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DEVELOPMENT AND VALIDATION OF ANALYTICAL QUALITY BY DESIGN-OPTIMIZED RP-HPLC METHODS FOR STABILITY-INDICATING QUANTIFICATION OF SACUBITRIL, VALSARTAN, THEIR RELATED IMPURITIES, AND ISOMERS IN PHARMACEUTICAL FORMULATIONS

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Abstract:

A robust and stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantification of Sacubitril and valsartan and its related impurities using an Analytical Quality by Design (AQbD) approach. The method employed Design of Experiments (DoE) to optimize critical chromatographic parameters, including buffer pH, acetonitrile content, tetrahydrofuran (THF) ratio, and column temperature, ensuring effective separation of Sacubitril and valsartan, its impurities, and isomers. Forced degradation studies under acidic, basic, oxidative, photolytic, and thermal conditions confirmed method specificity and stability-indicating capability. Validation results demonstrated excellent linearity ($r^2 \geq 0.997$), precision (%RSD <5%), accuracy (recoveries within 95–105%), and robustness within a defined Method Operable Design Region (MODR). Additionally, an RP-HPLC method for isomer quantification was developed using a chiral column, achieving baseline

separation with high specificity and reproducibility. The validated methods are suitable for routine quality control and stability testing of Apixaban formulations, supporting regulatory compliance.
Keywords: *Sacubitril and Valsartan, RP-HPLC, Isomers, Analytical Quality by Design and pharmaceutical quality assessment.*

1. INTRODUCTION:

Sacubitril and valsartan (SCB/VLS) are key components of Entresto®, a combination drug widely used for treating heart failure. Accurate quantification of these active pharmaceutical ingredients and their related impurities is critical to ensure drug safety, efficacy, and regulatory compliance. The development of robust, stability-indicating analytical methods is essential for routine quality control and stability studies. This study presents the development, optimization, and validation of two reverse-phase high-performance liquid chromatography (RP-HPLC) methods [3]: One for related substances including impurities of sacubitril and valsartan, and another for the separation and quantification of their isomers. Employing an Analytical Quality by Design (AQbD)[4] approach, these methods were systematically optimized and validated to provide precise, accurate, and specific analysis suitable for pharmaceutical quality assessment.

2. MATERIALS AND METHODS:

A. SACUBITRIL AND VALSARTAN RELATED SUBSTANCES METHOD

2.1 Chemicals and Reagents

Impurity B and Impurity C, which are associated with valsartan, were obtained from the US Pharmacopoeia and had a potency of more than 98.0%. The USP reference standard was used to develop the valsartan in-house working standard. These sacubitril impurities were obtained from AET labs in Hyderabad, Telangana, India: biphenyl ethyl ester, diacid, methyl ester, cyclic, TSV-ene, n-propyl ester, sacubitril lactam, malic analog, biphenyl-boc-acid, dehydro, succiniamide, and biphenyl-boc-ethyl-ester. Sigma-Aldrich and Merck, both situated in the United States, provided potassium dihydrogen phosphate as an analytical reagent. Acetonitrile, phosphoric acid, triethylamine (TEA), and tetrahydrofuran (THF) were acquired from Honeywell (United) in HPLC grade. We purchased EntrestoTM film-coated tablets from a neighboring supermarket. The strengths were 97 mg/103 mg, 49 mg/51 mg, and 24 mg/26.

2.2 Instrumentation

All chromatographic studies were carried out using a Waters Alliance 2695 HPLC system, which included a quaternary pump, autosampler, and column oven, as well as a Waters 2487 UV detector or a 2489 PDA detector (Waters Corporation, Milford, USA). Empower™ 3 software was used for system control and data collecting. Samples were prepared using a vacuum microfiltration system with 0.22-µm PTFE membranes and an ultrasonic bath. A Mettler Toledo ultra-microbalance with a precision of 1-2000 mg was used to measure mass.

2.3 Chromatographic Conditions for Related Substances Method

The HPLC analysis employed a Phenomenex Gemini-NX C18 column (150 × 4.6 mm, 3 µm). The buffer consisted of 2 mM TEA and 0.02 mM phosphate adjusted to pH 3.3 ± 0.05 with phosphoric acid.

Mobile phase A: buffer: acetonitrile (95:05, v/v)

Mobile phase B: acetonitrile: water: THF (93.5: 5: 3.5, v/v/v)

A gradient program was employed at a flow rate of 1.5 mL/min with the column oven set at 30°C. Detection was performed at 254 nm, and the injection volume was 20 µL. The total runtime was 35 minutes.

2.4 METHOD DEVELOPMENT FOR SACUBITRIL AND VALSARTAN RELATED SUBSTANCES METHOD

(Preparation of Diluent, Standard, Placebo, and Sample Solutions)

2.4.1 Diluent

A 50:50 (v/v) mixture of water and acetonitrile was degassed and equilibrated to room temperature before use.

2.4.2 Standard Solution

Accurately weighed quantities of SCB (21.80 mg) and VLS (23.50 mg) were transferred to a 200-mL volumetric flask. After initial dissolution in sonicated diluent, the volume was adjusted to yield a working concentration equivalent to 1% of the label claim.

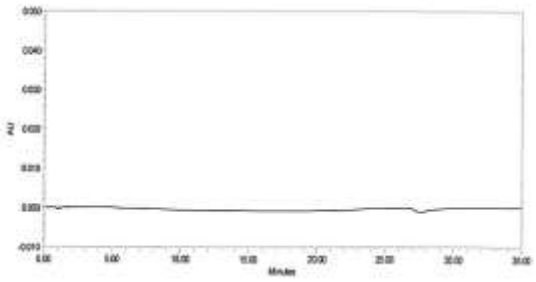

Fig.1 Diluent (Blank) solution chromatogram

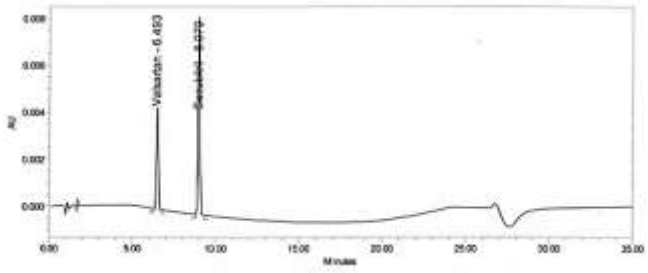

Fig.2 Standard solution chromatogram

2.4.3 Placebo Preparation

Placebo equivalent to 200 mg of SCB/VLS was dispersed in diluent under controlled agitation and sonication. The resulting solution was diluted to volume, filtered through 0.45-µm PTFE filters, and injected.

2.4.4 Sample Preparation

Ten tablets were weighed and transferred to a 250-mL flask. Following dispersion in diluent under agitation and sonication, the solution was volume-made, further diluted to 100 ppm, filtered, and transferred for analysis.

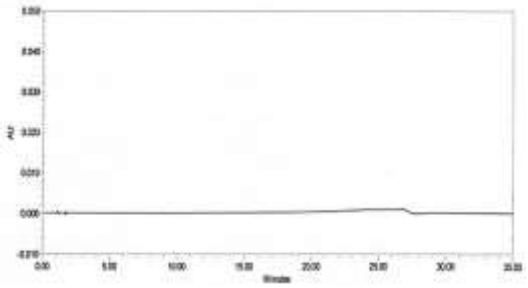

Fig.3 Placebo preparation chromatogram

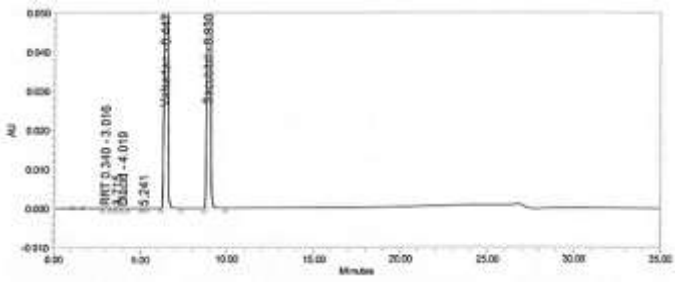

Fig.4 Sample solution chromatogram

3. MATERIALS AND METHODS:

B. SACUBITRIL AND VALSARTAN ISOMER METHOD:

3.1 Chemicals and Reagents

Valsartan analogue Compound-A (98% purity) was obtained from the United States Pharmacopeia. Sacubitril isomer reference standards were supplied by Mylan Laboratories (Hyderabad, India). HPLC-grade methanol, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Merck (India). Purified water was generated using a Milli-Q IQ-7000 system.

3.2 Instrumentation

Isomer separation was performed using the same liquid chromatographic platform employed for the SCB/VLS impurity method. Optimal chiral resolution was achieved using a Chiralcel® OJ-RH column (150 × 4.6 mm, 5 µm).

3.3 Chromatographic Conditions

Isomer separation was performed using a 60-min gradient method with 0.1% TFA in water (A) and methanol–acetonitrile 80:20 (B) at 0.8 mL/min, 35°C, and 254 nm detection. The run began at 70:30 (A:B), shifted to 50:50, then to 10:90, and returned to initial conditions. Injection volume was 20 µL.

3.4 METHOD DEVELOPMENT FOR SACUBITRIL AND VALSARTAN ISOMER METHOD:

3.4.1 Preparation of Standard Solution

Sacubitril sodium (21.80 mg) and valsartan (23.50 mg) were accurately weighed into a 200-mL volumetric flask, dissolved with 160 mL of sonicated diluent, cooled to room temperature, and diluted to volume. A further 50-mL dilution produced a working standard corresponding to approximately 1% of the sample concentration. The representative chromatogram is shown in following figure 5.

3.4.2 Preparation of Sample Solution

The sample stock prepared for the related-substances method was used for isomer analysis. Further dilution yielded ~200 ppm of sacubitril and valsartan. Samples were filtered through a 0.45-µm PTFE membrane before injection. The chromatogram of the sample preparation is shown following figure 6.

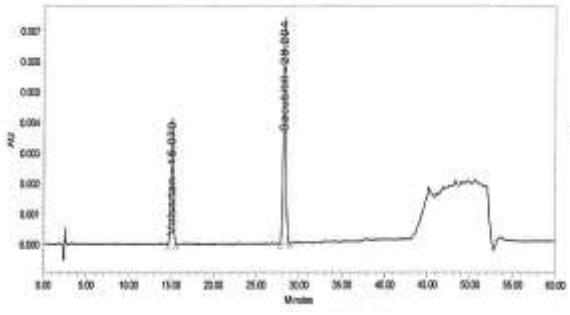

Fig.5 Standard solution chromatogram

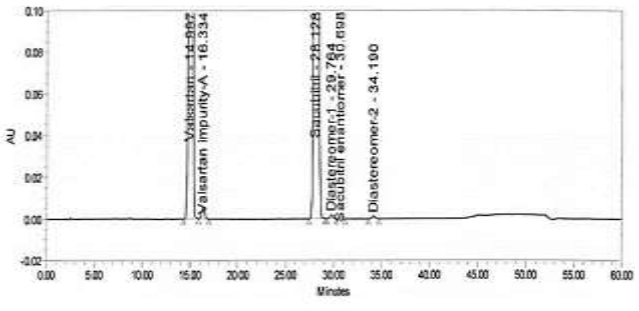

Fig.6 Sample solution all isomers

4. METHOD OPTIMIZATION:

A. SACUBITRIL AND VALSARTAN RELATED SUBSTANCES METHOD

4.1 Method Optimization

A stability-indicating RP-HPLC method was developed for the quantification of sacubitril (SCB), valsartan (VLS), and their related impurities in tablets. Physicochemical properties—including solubility in anhydrous ethanol, UV absorption, pKa (4.6 for SCB; 4.37 for VLS), hygroscopicity, and melting point—were evaluated prior to method development. Gradient RP-HPLC was selected to resolve structurally diverse impurities. Initial trials using acetonitrile and 0.1% TFA were insufficient to separate the acid impurity from VLS Impurity-B. Buffer pH was identified as a critical factor, and addition of THF to Mobile Phase B was essential for resolving VLS from SCB methyl ester. Optimization of mobile-phase composition, gradient program, column type, and temperature resulted in complete resolution of all impurities.

4.2 System Suitability

System suitability was verified using six replicate injections of SCB/VLS working standard. All parameters met acceptance criteria, with %RSD of 0.61% (SCB) and 0.70% (VLS). Peak symmetry, resolution, and theoretical plates were within analytical standards for all analytes.

Table 1 System suitability test (SST) results for SCB/VLS and impurities

Analyte	RT (min)	Resolution	Symmetry Factor	Plate Count (N)	%RSD	RRF
BPEE	4.125	NA	0.99	4,992	0.91	0.97
DIA	4.891	5.01	1.1	5,028	1.32	0.95
IMP-B	4.273	1.99	1.13	5,476	1.81	0.91
VLS	6.197	9.98	1.07	11,703	0.7	1
MTE	6.731	2.83	1.04	15,263	4.41	1.04
CYC	7.915	5.41	1.05	19,074	1.5	1.63
SCB	8.583	4.03	1.07	23,500	0.61	1
ENE	9.138	2.86	1.09	27,735	4.44	1.06
n-PRE	10.786	8.55	1.06	37,162	1.24	1.06
LAA	11.254	2.21	0.99	38,495	4.41	0.97
MAA	12.038	4.12	1.02	40,055	4.73	1.05
BBA	14.03	5.27	1.04	48,428	1.34	0.97
DEH	14.765	8.12	1.01	61,136	2.49	0.89
IMP-C	15.233	1.69	1.07	66,049	2.85	0.88
BBEE	20.26	25.3	1.02	141,280	2.59	0.91

4.3 Specificity and Forced Degradation

Specificity was demonstrated by evaluating peak purity and retention-time consistency for all known impurities and degradation products using RP-HPLC with PDA detection. Blank, placebo, standard, spiked, stressed, and test preparations were injected to assess interference and confirm analyte identity. Mass balance was verified using the corresponding assay method for each degradation sample.

Forced degradation studies showed that Sacubitril (SCB) is highly susceptible to acid- and base-induced hydrolysis, while valsartan (VLS) remains stable under these conditions. In contrast, VLS exhibited marked sensitivity to oxidative stress, whereas SCB and VLS were stable under thermal and photolytic conditions. PDA peak purity (PA, PT, PF) confirmed that all degradation peaks were spectrally homogeneous and free from co-elution, demonstrating the method's ability to detect the parent drugs and all related impurities with high specificity.

Table2 Forced Degradation Results for Sacubitril (SCB)

Stress Condition	Details	% Impurities	% Assay	Mass Balance	PA	PT	PF
Initial	–	0.06	100	100.1	0.184	0.436	Pass
Acidic	0.5 N HCl, 6 h, 60°C	0.36	99.2	99.6	0.146	0.427	Pass
Basic	0.5 N NaOH, 2 h, 60°C	8.12	90.9	99	0.208	0.343	Pass
Peroxide	5% H_2O_2, RT, 4 h	0.33	99.1	99.4	0.192	0.413	Pass
Photolytic	1200 W/m², 200 million lux h	0.05	100.2	100.3	0.161	0.418	Pass
Thermal	80% RH, 24 h	0.09	99.8	99.9	0.183	0.421	Pass

Table 3 Forced Degradation Results for Valsartan (VLS)

Stress Condition	Details	% Impurities	% Assay	Mass Balance	PA	PT	PF
Initial	–	0.09	100.2	100.3	0.121	0.386	Pass
Acidic	0.5 N HCl, 6 h, 60°C	0.09	100.3	100.4	0.127	0.384	Pass
Basic	0.5 N NaOH, 2 h, 60°C	0.05	100	100.1	0.115	0.337	Pass

Peroxide	5% H_2O_2, RT, 4 h	0.94	97.7	98.6	0.124	0.36	Pass
Photolytic	1200 W/m², 200 million lux h	0.09	99.7	99.8	0.125	0.377	Pass
Thermal	80% RH, 24 h	0.1	100.1	100.2	0.114	0.382	Pass

PA: Purity angle; PT: Purity threshold; PF: Purity flag.

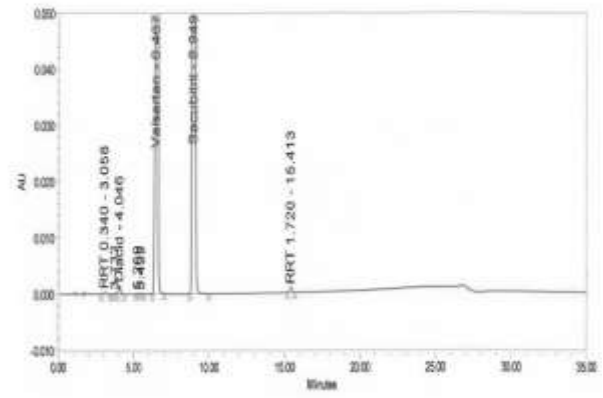

Fig.7 SCB/VLS Acid sample chromatogram

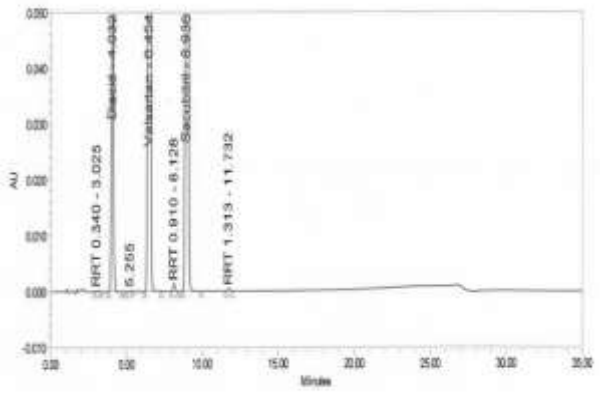

Fig.8 SCB/VLS Base sample chromatogram

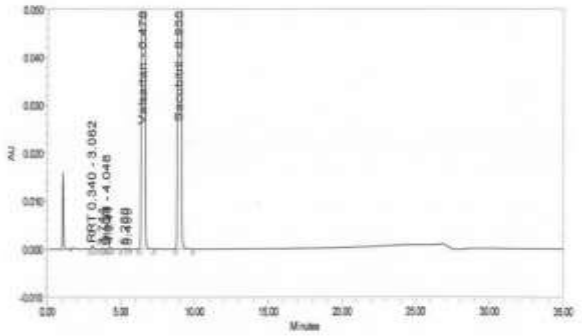

Fig.9 SCB/VLS Peroxide sample Chromatogram

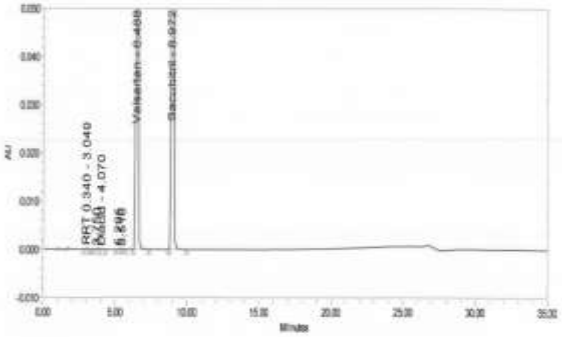

Fig. 10 SCB/VLS Photolytic sample Chromatogram

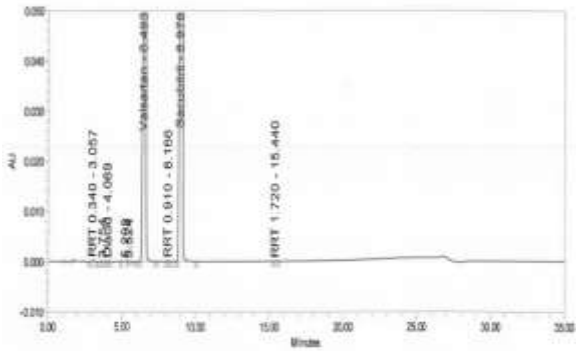

Fig. 11 SCB/VLS Thermal Chromatogram

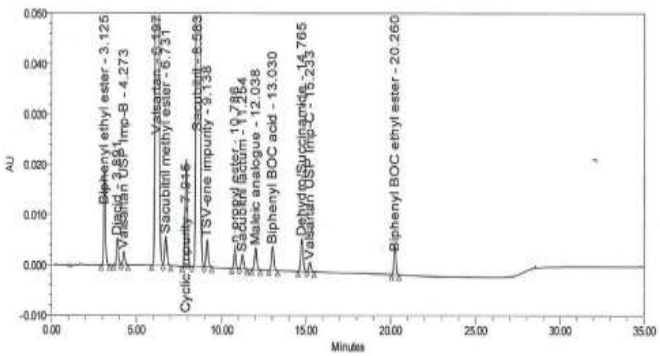

Fig.12 SCB/VLS with all Impurity mixture Chromatogram

5. METHOD VALIDATION:

5.1 Linearity

Linearity was evaluated for sacubitril (SCB), valsartan (VLS), and all specified impurities—biphenyl ethyl ester (BPEE), diacid (DIA), impurity-B (IMP-B), sacubitril methyl ester (MTE), cyclic impurity (CYC), ene impurity (ENE), n-propyl ester (n-PRE), lactam analogue (LAA), malic analogue impurity (MAA), biphenyl-BOC acid (BBA), dehydro impurity (DEH), impurity-C (IMP-C), and biphenyl-BOC ethyl ester (BBEE). Calibration curves were constructed from LOQ to 150% of the specification level. All analytes demonstrated excellent linearity, with correlation coefficients ($r^2 \geq 0.997$). Slopes and intercepts showed consistent detector response proportional to concentration.

Table 4 Linearity results of VLS, SCB and all related impurities

Analyte	Concentration Range (ppm)	Correlation Coefficient (r^2)	Slope	Intercept	Y-bias at Specification Level
VLS Impurity-B	0.02017–0.30260	1	51655.57	–25.53	–0.25
VLS Impurity-C	0.02017–0.30260	1	49612.11	74.44	0.74
Biphenyl ethyl ester	0.02018–0.30269	1	118311.98	29.26	0.12
Methyl ester	0.02006–0.30089	0.9999	106631.56	70.8	0.33
Cyclic impurity	0.02000–0.29996	0.9989	167915.59	190.4	0.57
Ene impurity	0.02000–0.30005	0.9995	108689.51	–406.19	–1.88
n-Propyl ester	0.02002–0.30036	0.9998	109177.98	–117.90	–0.54
Maleic analogue	0.02010–0.30156	0.9998	108172.99	–38.66	–0.18
Lactam impurity	0.02011–0.30167	0.999	99515.33	441.25	2.2
Biphenyl BOC acid	0.02044–0.30662	0.9997	99721.62	36.59	0.18
Dehydro impurity	0.02006–0.30093	0.9977	91474.69	–204.67	–1.09

Biphenyl BOC ethyl ester	0.02000–0.29997	0.9998	93818.28	167.69	0.87
Diacid impurity	0.02022–1.51665	0.9995	97715.3	–129.82	–0.13
Valsartan (VLS)	0.02002–1.50120	0.9974	56594	–1730.57	–4.32
Sacubitril (SCB)	0.02001–1.50075	0.9985	102861.61	–1147.13	–1.19

5.2 Precision

Precision was assessed through repeatability (intra-day) and intermediate precision (inter-day, analyst-to-analyst, system-to-system). Six replicates at specification level were analyzed. All analytes demonstrated %RSD values within acceptable limits (<5%). SCB and VLS showed excellent repeatability (%RSD 0.61% and 0.70%, respectively), confirming stable system performance.

Table 5 Precision results for SCB, VLS, and related impurities

Analyte	Repeatability (% Mean)	Repeatability (%RSD)	Intermediate Precision (% Mean)	Intermediate Precision (%RSD)	Between-Analyst Mean	Between-Analyst %RSD
VLS Impurity-B	0.213	0.97	0.216	0.97	0.215	1.11
VLS Impurity-C	0.222	0.97	0.246	0.28	0.234	5.37
Biphenyl ethyl ester	0.209	4.81	0.243	1.38	0.226	8.2
Methyl ester	0.197	4.13	0.205	0.23	0.201	4.06
Cyclic impurity	0.202	4.13	0.202	1.24	0.202	2.91
Ene impurity	0.19	5.59	0.202	2.84	0.196	5.32
n-Propyl ester	0.193	2.56	0.204	2.07	0.199	4.75
Maleic analogue	0.194	2.02	0.206	1.59	0.2	4.7

Lactam impurity	0.208	2.51	0.218	1.78	0.213	4.11
Biphenyl BOC acid	0.208	4.78	0.208	0.96	0.208	4.29
Dehydro impurity	0.225	4.1	0.209	1.07	0.217	5.03
Biphenyl BOC ethyl ester	0.221	7.51	0.21	4.26	0.215	6.28
Diacid impurity	1.059	1.7	1.043	0.64	1.051	1.46

5.3 Accuracy

Accuracy was confirmed by recovery studies at LOQ, 100%, and 150% of specification. Mean recoveries for all impurities were within 95–105%, indicating reliable quantification across concentration ranges. Sacubitril and valsartan showed consistent recoveries with no matrix interference.

Table 6 Accuracy results for SCB, VLS, and all impurities (LOQ, 100% and 150%)

Impurity	**Mean % Recovery at LOQ**	**Mean % Recovery at 100%**	**Mean % Recovery at 150%**	**Overall Avg. % Recovery**
VLS Impurity-B	101.3	101.5	102	**101.6**
VLS Impurity-C	101.5	100	101.6	**101**
Biphenyl ethyl ester	101.2	101.6	99.3	**100.7**
Methyl ester	104.9	101.1	100.5	**102.2**
Cyclic impurity	102	99	102.9	**101.3**
Ene impurity	99.3	94.8	100	**98**
n-Propyl ester	99.1	101.3	102.7	**101**
Maleic analogue	101.7	97.6	104.2	**101.2**
Lactam impurity	102.8	105.1	102.1	**103.3**
Biphenyl BOC Acid	104.7	104.4	104.5	**104.5**
Dehydro impurity	100.7	100.4	102.6	**101.2**
Biphenyl BOC Ethyl ester	101.9	104.6	104.2	**103.6**

Diacid impurity	106.8	105.5	104.9	**105.7**

5.4 LOD, LOQ, and Correction Factors

LOD and LOQ were established by signal-to-noise criteria (S/N ≈ 3 and 10). LOD values ranged from 0.004–0.006 ppm, whereas LOQ ranged from 0.012–0.018 ppm. Relative response factors (RRFs) were calculated for each impurity using SCB and VLS as reference standards. All RRFs were stable and suitable for routine application.

Table 7 Correction Factor, LOD, LOQ and Slope for All Impurities

Sr. No.	Name	Correction Factor	LOD (ppm)	LOQ (ppm)	Slope
1	BPEE	1.03	0.006	0.018	99,721.61552
2	DIA	1.05	0.006	0.018	97,715.30417
3	IMP-B	1.1	0.006	0.018	51,655.56505
4	VLS	1	0.006	0.018	56,594.00434
5	MTE	0.96	0.005	0.015	106,631.5602
6	CYC	0.61	0.004	0.012	167,915.5949
7	SCB	1	0.006	0.018	102,861.6055
8	ENE	0.95	0.004	0.012	108,689.5117
9	n-PRE	0.94	0.005	0.015	109,177.9824
10	LAA	1.03	0.006	0.018	99,515.33403
11	MAA	0.95	0.005	0.015	108,172.9875
12	BBA	1.03	0.005	0.015	99,721.61552
13	DEH	1.12	0.004	0.012	91,474.68803
14	IMP-C	1.14	0.005	0.015	49,612.10775
15	BBEE	1.1	0.005	0.015	93,818.27845

6. ROBUSTNESS BY AQBD APPROACH

A robustness evaluation was performed using an Analytical Quality by Design (AQbD) approach. Instead of varying one parameter at a time, a multivariate assessment was executed by simultaneously changing the critical chromatographic variables using a Response Surface Methodology (RSM) design in Design-Expert® software.

Twenty-five experimental runs were generated to study the influence of four critical method parameters—Buffer, pH (4.2–4.4), Acetonitrile content in mobile phase-A (35–65%), Tetrahydrofuran (THF) ratio in mobile phase-B(15–35 mL), and Column temperature (25–35 °C)—on four chromatographic resolution responses:

- RS1: Acid impurity vs. VLS impurity-B

- RS2: Methyl ester vs. VLS
- RS3: Cyclic impurity vs. SCB
- RS4: Ene impurity vs. SCB

The models (statistically significant after reduction) revealed that buffer pH had the strongest effect on peak separation, especially RS2 (variation: 2.5–4.5). THF ratio primarily affected RS3 and RS4, whereas acetonitrile content produced only minor resolution changes. Maintaining buffer pH at 4.30 ± 0.05 is essential for consistent peak resolution and chromatographic baseline quality.

Table 8 AQbD Robustness: Design of Experiments (DoE) Input Matrix

Run	Std No.	Buffer pH	% ACN (A)	THF (mL, B)	Column Temp (°C)
1	14	4.4	35	35	35
2	11	4.2	65	15	35
3	10	4.4	35	15	35
4	7	4.2	65	35	25
5	8	4.4	65	35	25
6	23	4.3	50	25	25
7	1	4.2	35	15	25
8	12	4.4	65	15	35
9	18	4.4	50	25	30
10	2	4.4	35	15	25
11	21	4.3	50	15	30
12	25	4.3	50	25	30
13	13	4.2	35	35	35
14	19	4.3	35	25	30
15	20	4.3	65	35	30
16	22	4.3	50	35	30
17	4	4.4	65	35	25
18	6	4.4	35	35	25
19	16	4.4	65	25	35
20	17	4.2	50	35	30
21	5	4.2	35	35	25
22	15	4.2	65	35	35
23	24	4.3	50	25	35
24	3	4.2	65	15	25
25	9	4.2	35	15	35

Table 9 AQbD Robustness: DoE Output Responses (Resolution Values)

(RS1–RS4 correspond to four critical pairwise peak separations)

Run	RS1	RS2	RS3	RS4
1	No resolution	4.63	2.98	2.06
2	1.64	2.77	2.57	2.08
3	No resolution	4.97	2.59	1.97
4	1.71	No resolution	2.81	2.44
5	0.94	4.01	2.49	2.28
6	1.3	4.02	2.85	2.67
7	1.75	2.46	2.74	2.6
8	No resolution	4.72	2.14	No resolution
9	No resolution	4.97	2.8	2.42
10	1.29	4.37	2.77	2.77
11	1.26	4.51	2.67	2.48
12	1.22	4.31	2.99	2.51
13	1.58	2.65	4.55	2.38
14	1.2	4.36	4.23	2.57
15	1.26	2.97	2.97	2.37
16	1.21	4.02	4.17	2.46
17	1.02	4.31	2.79	2.62
18	1.19	2.99	4.41	2.81
19	No resolution	4.93	2.59	2.09
20	1.88	1.95	4.38	2.51
21	1.71	2.12	4.34	2.66
22	1.58	2.46	4.04	2.14
23	1.15	4.59	4.02	2.32
24	1.78	2.24	2.3	2.4
25	1.62	4	4.12	2.32

Interpretation:

1. **RS1** (Acid impurity vs. VLS-B) highly sensitive to pH variations; several conditions produced no resolution when pH deviated from optimum.
2. **RS2** (Methyl ester vs. VLS) Most pH-dependent response; resolution varied from 2.5 to 4.5, confirming pH as a critical parameter.
3. **RS3** (Cyclic vs. SCB) strongly influenced by THF concentration, particularly when THF increased to 35 mL.
4. **RS4** (Ene vs. SCB) Minimal sensitivity to pH (2.3–2.6 range), but affected by THF and temperature interactions.
5. **ACN (%)** Had negligible to moderate effect on resolution for all four impurity pairs.

7. CRITICAL METHOD PARAMETERS ESTABLISHED

- Buffer pH: 4.30 ± 0.05
- Mobile Phase-A ACN concentration: 35–50%
- Mobile Phase-B THF ratio: 25–30 mL
- Column temperature: 30 °C recommended for stability.

7.1 The DoE Data:

- The DoE results were used to define a robust control strategy with pH maintained at 4.30 ± 0.05 and THF quality strictly controlled as a critical material attribute.
- All chromatographic responses were recorded, modeled, and statistically evaluated. The initial full quadratic models were subjected to reduction, and statistically significant terms ($p < 0.05$) were retained to develop improved and predictive models.
- The multivariate results revealed that buffer pH was the most influential CMP, particularly for RS2 (methyl ester vs. VLS), where resolution varied substantially between 2.5 and 4.5 across the tested pH range. Deviations from the optimized pH conditions led to partial or complete co-elution for RS1 and RS2, confirming pH sensitivity for these impurity pairs. In contrast, RS4 (ene impurity vs. sacubitril) exhibited minimal pH dependency, with resolution values remaining within 2.3–2.6.
- Changes in THF concentration significantly affected RS3 and RS4, indicating that the organic modifier in mobile phase-B plays a crucial role in modulating interactions among late-eluting impurities. Conversely, variations in acetonitrile composition had a relatively minor effect on resolution across all impurity pairs. Column temperature showed moderate influence but did not compromise method performance within the tested range.
- Based on the model predictions and response surface analyses, a robust Method Operable Design Region (MODR) was established, within which chromatographic resolution consistently met system suitability criteria. The MODR identifies buffer pH 4.30 ± 0.05, THF 25–30 mL, and acetonitrile 35–50% as optimal operating conditions, with a recommended column temperature of 30 °C to ensure peak shape and baseline stability.

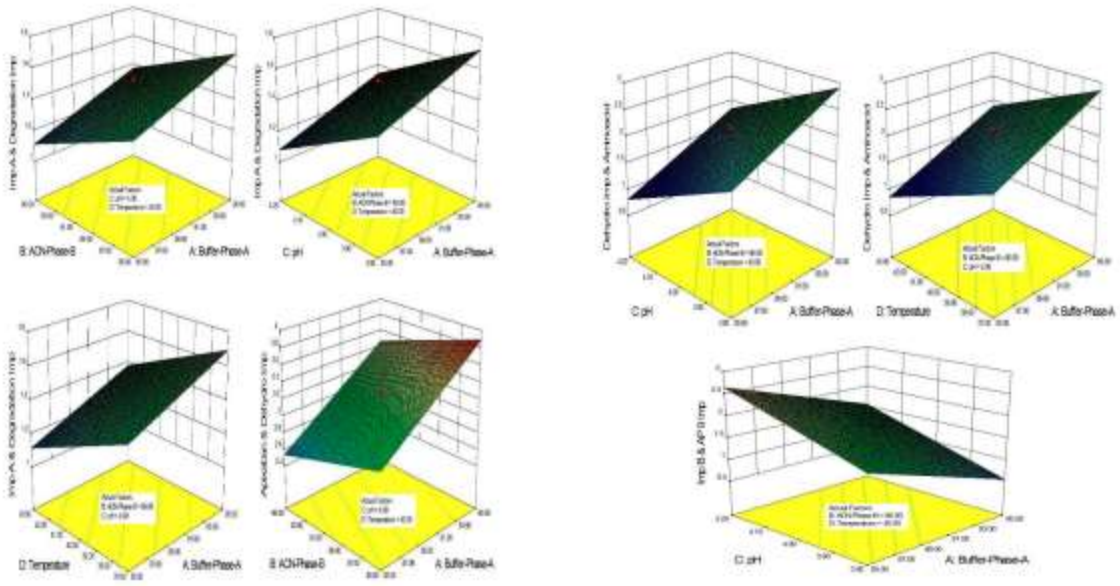

Fig. 13 DoE plots for experiments

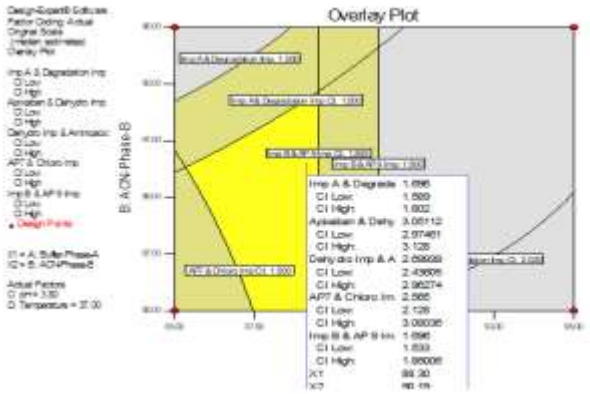

Fig. 14 Design space and overlay plot

Table 10 DoE factors and levels

Factor	Low	Center	High
pH	4.2	4.3	4.4
ACN (%)	5	15	25
THF (%)	10	15	20
Temperature (°C)	25	30	35

Table 11 Summary of critical responses from DoE

Response	Acceptance Criterion	Influence Rank
Resolution (VLS vs MTE)	≥2.0	pH > THF > ACN
Resolution (SCB vs ENE)	≥2.0	THF > pH > Temperature
Peak symmetry	0.9–1.2	Temperature > ACN
Plate count	>5000	Temperature > pH

8. STABILITY ASSESSMENT OF SACUBITRIL/VALSARTAN ORAL SUSPENSION

Sacubitril/Valsartan (SCB/VLS; Entresto®) received FDA approval in 2019 for treating symptomatic heart failure in pediatric patients ≥1 year of age with systemic left ventricular systolic dysfunction. Because children weighing <40 kg require a liquid formulation, an oral suspension is prepared extemporaneously from Entresto® tablets. As per the product information leaflet (PIL), the suspension is stored in amber PET or glass bottles for up to 15 days, refrigerated when above 25 °C, and shaken prior to use.

A stability study was conducted to evaluate impurity progression in the extemporaneously prepared SCB/VLS suspension over 28 days. Results are presented in Table 12.

Table 12 Stability Data of SCB/VLS Oral Suspension

Test	Specification	Initial	15 Days	28 Days
Sacubitril				
Diacid impurity	NMT 0.5%	BDL	0.2	0.49
Unspecified impurity	NMT 0.2%	BDL	0.2	0.49
Valsartan				
Unspecified impurity	NMT 0.2%	BDL	BDL	BDL
Total impurities	NMT 1.0%	BDL	0.4	0.98

Values indicate a notable increase in an unspecified impurity in the Sacubitril component, reaching close to allowable limits by Day 28.

8.1 LC–MS/MS Method for Impurity Identification

An LC–MS/MS–compatible analytical method was developed to elucidate the identity of the unknown impurity increasing during suspension storage. The reference sample was prepared from 49 mg/51 mg tablets.

8.1.1 Instrument Method Parameters

Chromatographic System

- Column: Phenomenex Gemini C18 (150 × 4.6 mm, 3 µm)
- Temperature: 30 °C
- Mobile Phase A: Water with formic acid, pH 4.3
- Mobile Phase B: Acetonitrile
- Flow rate: 1.0 mL/min
- Injection volume: 20 µL
- Mode: Gradient elution
- Run time: 35 min

B. Mass Spectrometer (API 4500, AB Sciex)

- Ion source: ESI-positive (TurboSpray)
- Scan mode: MRM and Q1 Scan
- MRM transition: m/z 394.3 → 348.3
- Dwell: 120 ms; CE: 15 V; DP: 35 V; EP: 10 V
- Curtain Gas: 30; GS1: 50; GS2: 0
- TEM: 500 °C; IonSpray: 5500 V

Table 13 LC–MS/MS Findings

Sample	RT of Impurity (min)	Sample Type	Q1 (m/z)	Q3 (m/z)
SCB/VLS oral suspension	17.21	Reference	394.2	348.3

The impurity consistently produced a precursor ion at m/z 394 and a product ion at m/z 348, matching the known fragmentation profile of succinamide impurity, a recognized degradation product of Sacubitril.

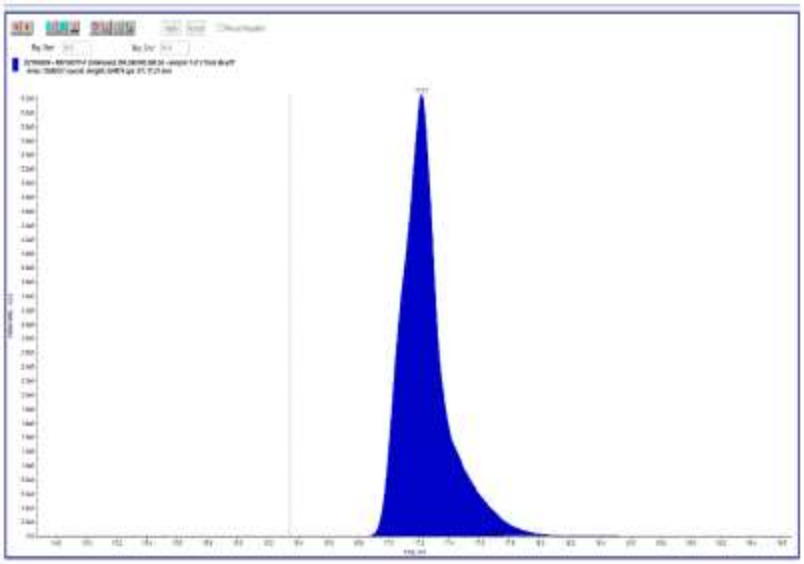

Fig.15 LC-MS/MS spectra unspecified Impurity in Oral suspension

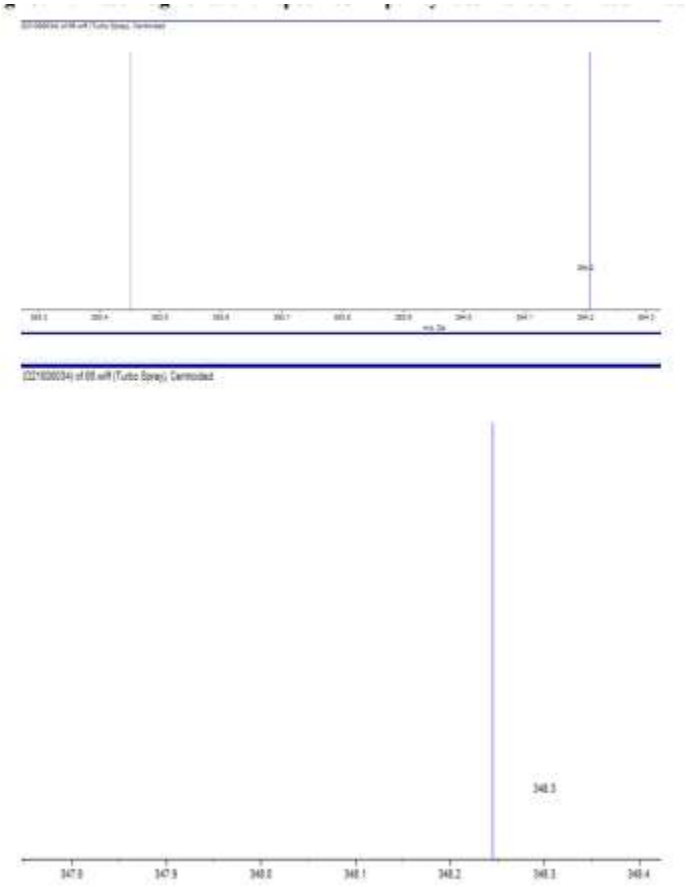

Fig.16 Mass fragments of unspecified Impurity is 394 and 348 in scan mode

8.1.2 Interpretation of LC–MS/MS Findings

- Comparison of the spectral data (Q1, Q3 mass transitions, retention time, and fragmentation pattern) with known reference standards confirmed that the increasing unidentified impurity in the oral suspension is Succinamide Impurity.
- The impurity progressively increases during storage and approaches specification limits by Day 28. Consequently, based on impurity growth kinetics and LC–MS/MS confirmation, the extemporaneously prepared suspension remains within acceptable limits only for 15 days, supporting the product information leaflet recommendations.
- The SCB/VLS oral suspension shows acceptable stability up to 15 days, beyond which succinamide impurity levels rise significantly. The LC–MS/MS method successfully identified the impurity, establishing the method's suitability for monitoring degradation in pediatric extemporaneous preparations. These findings reinforce the recommended 15-day shelf life for the suspension and emphasize the need for controlled storage conditions.

9. FINAL RISK ASSESSMENT AND CONTROL STRATEGY

A structured risk assessment was performed following ICH Q9 (Quality Risk Management) and AQbD principles to identify material attributes and method parameters that may influence the performance of the SCB/VLS related-substances method. The assessment incorporated evidence gathered during method development, DoE studies, robustness evaluation, and system suitability performance. Based on this analysis, risks were classified as High, Medium, or Low, and corresponding control strategies were defined.

9.1 Risk Assessment of Material Attributes

Table 14 Risk Assessment of Material Attributes

Sr. No.	Material Attribute	Purity	Grade	Make	Criticality	Justification
1	THF	High	High	High	High	Chromatogram baseline was highly sensitive to THF make and grade; significant variation observed during development.
2	Potassium dihydrogen phosphate	Low	Low	Low	Low	Evaluated during method development; no further impact observed on chromatographic behavior.

3	Acetonitrile	Low	Low	Low	Low	ACN quality did not significantly affect resolution or detection at selected wavelength.

9.2 Risk Assessment of Method Parameters

Table 15 Risk Assessment of Method Parameters

Sr. No.	Method Attribute	Parameter Influence	Risk	Justification
1	HPLC Column	Robustness, specificity, chromatography	Low	Column chemistry did not critically influence separation of SCB/VLS and impurities.
2	Flow Rate	(Part of general chromatographic conditions)	Low	No significant impact within evaluated ranges.
3	Column Temperature	(Final optimized condition)	Low	Stable method performance across tested ranges; temperature not a major driver of peak resolution.
4	Solution stability	Precision, accuracy	Medium	SCB/VLS analytical solutions were stable for up to 48 h; moderate risk if stability window exceeded.
5	Needle-wash solvent and wash mode	Chromatography, system suitability	Low	Optimized diluent ensures minimal carryover; risk classified as low.

9.3 Final Control Strategy

Based on the established Method Operable Design Region (MODR), robustness data, and risk ranking, the following controls are required to ensure consistent method performance.

Table 16 Final Control Strategy for SCB/VLS Related-Substances Method

Sr. No.	Control Variable	Final Condition	Control Requirement
1	THF Quality	HPLC-grade from qualified vendor	THF significantly affects chromatographic baseline; test procedure should specify vendor/make and catalogue number.
2	Mobile Phase Buffer pH	pH 4.30 (±0.05)	Critical for resolution between VLS and methyl-ester impurity; requires strict pH control and documentation.

The risk assessment identified THF quality and buffer pH as the only materially critical attributes for the SCB/VLS related-substances method. All other parameters demonstrated low to moderate

risk due to the method's intrinsic robustness confirmed through AQbD design studies. The final control strategy—involving tight control of buffer pH and specification of THF source—ensures consistent peak resolution, acceptable system suitability, and reproducible quantification of impurities during routine quality control.

10. METHOD DEVELOPMENT AND VALIDATION OF SCB/VLS ISOMER

10.1 Chromatographic Conditions Development

The objective of the study was to develop a robust RP-HPLC method for the quantification of SCB/VLS isomers. Although isomer analysis is frequently carried out using normal-phase chromatography, such methods require system equilibration time and pose challenges when switching back to reverse phase. To ensure operational simplicity and broader applicability, a reverse-phase HPLC method was optimized. The method demonstrated consistent elution and adequate resolution of all sacubitril and valsartan isomers without the limitations associated with normal-phase systems.

10.2 Specificity

Specificity was established by injecting blank, placebo, standard, spiked, and stressed samples under acid, base, peroxide, photolytic, and thermal conditions. All isomers were well resolved with no co-eluting peaks, confirming a stability-indicating nature.

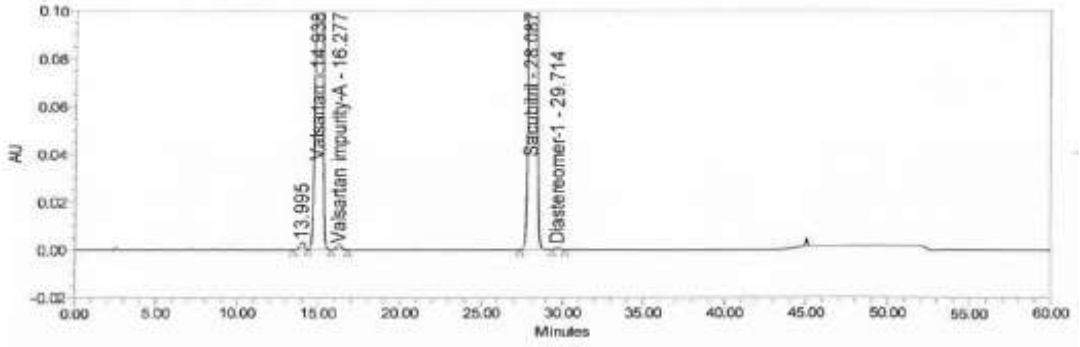

Fig. 17 Chromatogram of SCB/VLS Acid sample in Isomer method

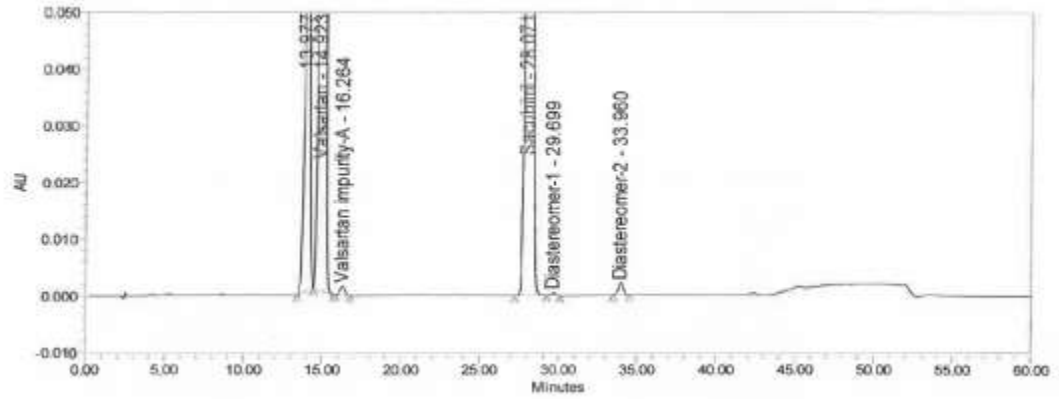

Fig. 18 Chromatogram of SCB/VLS Base sample in Isomer method

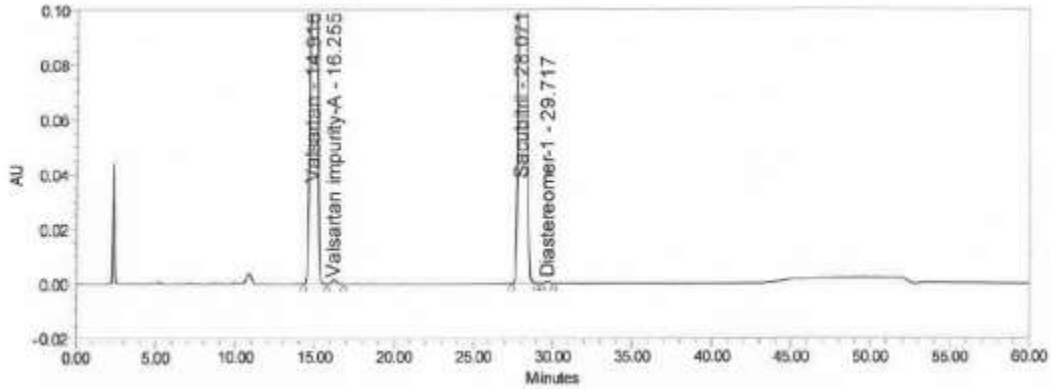

Fig.19 Chromatogram of SCB/VLS Peroxide sample in Isomer method

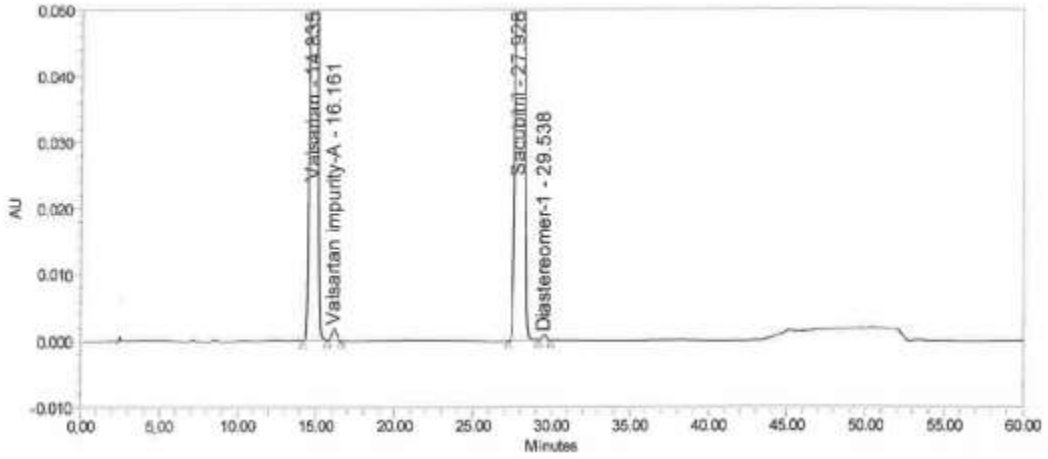

Fig. 20 Chromatogram of SCB/VLS Photytic sample in Isomer method

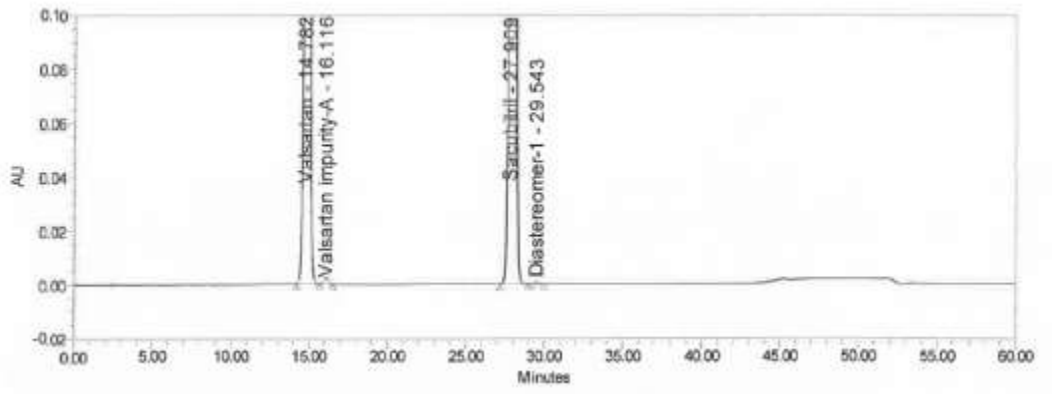

Fig. 21 Chromatogram of SCB/VLS Thermal sample in chiral purity method

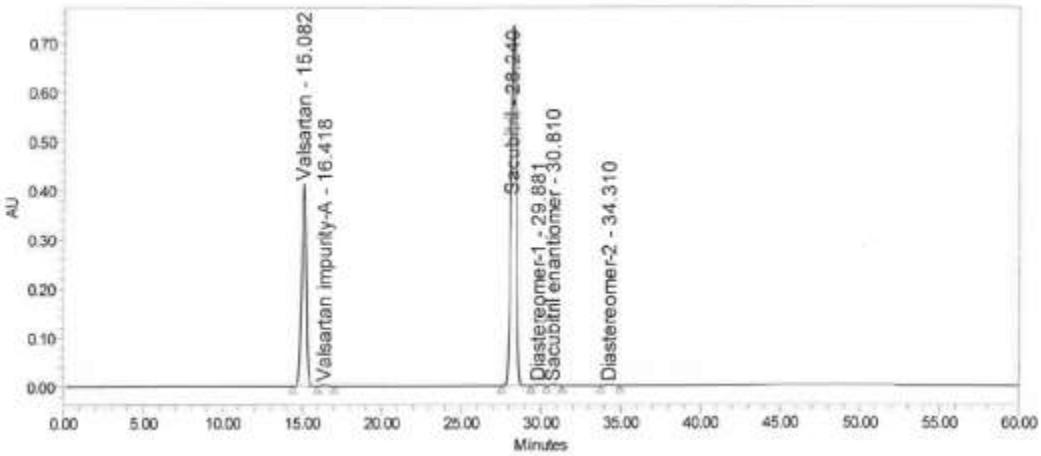

Fig. 22 Chromatogram of SCB/VLS with all isomers in chiral purity method

10.3 Linearity

Linearity for each isomer was evaluated at seven concentration levels, covering LOQ to 150% of specification. All analytes showed excellent correlation coefficients (r > 0.996).

Table 17 Linearity for VLS and VLS Impurity-A

Sr. No.	VLS (ppm)	Peak area	VLS Impurity-A (ppm)	Peak area
1	0.09915	4427	0.09887	3982
2	0.49576	21918	0.49439	20017
3	0.99153	43246	0.98879	39758
4	1.58644	64482	1.58206	59271
5	1.98306	86598	1.97758	79822
6	2.37967	107961	2.37309	98839
7	2.97459	130360	2.96637	119000

Correlation coefficient: 0.9985 (VLS), 0.9986 (VLS Imp-A) Slope: 44205.76 (VLS), 40557.15 (Imp-A)

Table 18 Linearity for SCB and SCB Diastereomer-1

Sr. No.	SCB (ppm)	Peak area	Diastereomer-1 (ppm)	Peak area
1	0.09826	7532	0.01985	2977
2	0.49134	37886	0.09925	7317
3	0.98269	75104	0.1985	14460
4	1.57229	112839	0.3176	21904
5	1.96537	150513	0.397	29459
6	2.3584	187685	0.4764	36619
7	2.94806	225894	0.5955	43879

Correlation coefficient: 0.9986 (SCB), 0.9977 (Dias-1)

10.4 Linearity for SCB Enantiomer and SCB Diastereomer-2: (Cleaned and standardized.)

10.5 Method Precision

Precision was evaluated through six replicate spiked samples at specification level. Intermediate precision was assessed using different days, columns, and instruments.

Table 20 Precision Results for VLS Impurity-A and SCB Diastereomer-1

Parameter	VLS Imp-A (Rep)	VLS Imp-A (IP)	SCB Dias-1 (Rep)	SCB Dias-1 (IP)
Average	1.031	1.097	0.193	0.211
SD	0.015	0.034	0.003	0.015
%RSD	1.49	4.14	1.65	7.27

10.6 Accuracy (Recovery)

Recovery was assessed at LOQ, 100%, and 150% levels. All results were within ICH Q2 acceptance.

Table 20 Accuracy Summary

Analyte	Avg. Recovery (%)
VLS Impurity-A	104.4 (LOQ), 101.7 (100%), 104.3 (150%)
SCB Diastereomer-1	106.5, 97.4, 98.8
SCB Enantiomer	91.7, 96.9, 98.0
SCB Diastereomer-2	98.8, 97.7, 99.3

10.7 LOD, LOQ and Correction Factors

Table 21 LOD, LOQ and CF Values

Sr. No.	Impurity	LOD (ppm)	LOQ (ppm)	Slope	CF
1	VLS Imp-A	0.02	0.06	40557.15	1.09
2	SCB Dias-1	0.02	0.06	73108.86	1.06
3	SCB Enantiomer	0.024	0.072	74017.76	1.05
4	SCB Dias-2	0.024	0.072	81876.24	0.95

10.8 Risk Assessment and Mitigation Strategy

A systematic risk assessment was performed to identify critical material attributes (CMA) and critical method parameters (CMP).

Table 22 Risk Assessment: Critical Material Attributes (CMA)

Material Attribute	Purity	Grade	Make	Overall Criticality	Justification
TFA	High	High	High	High	Essential for chromatographic resolution and peak shape.
Methanol, ACN, Water	Medium	Medium	Low	Medium	No further experiments required; consistent performance.

Table 23 Risk Assessment: Critical Method Parameters (CMP)

Method Attribute	Parameter Influence	Risk Level	Justification
Chromatographic conditions	Robustness, specificity	Medium	Variations may affect VLS/Imp-A resolution.
Mobile phase preparation	Peak separation	Medium	Controlled via GLP; training required.
Standard preparation	Accuracy, Precision	High	Standards and isomers are hygroscopic.
Sample preparation	Accuracy, Precision	Low	Whole-tablet dropping minimizes risk.

10.9 Control Strategy

Table 24 Final Control Strategy

Control Variable	Final Requirement	Rationale
TFA grade and make	Use same grade and supplier as in validation; specify catalog number	TFA critically affects peak shape and selectivity.
Handling of hygroscopic standards/isomers	Use validated supplier; recommend single-use vials	Prevents moisture uptake and weighing errors.

11. Summary of Analytical Methodologies

A. Sacubitril and Valsartan Related Substances Method

A comprehensive Design of Experiments (DoE)-driven Analytical Quality by Design (AQbD) strategy was employed to develop a robust RP-HPLC method for the quantification of related impurities in Sacubitril (SCB) and Valsartan (VLS) tablet dosage forms. The method development process focused on defining the Analytical Target Profile (ATP) and identifying critical method parameters (CMPs) through systematic risk assessment. Environmental and process conditions—including handling, manufacturing, packaging, and storage—were considered, as these factors can generate impurities influencing the drug product quality.

A multi-factor robustness study, executed within the DoE framework, demonstrated that the method consistently resolved all specified impurities under a wide range of chromatographic conditions. Key factors influencing critical separations included buffer pH, organic solvent composition, and THF quality. The final optimized method was found to be stability-indicating, with efficient resolution of process-related and degradation impurities, while maintaining a relatively short run time. The validated method showed high precision, accuracy, and ruggedness, making it suitable for routine quality control, stability studies, and regulatory submissions.

B. Sacubitril and Valsartan Isomer Method

A dedicated RP-HPLC method was also developed to quantify Valsartan enantiomer and Sacubitril isomers, leveraging the reverse-phase platform for improved operational simplicity. Unlike conventional approaches that often require chiral or normal-phase chromatography with lengthy equilibration times, the present method achieves complete isomer separation on an RP column without altering the chromatographic mode.

Importantly, the same sample preparation used for related substances analysis was applied, enabling unified workflow and improved laboratory efficiency. Evaluation of stressed, spiked, and placebo samples confirmed excellent specificity, with clear separation of all isomeric species. The method exhibited strong linearity, reproducibility, and recovery across the tested concentration range. As a result, it provides a robust, user-friendly, and regulatory-compliant approach for routine QC quantification of SCB/VLS isomers, including formulation-specific degradation products such as the Valsartan enantiomer

12. Result and Discussion:

The RP-HPLC method for related substances was developed using a Phenomenex Gemini-NX C18 column with a gradient elution program optimized through a Design of Experiments (DoE) approach. Key chromatographic parameters such as buffer pH, acetonitrile content, tetrahydrofuran (THF) ratio, and column temperature were evaluated for their impact on resolution. Buffer pH (4.30 ± 0.05) and THF concentration (25–30 mL) were identified as critical method parameters (CMPs) affecting peak separation, particularly between valsartan impurity-B and methyl ester impurity. System suitability tests confirmed consistent performance with %RSD values below 1% for sacubitril and valsartan, and all impurities demonstrated acceptable peak symmetry, resolution, and plate counts. Forced degradation studies established the method's stability-indicating capability, showing sacubitril's susceptibility to acid and base hydrolysis and valsartan's sensitivity to oxidative stress, with clear resolution of degradation products. Linearity was excellent across all analytes ($r^2 \geq 0.997$), and precision studies showed repeatability and intermediate precision within acceptable limits (%RSD <5%). Recovery studies confirmed accuracy with mean recoveries between 95–105%. Limits of detection and quantification were suitably low, supporting sensitive impurity detection. Robustness assessment via AQbD multivariate studies demonstrated method resilience within the defined Method Operable Design Region (MODR). The final control strategy emphasized strict control of buffer pH and THF quality to maintain consistent chromatographic performance.

The isomer separation method utilized a Chiralcel® OJ-RH column on the same chromatographic platform, optimized for reverse-phase operation to simplify workflow and avoid equilibration issues inherent in normal-phase methods. The method achieved clear resolution of sacubitril and valsartan isomers with excellent specificity, as confirmed by analysis of blank, placebo, spiked, and stressed samples. Linearity was robust ($r > 0.996$), and precision and accuracy met ICH Q2 criteria. Risk assessments identified trifluoroacetic acid (TFA) grade as a critical material attribute, necessitating controlled sourcing and handling of hygroscopic standards to ensure method reliability. The unified sample preparation for both related substances and isomer analysis enhances laboratory efficiency.

13. CONCLUSION:

The developed RP-HPLC methods for sacubitril and valsartan related substances and isomers demonstrate robust, precise, and accurate quantification suitable for pharmaceutical quality control. The related substances method, optimized through AQbD principles, reliably separates all specified impurities and degradation products with a short runtime and high sensitivity. The isomer method provides effective chiral separation on a reverse-phase platform, facilitating streamlined analysis without compromising resolution. Stability-indicating capabilities were confirmed via forced degradation studies, and comprehensive validation affirmed linearity, precision, accuracy, and robustness within defined operational parameters. The final control strategies emphasize critical material attributes and method parameters, ensuring consistent and reproducible performance. These validated methods support regulatory compliance and stability monitoring for sacubitril/valsartan formulations, including pediatric oral suspensions, thereby reinforcing product quality and patient safety.

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