4789.000	600.000	601.000
6	4877.000	4792.000	609.000	603.000
n	6	6	6	6
Mean	4827.8333	4791.1667	604.6667	601.6667
SD	33.20492	18.50856	5.57375	3.98330
% CV	0.69	0.39	0.92	0.66
%Mean Accuracy	100.58	99.82	100.78	100.28
% Mean Stability	99.24		99.50	

Matrix samples stability at -80±5 °C for 37days

Table no 22: Matrix samples stability at -80±5 °C for 37 days

Long Term Analyte Stability in Matrix for Sacubitril				
S. No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	4800.000	4800.000	600.000	600.000
	Nominal Concentration Range (ng/mL)			
	(4,080.000-5,520.000)	(4,080.000-5,520.000)	(510.000-690.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	4805.000	4725.000	616.000	589.000
2	4796.000	4791.000	605.000	601.000
3	4820.000	4805.000	592.000	598.000
4	4871.000	4801.000	620.000	595.000
5	4816.000	4799.000	609.000	598.000
6	4821.000	4801.000	615.000	601.000
n	6	6	6	6
Mean	4821.5000	4787.0000	609.5000	597.0000
SD	26.09789	30.72458	10.09455	4.51664
% CV	0.54	0.64	1.66	0.76
%Mean Accuracy	100.45	99.73	101.58	99.50
% Mean Stability	99.28		97.95	
Long Term Analyte Stability in Matrix for Valsartan				
S. No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	3200.000	3200.000	600.000	600.000
	Nominal Concentration Range (ng/mL)			
	(2,720.000-3,680.000)	(2,720.000-3,680.000)	(510.000-690.000)	(510.000-690.000)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	3162.000	3198.000	595.000	588.000
2	3271.000	3156.000	602.000	591.000
3	3225.000	3185.000	598.000	595.000
4	3211.000	3198.000	603.000	598.000
5	3182.000	3185.000	596.000	601.000
6	3177.000	3125.000	601.000	593.000
n	6	6	6	6
Mean	3204.6667	3174.5000	599.1667	594.3333
SD	39.88316	28.69669	3.31160	4.71876
% CV	1.24	0.90	0.55	0.79
%Mean Accuracy	100.15	99.20	99.86	99.06
% Mean Stability	99.06		99.19	

Summary

VALIDATION RESULTS OF Sacubitril and Valsartan				
Analyte Parameters	**Sacubitril and Valsartan**	**Internal standard**	**Acceptance Criteria**	
	%Nominal precision		%	Precision
Biological Matrix	Rabbit Plasma	Rabbit Plasma	N/AP	N/AP
Analytical Range	0.4ng/ml-8µg/ml of Valsartan & 0.2 µg/ml -4 µg/ml of Sacubitril	N/AP	N/AP	N/AP
Minimum Quantifiable	8 µg/ml of Valsartan_ 4 µg/ml of Sacubitril	N/AP	N/AP	≤ 20%
Matrix Effect LQC HQC	99.51%& 101.81% of Sacubitril & 100.71%& 101.46% of Valsartan	N/AP	85% - 115%	≤ 15%
Coefficient of correlation	0.999	N/AP	r2 ≥ 0.98	
Accuracy and Precision for Sensitivity	100.0%	N/AP	80% - 120%	≤ 20%
Within Batch Accuracy and Precision	101.52% ,99.42% , 99.93 % of Valsartan & 100.71% ,101.48%, 100.18 % of Sacubitril	N/AP	85%-115% (L, M1, M2, H)80%-120%	≤15%% (L, M1, M2,H) ≤20%(LL

Conclusion

A simple, accurate, precise method was developed for the estimation of the Sacubitril and Valsartan in Rabbit plasma using the Emtricitabine as internal standard. Retention time of Sacubitril and Valsartan was found to be 1.196min (IS) and 1.528min of Sacubitril and 1.799 min of Valsartan. Which reach the level of both drugs possibly found in Rabbit plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range. The proposed method is simple, rapid, accurate, precise, and appropriate for pharmacokinetic and therapeutic drug monitoring in the clinical laboratories.

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