A SYNOPSIS
on

STUDY ON DEGRADATION BEHAVIOUR AND IMPURITY PROFILING OF DRUGS AND THEIR FORMULATION USED IN THE TREATMENT OF CARDIOVASCULAR DISORDERS

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STUDY ON DEGRADATION BEHAVIOUR AND IMPURITY PROFILING OF DRUGS AND THEIR FORMULATION USED IN THE TREATMENT OF CARDIOVASCULAR DISORDERS

Abstract

Sacubitril/Valsartan (SAC/VAL) is a combination drug used for the treatment of heart failure. VAL underwent significant degradation under acid hydrolytic, oxidative, photo and thermal stress conditions, while it was stable under base hydrolytic stress condition. SAC underwent significant degradation under hydrolytic (acid and base), oxidative stress conditions, while it was stable under photo and thermal stress condition. A total of six degradation products (DPs) were obtained. A simple, selective and reliable RP-HPLC method has been developed for the separation of VAL, SAC and its DPs using Acquity Zorbax SB- C8 column (150 mm x 4.6 mm, 5 µm) with mobile phase consisting of ammonium acetate (0.02 M, pH 3.0) buffer and acetonitrile (55:45, v/v). Chromatographic analysis was performed at flow rate of 1.0 mL/min using a PDA detector at a wavelength of 254 nm. The method was validated as per ICH Q2 (R1) guideline and all the validation parameters were found within the acceptance criteria. The forced degradation study for SAC/VAL showed that the drugs were prone to acidic, alkaline, oxidative stress conditions. All the degradation products were separated from each other, SAC/VAL and their degradation products showing the stability indicating criteria of the method. The major DP of valsartan (VAL D-5) was characterized using LCMS based on mass fragmentation pattern and accurate m/z values. The major DPs of sacubitril (SAC D-2 and SAC D-3) were characterized by LCMS and extensive NMR (including 2D) spectroscopic methods. Study of NMR spectra and Mass spectrometry confirmed the suggested structures for DPs of sacubitril.

Introduction

The ICH stability testing guideline requires the drug to be subjected to stress decomposition studies followed by identification and characterization of the degradation products. In parallel, the ICH guideline on impurities necessitates characterization of all degradation products formed in drug products. The hyphenated techniques are in focus for the purpose, among which LC-MS tools have been explored more strongly due to their potential to directly characterize small quantities of degradation products.

Heart failure is a complex clinical syndrome, which occurs due to the reduced ability of the heart to pump an adequate supply of the blood throughout the body. It has become a growing epidemiologic problem and a leading cause of morbidity and mortality. Globally, around 26 million people suffer from heart failure. A strategy was finalized for elucidation of structures of degradation products present in minute amounts. An endeavour of the present study was to validate this strategy by its further application to forced decomposition samples of combination of sacubitril, a novel neprilysin inhibitor, with valsartan, an angiotensin receptor blocker.[1-5]

Valsartan is chemically designated as N-(1-oxopentyl)-N-({2′-(2H-tetrazol-5-yl) [1,1′-biphenyl]-4-yl}methyl)-l-valine (VAL, Fig. 1a) while Sacubitril calcium is chemically designated as 4 ((2S,4R)-1-(biphenyl-4-yl)-5-ethoxy-4-methyl-5-oxopentan- 2-ylamino)-4-oxobutanoic acid calcium salt (SAC, Fig. 1b). SAC is a prodrug that is hydrolyzed by esterases to sacubitrilat (Fig. 1c). Sacubitrilat suppresses neprilysin enzyme which is responsible for the disintegration of two blood pressure-lowering natriuretic peptides. These peptides raise the level of cyclic guanosine-3,5-monophosphate, cause diuresis, natriuresis and vasodilation and also possess further antisympathetic and antifibrotic effects. In addition, neprilysin enzyme assists in angiotensin breakdown. Concurrent inhibition of the renin–angiotensin–aldosterone system gives the entire advantages and benefits of neprilysin inhibition. Valsartan acts by selectively blocking an angiotensin II receptor subtype 1, present in the adrenal gland and vascular smooth muscle. In addition, valsartan hinders angiotensin II effect in the renin angiotensin system and also retards the advancement of chronic heart failure.[6-11]

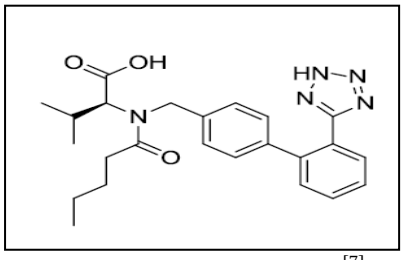


Fig.1a Structure of Valsartan[7]

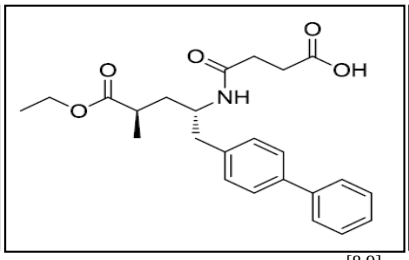


Fig.1b Structure of Sacubitril[8,9]

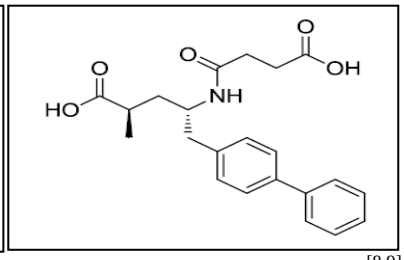


Fig.1c Structure of Sacubitrilat[8,9]

The degradation behaviour of valsartan along with other drugs was reported [12-33] but none of the degradation products were characterized. Hence it was of interest to study the same. The research work involved the following steps: (1) subjecting of drugs to ICH prescribed hydrolysis, oxidative, photolytic and thermal stress; (2) separation of degradation products on an HPLC column; (3) establishment of fragmentation pattern of the drug using MS/TOF, MS^n and H/D exchange studies; (4) characterization of degradation products from LC-MS/TOF and online H/D exchange data and NMR studies; and (5) justification of elucidated structures.

Definition of the Problem

- FDA has approved SACUBITRIL/VALSARTAN Tablets on July 2015.
- Brand Name in the Market is ENTRESTO manufactured by Novartis.
- ENTRESTO is a combination of sacubitril, a neprilysin inhibitor, and valsartan, an angiotensin II receptor blocker, indicated to reduce the risk of cardiovascular death and hospitalization for heart failure in patients with chronic heart failure.
- Based on literature review, several reported methods were found including stability indicating method for simultaneous estimation of sacubitril and valsartan but not a single reported method was found for estimation of both drugs and its impurities in the formulation.

Objective and Scope of work

- Development of RP-HPLC method for simultaneous estimation of valsartan and sacubitril.
- Validation of developed method according to ICH Q2 (R1) guideline.
- Study of force degradation behaviour: To perform Acid degradation, Base degradation, Oxidative degradation, Photo degradation, Thermal degradation.
- Identification, Isolation and structure elucidation of impurities by LC/MS and NMR studies.
- Characterization of DPs of valsartan and sacubitril.

Original contribution by the thesis

- A simple, precise, accurate and stability indicating reverse phase HPLC methods for determination of SAC and VAL in routine analysis which are validated as per ICH guideline and developed method resolves the drugs from its degradation products.

- Identification, Isolation and structure elucidation of DPs of VAL and SAC by LC/MS and NMR studies.

Methodology of Research and Results

Drugs and reagents

Pure standard of VAL was obtained as a gift sample from Lincoln Pharmaceutical Ltd., Gujarat, India and SAC from Cipla Pharmaceutical Ltd., Mumbai, India. Analytical reagent (AR) grade sodium hydroxide (NaOH) was purchased from Ranbaxy Laboratories, hydrochloric acid (HCl) from LOBA Chemie Pvt. Ltd. (Mumbai, India), and hydrogen peroxide (H_2O_2) from s.d.Fine-Chem Ltd. (Boisar, India). Buffer salts of AR grade and all other chemicals were bought from local suppliers. HPLC grade acetonitrile (ACN) was procured from J.T. Baker (Phillipsburg, NJ, USA). Ultra pure water was obtained from a milli Q purification system. Deuterated water (D2O, 99.9%) was obtained from Aldrich (St Louis, MO, USA).

Apparatus and equipment

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for solution degradation studies. A Dri-Bath (Thermolyne, IA, USA) was used for solid state thermal stress study. UV radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany) were used to measure visible illumination and UV energy, respectively. pH/Ion analyzer (MA 235, Mettler Toledo, Schwerzenbach, Switzerland) was used to check and adjust the pH of buffer solutions. Other smaller equipment used were sonicator (3210, Branson Ultrasoincs Corporation, Danbury, CT, USA), precision analytical balance (AG 135, Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf, Hamburg, Germany). The degradation behaviour of the drug was studied on a liquid chromatography (HPLC) system equipped with a photodiode array detector and controlled by Empower software ver. 2 (Seperation Modules 2695,Waters, USA). MS^n studies were carried out on an LTQ XL MS 2.5.0 system (Thermo, San Jose, CA, USA). The same was controlled by Xcalibur (version 2.0.7 SP1) software. LC–MS/TOF results were obtained on a system in which HPLC (1100, Agilent Technologies, Waldbronn, Germany) was hyphenated to MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), using Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. The TOF instrument was also used for H/D exchange study on the drug, while MS^n system was employed for online H/D exchange investigations on the degradation products. In all the studies, the separations were achieved on a Zorbax SB C-8 (150 mm× 4.6 mm i.d., particle size 5 μm) column.

Sample preparation

Preparation of stock solutions for the standard drugs

The mixture of water and acetonitrile (50:50, %v/v) was used as a diluent in the preparation for samples. Accurately weighed 20 mg of SAC and 20 mg of VAL standards were transferred to volumetric flask of 100 mL. Around 50 mL of the diluent was added and sonicated for 15 minutes to dissolve the drugs completely. The volume was made up to the mark with the diluent to obtain the stock solution of 200 μg/mL of SAC and 200 μg/mL of VAL.

Preparation of standard solutions

Precise and appropriate 1 mL volume from the stock solution for SAC and VAL was transferred into the 10 mL volumetric flask and diluted with the diluent to obtain a solution containing SAC (20 μg/mL) and VAL (20 μg/mL) for the assay method.

Preparation of SAC/VAL sample solution

A Quantity of the tablet powder equivalent to 24 mg of the SAC and 26 mg VAL complex sample was weighed accurately and transferred to 100 mL of volumetric flask. About 50 mL of the diluent was added and sonicated for 15 minutes. The volume was made up to the mark. The resulting sample stock solution was filtered using Whatman filter paper, discarding the first few milliliters. One mL of Aliquot from the prepared stock solution was transferred into 10 mL volumetric flask and the volume was adjusted upto the mark with the diluents. This solution was used for the assay method.

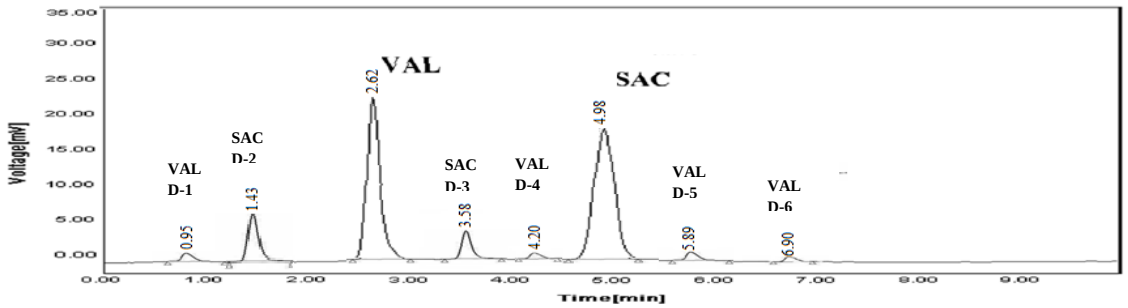
HPLC method development and optimization

Various trials were conducted for the method development. Final chromatographic separation was achieved on Zorbax SB C8, (150 × 4.6) mm; 5 μm column. In the optimized method, the mobile phase consisted of a mixture of 0.02 M ammonium acetate buffer and acetonitrile (55:45, %v/v) and pH of the mobile phase was set at 3.0 using glacial acetic acid. The flow rate was set at 1.0 mL/minute, the injection volume was kept 20 μl. The samples were scanned in the range of 200–400 nm using a PDA detector and the monitoring wavelength was set at 254 nm.

Stress studies

For Valsartan and Sacubitril, Acidic and alkaline hydrolysis were carried out in 2N HCl and 2N NaOH, respectively. All the hydrolytic studies were conducted at 75°C for 1 hour. The oxidative study was carried out in 15% H_2O_2 at room temperature for 5 hours. Photolytic studies on the drug in the solid and solution state were carried out by exposure to a combination of UV lamps in a photostability chamber for 24 hours. A parallel blank set was

kept in the dark for comparison. For thermal stress testing, the drugs were sealed in glass vials and placed in the thermostatic block at 121°C for 3 days. After subjecting the samples to stress studies, they were withdrawn at suitable time intervals and diluted with mobile phase to make final concentration for VAL 20 µg/mL and SAC 20 µg/mL before LC injection. All the prepared sample solutions were filtered through 0.22 µm nylon syringe filters prior to the injection in HPLC. From the individual stress studies of Valsartan and Sacubitril, identify the degradants of valsartan are found to be DP-1, DP-4, DP-5 and DP-6 named VAL D-1, VAL D-4, VAL D-5 and VAL D-6 respectively while degradants of Sacubitril are found to be DP-2 and DP-3 named SAC D-2 and SAC D-3 respectively shown in Figure 2.



Analytes	Valsartan	Valsartan	Valsartan	Valsartan	Sacubitril	Sacubitril
DP Peaks	VAL D-1	VAL D-4	VAL D-5	VAL D-6	SAC D-2	SAC D-3
Stress Conditions	Alkaline	Oxidative	Oxidative and Photo	Oxidative and Photo	Acid and Alkaline	Acid

Figure 2 Chromatogram showing separation of DPs (1-6), Valsartan and Sacubitril in the mixture of stressed samples[5]

Analytical method validation

Analytical method validation was performed to prove the reliability and consistency of the results within the scope of its intended use. The parameters including precision, accuracy, linearity, LOD, LOQ, specificity, and robustness were evaluated as per ICH Q2 guideline. The results of method validation are summarized in Table 1.

Table 1. Summary of validation parameters

VALIDATION PARAMETERS	**SAC**	**VAL**
Linearity	12-36 µg/mL	13-39 µg/mL
Accuracy (% Recovery) (n=3)	99.10-99.84 %	100.81-100.96%
Precision (% RSD)		
Repeatability (n=6)	0.10	0.21
Intraday (n=3)	0.10 - 0.14	0.10 - 0.23
Interday (n=3)	0.11 - 0.20	0.15 - 0.31
LOD (µg/mL)	0.0797	0.1615

LOQ (µg/mL)	0.2416	0.4893
Robustness (% RSD)		
Change in Flow Rate	0.12	0.12
Change in pH	0.11	0.14

Estimation of Sacubitril and Valsartan in the marketed formulation by the proposed Stability indicating RP-HPLC method:

Applicability of the proposed method was tested by analyzing the commercially available formulation. The assay data are shown in the Table 2.

Table 2. Analysis of marketed formulation

SACUBITRIL		**VALSARTAN**	
Label claim (mg)	**Amt found in assay (µg)**	**Label claim (mg)**	**Amt found in assay (µg)**
24	24.32	26	26.56
	24.53		25.81
	24.89		26.24
	24.72		25.62
	24.87		25.97
Mean Amt Found (mg) ± SD	24.67 ± 0.24	Mean Amt Found (mg) ± SD	26.04 ± 0.37
% RSD	0.98	% RSD	1.42
% labelled claim	102.79	% labelled claim	100.15
Standard Limit	90 - 110	Standard Limit	90 - 110

MS/TOF, MS^n and H/D exchange studies on the drug

In order to establish a comprehensive fragmentation pathway of the drugs, MS/TOF studies were performed in ESI positive mode in the mass range of 50 to 1500 Da. High purity nitrogen was used as a nebulizer as well as an auxiliary gas. Mass parameters were optimized. The drugs were further subjected to multistage mass studies (MS^n) in ESI positive mode. Fragmentation of various precursor ions formed in MS^n studies was achieved at different collision energies. This was followed by conduct of H/D exchange studies on the drug, wherein the drugs solutions were prepared in a mixture of CH_3CN and D_2O.

LC-MS/TOF studies on degradation products

The stressed samples were subjected to LC-MS/TOF studies using the developed LC method having ammonium acetate at same buffer concentration and pH. The identity of each degradation product was established with the help of LC-MS/TOF accurate mass values, and comparison of fragmentation profiles with the drugs.

Nuclear Magnetic Resonance Studies

For Sacubitril, Both degradation impurities were dissolved individually in DMSO-d6 solvent and recorded the spectra of ^1H, ^{13}C, ^1H-^1H COSY, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC of these

degradant products from 400 MHz w.r.t. ^{1}H NMR spectrometer equipped with the multinuclear probe. Methylated silanes like SiMe4 (TMS) [(Me = CH_3)] were taken as an internal reference compound and relatively this, ^{1}H and ^{13}C nuclear chemical shifts are reported using ppm units with a reference SiMe4 (TMS). The spectra were recorded by referencing Tetra Methyl Silane to δ 0.0 ppm in both ^{1}H and ^{13}C NMR, and δ 2.5 ppm, δ 39.50 ppm in ^{1}H, ^{13}C NMR respectively.

Results and Discussion

Degradation behaviour of valsartan and sacubitril

The Valsartan degraded an extent of 15.95%, 20.08%, 10.04% and 12.21% under acid, oxidative, photo and thermal conditions, respectively to form one degradation product (VAL D-5). The Sacubitril degraded an extent of 32.33%, 36.71% and 2.98% under acid, alkali and oxidative conditions, respectively to form two degradation products (SAC D-2 and SAC D-3). The Valsartan was stable under stress condition, including hydrolysis in alkali; exposure to UV light while the Sacubitril was stable under photo and thermal stress conditions.

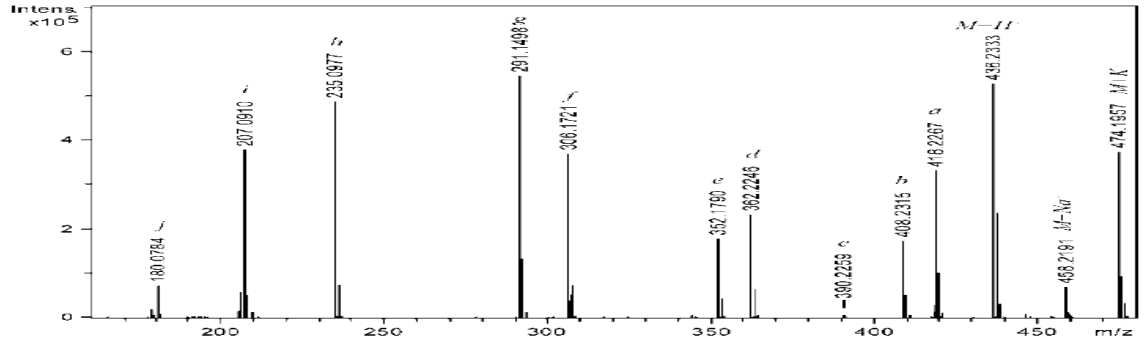


Figure 3 Line spectrum of valsartan obtained in LC-MS/TOF study.

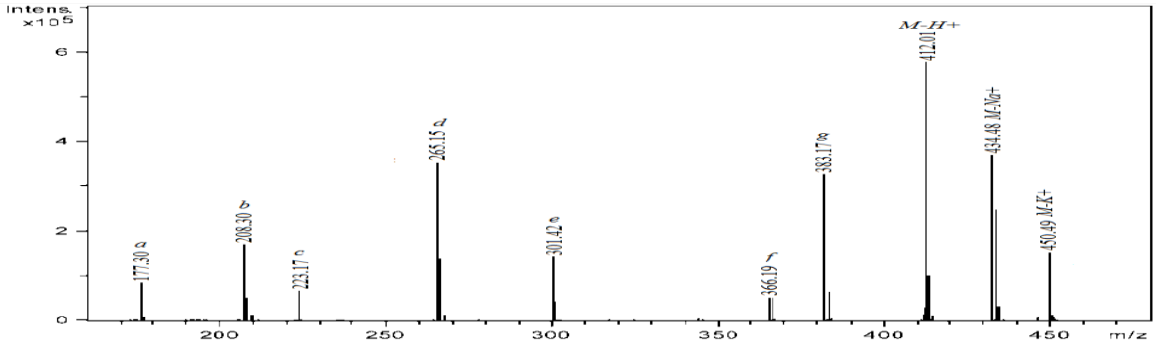


Figure 4 Line spectrum of sacubitril obtained in LC-MS/TOF study.

Mass fragmentation behaviour of the valsartan and sacubitril

Figure 3 and 4 shows line spectrum of the valsartan and sacubitril obtained from MS/TOF studies, respectively. In total, ten fragments (labelled 'a-j') were formed from valsartan ($M+H^{+}$), apart from Na and K adducts and seven fragments (labelled 'a-g') were formed from

sacubitril ($M+H^+$), apart from Na and K adducts. The accurate mass of each fragment was used to determine the most probable molecular formula (Table 3), taking the help of elemental composition calculator. Subsequently, MS^n studies were performed on the valsartan to determine the origin of each fragment (Table 4). This was followed by proposition of tentative structures to each, also taking into account data from H/D exchange studies, and calculated values of ring plus double bonds (RDBs). The fragmentation behaviour of valsartan correlated to data in Tables 3 and 4 is outlined in Figure 5. The figure shows existence and involvement of three possible protonated forms of the precursor [m/z 436] in the valsartan fragmentation pathway. The precursor with protonation at the carboxylic group produced a daughter ion of m/z 418, whereas those charged at amide nitrogen and tetrazole nitrogen reduced to ions of m/z 408 and 352, respectively. The ion of m/z 418 further fragmented in MS^3 step into an ion of m/z 390, which on MS^4 resulted in two parallel daughter fragments of m/z 362 and 306. The ion of m/z 362, on further MS^4 and MS^5 analyses fragmented to m/z 347 → 291, while the one of m/z 306 followed the pathway m/z 235→ 207→ 180.

Table 3 Interpretation of MS/TOF, H/D exchange and MS^n data of fragments of Valsartan[6]

Peak no.	MS/TOF data	Best possible molecular formula	Exact mass of most probable structure	Error in ppm	RDB	Possible parent fragment	Difference from parent ion	Possible molecular formula for loss		H/D Exchange data	No. of labile hydrogens	
								L1	L2		M+H+	M+
M+H$^+$	436.2333	$C_{24}H_{30}N_5O_3$	436.2343	-2.2	12.5	-	-	-	-	439	3	2
a	418.2267	$C_{24}H_{28}N_5O_2$	418.2238	6.9	13.5	M+H+	18.0066	H_2O	-	419	1	0
b	408.2315	$C_{24}H_{30}N_3O_3$	408.2282	8.0	11.5	M+H+	28.0018	N_2	-	411	3	2
c	390.2259	$C_{23}H_{28}N_5O$	390.2288	-7.4	12.5	a	28.0008	N_2	CO	391	1	0
d	362.2246	$C_{23}H_{28}N_3O$	362.2227	5.2	11.5	b,c	-	CH_2O_2, N_2	-	363	1	0
e	352.1790	$C_{19}H_{22}N_5O_2$	352.1768	6.2	11.5	M+H+	84.0543	$C_3H_6N_3$	C_5H_8O	356	4	3

f	306.1721	$C_{18}H_{20}N_5$	306.1713	2.6	11.5	c,e	-	C_5H_8O, CH_2O_2	$C_3H_6N_3$	308	2	1
g	291.1498	$C_{19}H_{19}N_2O$	291.1492	2.0	11.5	*	-	-	-	292	1	0
h	235.0977	$C_{14}H_{11}N_4$	235.0978	-0.4	11.5	f	71.0743	C_4H_9N	-	236	1	0
i	207.0910	$C_{14}H_{11}N_2$	207.0917	-3.3	10.5	h	28.0067	N_2	-	208	1	0
j	180.0784	$C_{13}H_{10}N$	180.0808	-13.3	9.5	*	-	-	-	181	1	0

*MS^n study could not be achieved

Table 4 MS^n fragmentation of Valsartan

MS^n	Precursor ion	Product ions
MS^2	436	418,408,362,352,291,235,207
MS^3	418	390,362,306,235,207
	408	362,291
	352	306,235,207
MS^4	390	362,347,306,235,207
	362	347[a],291[a]
	306	235,207,180
MS^5	235	207[a], 180

[a] Fragments had low intensity, so could not be captured for further MS^n

LC-MS/TOF and on-line H/D exchange studies on degradation product of valsartan (VAL D-5)

Subsequent to establishment of mass fragmentation pattern for the valsartan, the VAL D-5 was also subjected to LC-MS/TOF analyses to elucidate their structures. The mass spectra obtained thereof are shown in Figure 6. The data of accurate masses, possible molecular formulae, major fragments and on-line H/D exchange for the VAL D-5 are enlisted in Table 5.

Characterization of VAL D-5

The VAL D-5 was characterized through systematic amalgamation of HRMS, mass fragmentation and on-line H/D exchange data.

VAL D-5

The HRMS value of VAL D-5 was 352.1762 (Figure 6, Table 5). Its major fragments had m/z of 306, 235, 207 and 180. All these fragments were also formed from the product ion of m/z 352.1768 in case of the Valsartan drug (Figure 5). This clearly meant that VAL D-5 had

the same structure as the drug fragment of equal mass. The same was supported by the presence of three labile hydrogens (as determined by H/D exchange study) and very small error of −1.7 ppm between exact and observed masses. The proposed structure of VAL D-5 is shown in Figure 7. The interpretation using Mass data and fragmentation data confirms that the degradation product VAL D-5 is named to be as 3-methyl-2-({[2'-(1H-1,2,3,4-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]methyl}amino)butanoic acid.

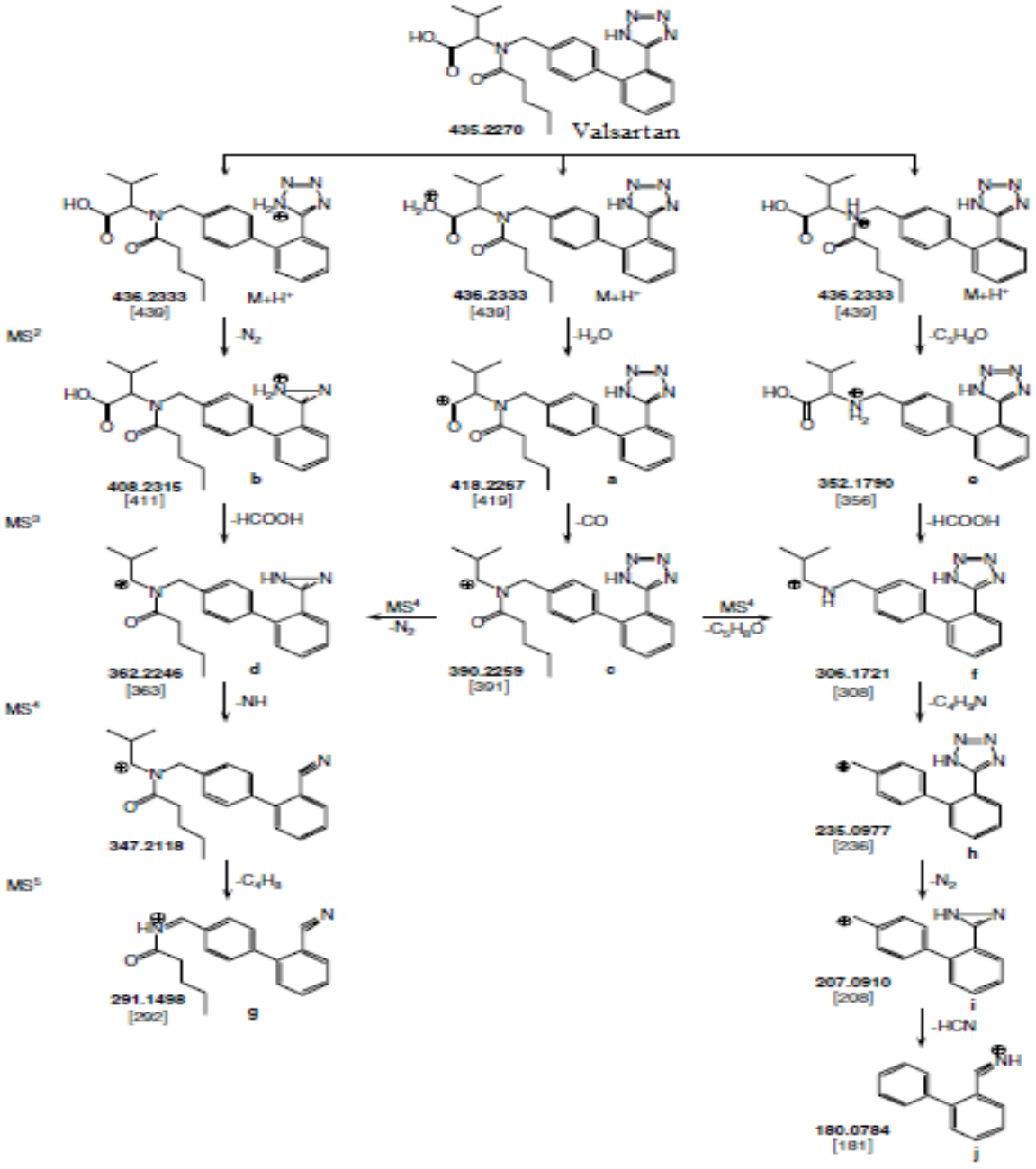


Figure 5 Fragmentation pathway of Valsartan. The value of obtained accurate mass is shown below each structure, along with the mass obtained in H/D exchange study (in square brackets). $M+H^+$ denotes the protonated form of precursor; while a-j represent fragments in the line spectrum of valsartan.

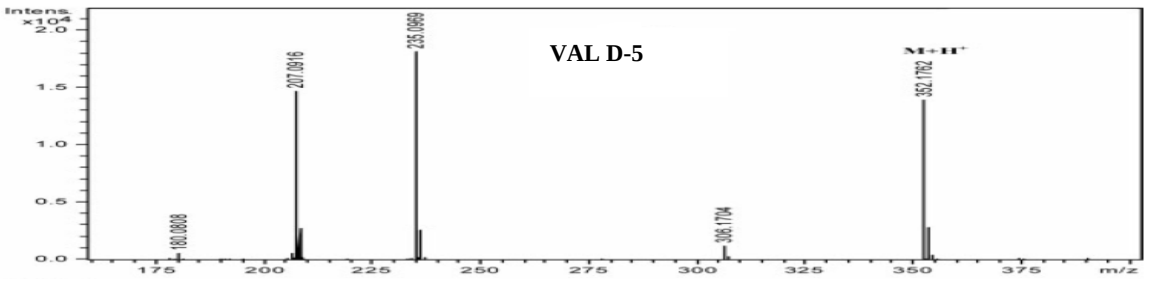


Figure 6 Line Spectra of Valsartan degradation product (VAL D-5)

Table 5 MS/TOF and online H/D exchange data of VAL D-5 along with their molecular formulae and major fragments

DPs	Accurate mass	Molecular formulae (Exact mass;RDB; error in ppm)	Major fragments (Chemical formulae; error in ppm)	Mass after H/D exchange in ESI +ve mode	Number of labile hydrogens
VAL D-5	352.1762	$C_{19}H_{22}N_5O_2^+$ (352.1768; 11.5; -1.7)	306.1704 ($C_{18}H_{20}N_5^+$; -2.9), 235.0969 ($C_{14}H_{11}N_4^+$; -3.8), 207.0916 ($C_{14}H_{11}N_2^+$; -0.4), 180.0808 ($C_{13}H_{10}N^+$; 0.0),	356	3

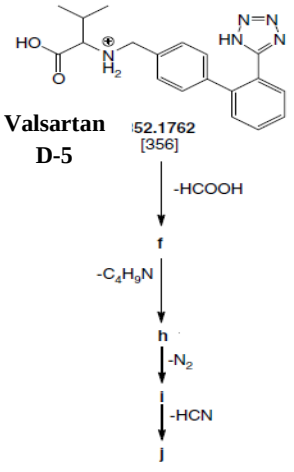


Fig.7a

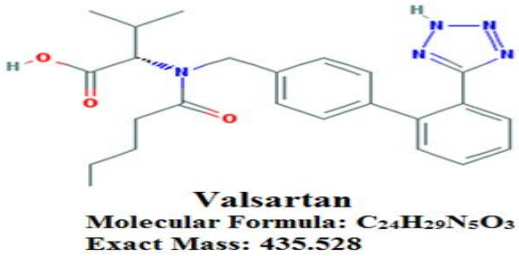


Fig.7b

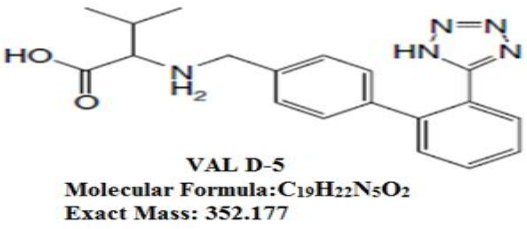


Fig.7c

Figure 7 Fragmentation pattern of DP of Valsartan (VAL D-5). The value of obtained accurate mass is shown below each structure along with the mass obtained in online H/D exchange study (7a) and Structure of Valsartan and Valsartan degradation compound (7b, 7c)

Isolation of Degradation Products of Sacubitril (SAC D-2 and SAC D-3)

For Sacubitril, Two base degradation products (SAC D-2 and SAC D-3) have been found in the acid and base degradation study and the same were identified with the LC-MS technique (as mentioned above section) and found the molecular ion peak at 383.17 and 265.15 corresponding to SAC D-2 & SAC D-3 respectively. After many injections, a few litters of organic solvents were collected by using an optimized condition in preparative HPLC (as mentioned above section). The peaks are almost baseline-separated, which leads to high purity and yield. At the end of a sequence of purification runs, the collected fractions evaporated from the fraction container into the laboratory air. Refer to the chromatograms of acid and base degradants product in (Fig. 8 and 9).

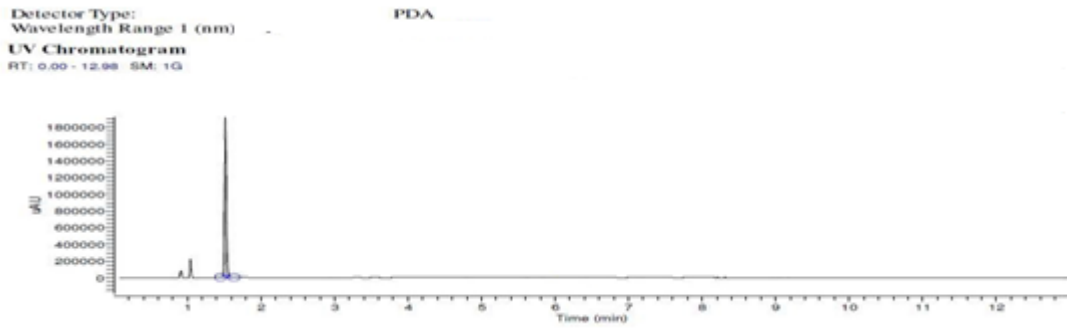


Figure 8 Chromatogram of acid and base Degradants Product (SAC D-2)

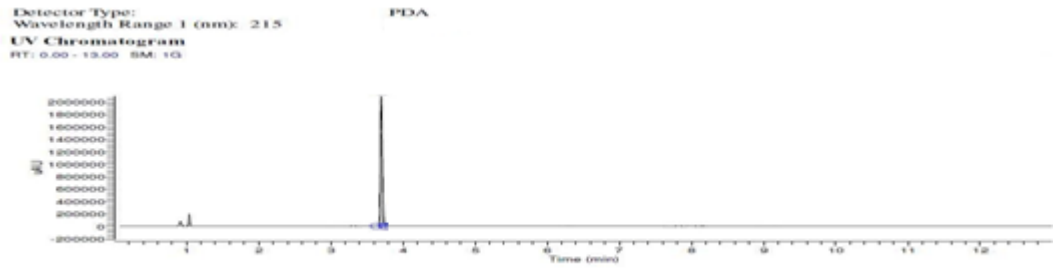


Figure 9 Chromatogram of acid Degradants Product (SAC D-3)

The Sacubitril was subjected to stress degradation as mentioned in stress studies, viz., acid hydrolysis, base hydrolysis, thermal, oxidative, and photolytic degradation. Based on the chromatographic data, it was observed that the Sacubitril calcium was degraded more extensively in acid and base hydrolysis. The degraded Sacubitril calcium impurities were isolated using preparative chromatography, and the isolated impurities were again injected to confirm the retention time. These two degradation products are tagged as SAC D-2 and SAC D-3. The Sacubitril calcium eluting at a retention time of 3.98 min and the degradants SAC D-2 and SAC D-3 elute at retention times of 1.51 min and 3.73 min, respectively. These impurities were isolated using preparative chromatography and then subjected to structural

elucidation using mass spectrometry and NMR spectroscopy to assign the structures. Refer to below (Table-6) for the δ values in ppm of NMR of Sacubitril calcium, SAC D-2 & SAC D-3.

Structure Elucidation of Sacubitril

The Mass spectroscopic analysis using positive polarity with Electro Spray Ionization technique showed the molecular mass as 412.01 Daltons (as M+H). Based on this mass data, the molecular mass of Sacubitril sodium was confirmed as 411 Daltons. The ^{1}H NMR spectrum exhibited 27 protons, of which 18 protons were from aliphatic chains and 9 protons are from the aromatic regions. Similarly, the ^{13}C NMR showed 24 carbons of which 9 carbons were from the aliphatic region and 15 carbons from the aromatic region. The ^{1}H-^{13}C correlation spectrum confirmed that there are 11 methyne, 5 methylene and 2 methyl groups present. Further confirmation of structure and assignments of ^{1}H and ^{13}C signals were established by NMR studies by correlating ^{1}H-^{1}H and ^{1}H-^{13}C data. Refer to table-6 below for details.

Structure Elucidation of SAC D-2

The Mass spectroscopic analysis using positive polarity with Electro Spray Ionization technique showed the molecular mass as 383.17 Daltons (as M+H). Based on this mass data the molecular mass of SAC D-2 was confirmed as 383 Daltons. The ^{1}H NMR spectrum of SAC D-2 exhibited 22 protons of which 13 protons are from the aliphatic region and 9 protons are from the aromatic region, one labile proton with broad signal arising from –NH group, and two labile protons with broad signal due to acidic protons are observed. Ethyl ester protons which were observed in Sacubitril proton NMR were absent in ^{1}H NMR of SAC D-1. ^{13}C NMR is complimenting the ^{1}H NMR in that 2 aliphatic carbons are absent in its spectrum when compared to Sacubitril drug ^{13}C NMR spectrum. This confirms that Ethyl ester in Sacubitril was converted into acid during base degradation. The ^{13}C Spectra of SAC D-2 exhibited 7 aliphatic carbons and 15 aromatic carbons. The ^{1}H-^{13}C correlation NMR data confirmed that SAC D-1 had 11 methyne, and 4 methylene, one methyl group present in the 2D-NMR spectrum. Further, the SAC D-2 subjected to Hetero nuclear Multi Bond correlation NMR spectroscopy (i.e. ^{1}H-^{13}C multi bond correlation) and interpretation from this study confirmed the assigned structure as 5-([1,1'-biphenyl]-4-yl)-4-(3- carboxypropanamido)-2-methylpentanoic acid.

Structure Elucidation of SAC D-3

The Mass spectroscopic analysis using positive polarity with Electro Spray Ionization technique showed the molecular mass as 265.15 Daltons (as M+H). Based on this mass data, the molecular mass of SAC D-3 was confirmed as 265 Daltons. The 1H NMR spectrum of

SAC D-3 exhibited 18 protons of which 9 protons are from the aliphatic region and 9 protons are from the aromatic region, and one labile proton with a broad signal arising from the –NH group was observed. ^{13}C NMR is complimenting to the ^{1}H NMR that 5 carbons detected in the aliphatic region and 13 carbons detected in the aromatic region. The ^{1}H-^{13}C single bond and multi bond correlation NMR study show that SAC D-2 had 11 methyne groups, 2 methylene groups, and one methyl group in the spectrum. The interpretation using Mass data and NMR data confirms that the isolated degradation product SAC D-3 is named to be as (3S)-5-([1,1'-biphenyl]-4-ylmethyl)-3-methylpyrrolidin-2-one.

Table 6 Molecular Masses, ^{1}H-NMR, and ^{13}C NMR, and Heteronuclear NMR Correlation Data of Sacubitril calcium and Degradation Products (SAC D-2 and SAC D-3)[6]

			EM-383.99		EM-265.09	
Assignment	SAC API		SAC D-2		SAC D-3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	7.51	129.2	7.45	128.9	7.46	129.9
2	7.41	127.6	7.34	127.2	7.35	127.2
3	7.51	129.2	7.45	128.9	7.46	128.9
4	7.52	127.9	7.65	126.5	7.66	126.5
5		140.8		140.1		139.9
6	7.52	127.9	7.65	126.5	7.66	126.5
7		138.0		137.8		138
8	7.40	127.7	7.58	126.3	7.60	126.47
9	7.35	130.1	7.27	129.9	7.32	130
10		137.5		138.1		137.3
11	7.35	130.1	7.27	129.9	7.36	130
12	7.40	127.7	7.58	126.3	7.60	126.47
13	2.7,2.4	45.8	2.73	40.3	2.68,2.82	41
14	3.92	51.5	3.99	48.5	3.74	52.1
15	1.6	37.9	1.37,1.80	37.8	1.63,1.99	33.7
16	8.28		7.79		7.76	
17		171.3		170.7	2.17	34
18	1.61	31.8	2.42	29.5		178.7
19	2.48	26.8	2.32	30.4	0.98	16
20						
21		177.3		174		
22						
23						
24	2.49	34.4	2.46	35.9		
25		176.2	1.07	18.1		
26	1.19	16.9		177.2		
27						

28						
29	4.21	61.6				
30	1.29	14.1				

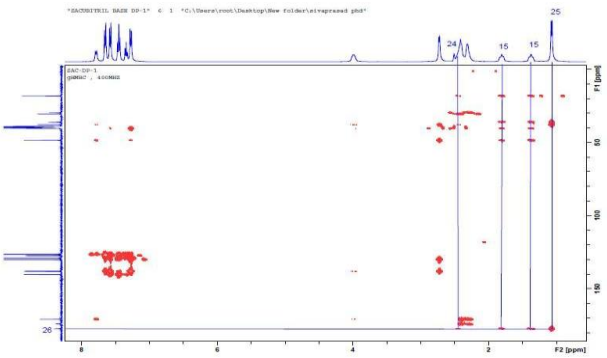


Figure 10 HMBC information of SAC D-2

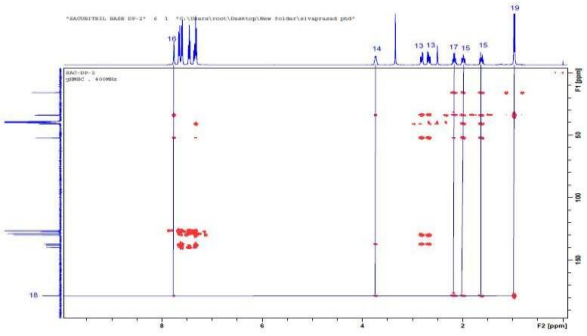


Figure 11 HMBC information of SAC D-3

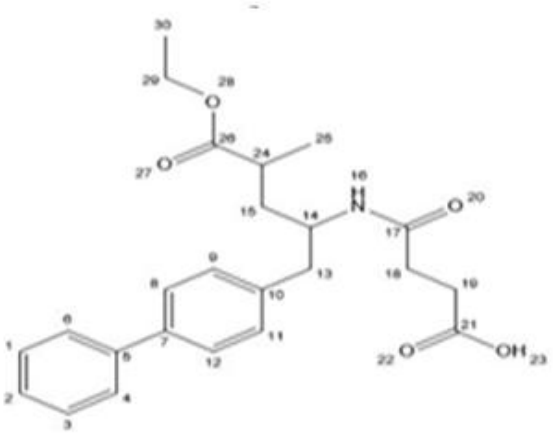


Fig.12a

Sacubitril

Chemical Formula: $C_{24}H_{29}NO_5$

Exact Mass: 411.20

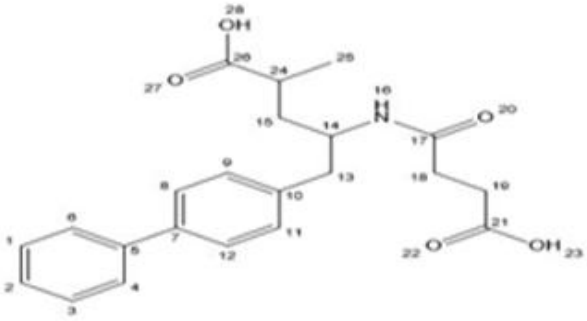
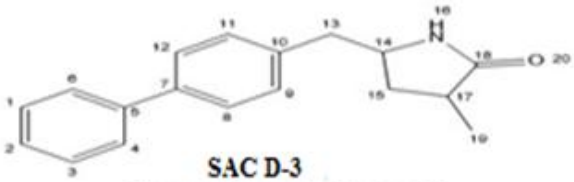


Fig.12b

SAC D-2

Chemical Formula: $C_{22}H_{25}NO_5$

Exact Mass: 383.17



SAC D-3

Chemical Formula: $C_{18}H_{19}NO$

Exact Mass: 265.15

Fig.12c

Figure 12 Structure of Sacubitril (12a) and Sacubitril degradation compounds (12b, 12c)

Achievements with respect to objectives

- RP-HPLC method for simultaneous estimation of valsartan and sacubitril were developed.
- Developed method was validated according to ICH Q2 (R1) guideline.
- Force degradation behaviour was performed on Acid degradation, Base degradation, Oxidative degradation, Photo degradation, Thermal degradation for SAC and VAL.

- DPs of SAC and VAL were identified, isolated and structure elucidate by LC/MS and NMR studies.
- DPs of valsartan and sacubitril were characterized.

Conclusion

The degradation behaviour of Valsartan and Sacubitril were explored under stress degradation conditions prescribed by ICH guidelines. The drugs underwent acid, base hydrolysis and oxidative, photo, thermal degradation conditions. To characterize the three degradation products, the strategy was employed. First a complete fragmentation pathway of the valsartan was established using MS^n and MS/TOF data. The DPs were then subjected to LC-MS/TOF analysis and structures were proposed based on their accurate mass and H/D exchange data, and also through comparison of fragmentation pathway of VAL D-5 to that of the valsartan. The degradation product of valsartan VAL D-5 was characterized successfully by LC and LC-MS techniques. Two degradation products, SAC D-2, and SAC D-3 were formed during acid and base hydrolysis of the Sacubitril drug substance. All the degradants were isolated & unambiguously characterized by LCMS and NMR techniques. Structure elucidation of the degradation product SAC D-2 and SAC D-3 were carried out by Mass spectrometry and ^{1}H, ^{13}C, and 2D-NMR techniques.

List of all publications arising from the thesis

Details of Paper Published in Journal

1. Priyanka Y, Hiral P, 2022, Stability indicating RP-HPLC Method for Simultaneous estimation of Valsartan and Sacubitril in Pharmaceutical Dosage Form, Research Journal of Pharmacy and Technology, 15(8), 3627-3633, ISSN No. 0974-3618.
2. Priyanka Y, Hiral P, 2023, Stability-Indicating Reverse Phase HPLC Method Development and Characterization of Degradation Products of Valsartan and Sacubitril by LC-QTOF-MS/MS and NMR Studies, Journal of Pharmaceutical Science and research, 15(2), 1007-1015, ISSN No. 0975-1459.

Details of Paper Presented in Conference

1. Priyanka Y, Hiral P, 2021, "RP-HPLC Method development and validation for Simultaneous estimation of Valsartan and Sacubitril in Pharmaceutical Dosage Form", International e-conference on Regulatory Trends of Biologics and Medical Devices in Covid-19 Pandemic organized by L.J.Institute of Pharmacy, Ahmedabad on 16th and 17th July 2021.

2. Priyanka Y, Hiral P, 2023, "stability-indicating reverse phase HPLC method development and characterization of degradation products of valsartan and sacubitril by LC-QTOF-MS/MS and NMR studies" 10th Indo-Caribbean International Conference on Global Trend in Pharmacy Practice and Pharmaceutical Sciences organized by Shree Swaminarayan Sanskar Pharmacy College, Zundal on 21st January 2023.

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