A NEW REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS ESTIMATION OF SERRATIOPEPTIDASE AND DICLOFENAC SODIUM IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

Objective: The objective is to study the development of a simple, rapid, specific, precise, and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of serratiopeptidase (SER) and diclofenac (DC) sodium in bulk and tablet formulation.

Methods: RP-HPLC method was developed for the simultaneous estimation of SER and DC sodium in tablet formulation. The separation was achieved by Kromasil C18 column (250 mm × 4.6 mm, 5 μm particle size) with phosphate buffer pH-7 and o-phosphoric acid:methanol:acetonitrile (5:4:1% v/v/v). Flow rate was maintained at 1 mL/min and UV detection was carried at 270 nm.

Result: For RP-HPLC method, the retention time for SER and DC sodium was found to be 3.3833 min and 8.1667 min, respectively. The method was validated for accuracy, precision, and specificity. Linearity for SER and DC sodium was in the range of 5–50 μg/ml.

Conclusion: The developed RP-HPLC method is simple, accurate, rapid, sensitive, precise, and economic. Hence, this method can be employed successfully for the estimation of SER and DC sodium in both bulk and tablet dosage forms.

Keywords: Serratiopeptidase, Diclofenac sodium, Methanol, Acetonitrile, Phosphate buffer pH-7, O-phosphoric acid, Reversed-phase high-performance liquid chromatography.

INTRODUCTION

Serratiopeptidase (SER) is a proteolytic enzyme (protease) produced by Enterobacter *Serratia* sp. E-15. This microorganism was originally isolated in the late 1960s from silkworm *Bombyx mori* L. (intestine). SER is present in the silkworm intestine and allows the emerging moth to dissolve its cocoon. SER is produced by purification from a culture of *Serratia* E 15 bacteria. SER is primarily indicated in the condition of hematoma, inflammation, edema osteoarthritis, and thrombosis. Diclofenac (DC) sodium is sodium 2-[(2, 6-dichlorophenyl)-amino] phenylacetate. It is a synthetic non-steroidal anti-inflammatory agent with analgesic, anti-inflammatory, and antipyretic activity. Its mechanism of action is associated principally with the inhibition of prostaglandin synthesis (specifically, inhibition of cyclooxygenase). DC sodium is administered to reduce inflammation and as an analgesic reducing pain in conditions such as arthritis or acute injury. It can also be used to reduce menstrual pain, dysmenorrhea (Figs. 1 and 2) [1-4].

Literature survey reveals few methods for the simultaneous estimation of SER and DC sodium in the combination considered in this work and other combinations. This initiates a need for more rigorously studied and competent reversed-phase high-performance liquid chromatography (RP-HPLC) method development and validation for SER and DC sodium in bulk and tablet dosage form.

METHODS

1. DC sodium (Zydus Cadila, PTC, Thane)
2. SER (Analab Fine Chemicals)
3. Marketed formulation: Emanzen-D (EMCURE Pharmaceuticals Pvt. Ltd.)
4. Acetonitrile (HPLC grade, Merck Ltd.)
5. Methanol (HPLC grade, Merck Ltd.)
6. Phosphate buffer pH- 7(HPLC grade-Merck Ltd.).

Instruments

- Model: Cyberlab LC-100 HPLC (binary gradient system)
- Injector: 20 μL
- Detector: UV detector
- Software: Workstation -100
- Analytical balance: Shimadzu, AX 200
- Sonicator: Dakshin (ultrasonicator)

Chromatographic condition

- Analytical column: Kromasil C18 column (250 mm × 4.6 mm, 5 μm particle size)
- Mobile phase: phosphate buffer (pH-7) with o-phosphoric acid (OPA):methanol:acetonitrile (ACN) (5:4:1% v/v/v)
- Injection volume: 20 μL
- Flow rate: 1 mL/min
- Detection wavelength: 270 nm
- Retention time: SER 3.38 min and DC 8.16 min.

Preparation of standard solutions

Preparation of diluent

Phosphate buffer pH 7 with OPA, methanol, and acetonitrile (5:4:1% v/v/v) mixtures was used as diluents and was filtered through 0.22 μm nylon filter under vacuum condition. Then, it was sonicated for 15 min and further used for the mobile phase preparation.

Preparation of SER and DC stock solution

Accurately weighed quantity of DC sodium equivalent to 10 mg and SER (10 mg) was transferred into two separate 10 mL volumetric flasks.
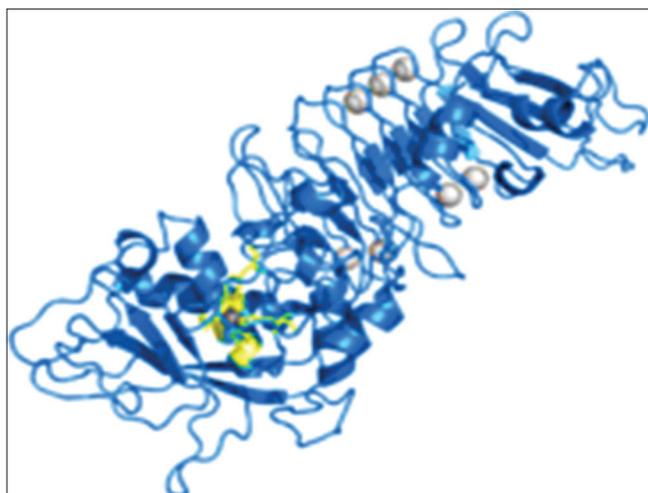
Fig. 1: Crystal structure of serralysin with coordinated zinc (gray) and calcium (white)
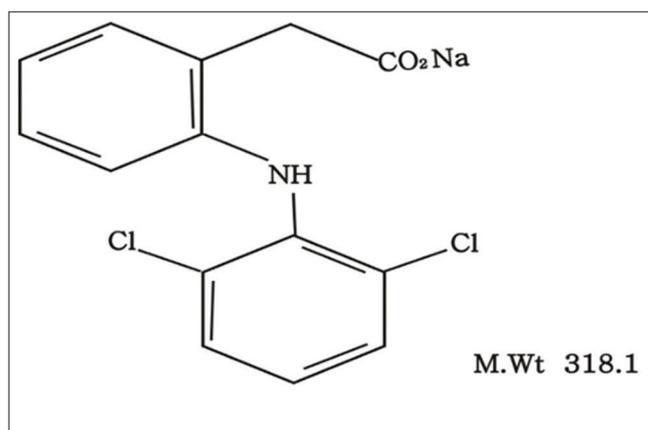

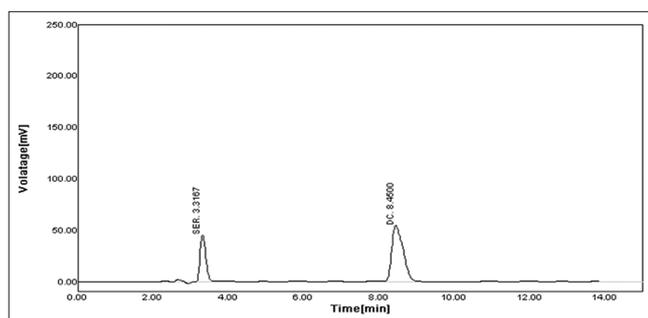**Fig. 2: Structure of diclofenac sodium**

Fig. 3: Reversed-phase high-performance liquid chromatography graph of bulk mixture (10 µg/mL SER and 50 µg/mL DC). Buffer (pH-7) with OPA:methanol:ACN (5:4:1 v/v/v), flow rate: 1.0 mL/min

The drug was dissolved in 10 mL of buffer pH-7.0 (HPLC grade) with shaking and sonicated for 10 min.

Preparation of tablet stock solution

An accurately weighed powder sample equivalent to 50 mg of DC and 10 mg of SER was transferred to 10 mL volumetric flask; 10 mL of phosphate buffer pH-7.0 was added and the flask was sonicated for 5 min. The solution was filtered through 0.45 µ membrane filter paper. From the prepared solution, 0.1 mL of the filtrate was transferred to 100 mL volumetric flask, and volume was made with mobile phase to get final concentration 50 µg/mL for DC and 10 µg/mL for SER.

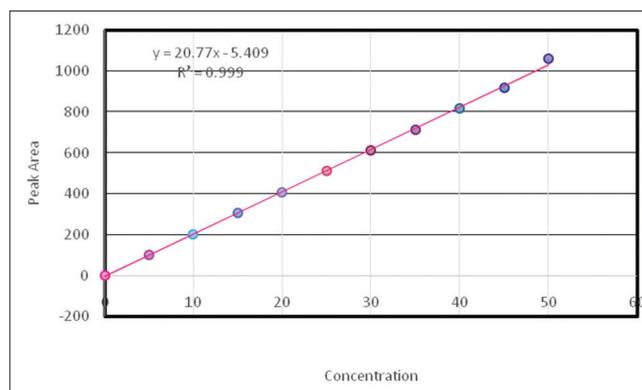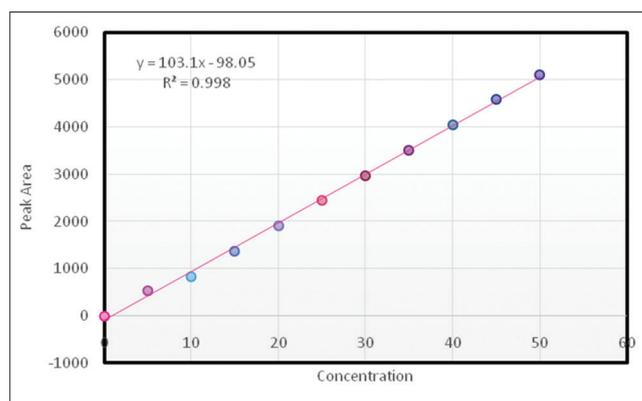**Fig. 4: Standard calibration curve of diclofenac**

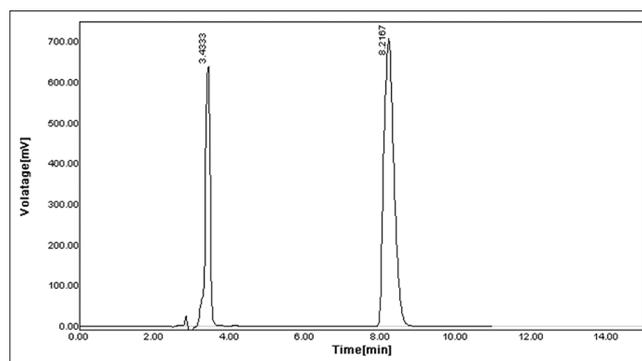**Fig. 5: Standard calibration curve of serratiopeptidase**

Fig. 6: Flow rate change - robustness: (1) Flow rate - 0.9 mL
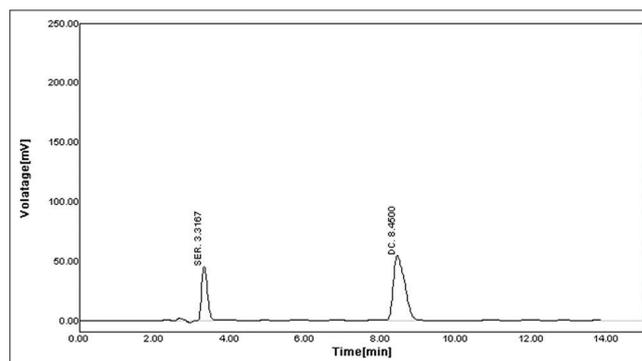

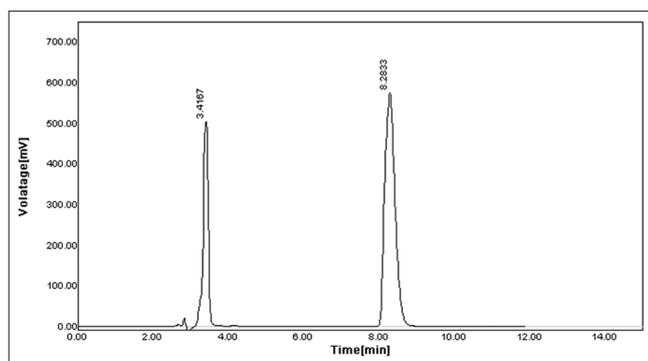**Fig. 7: Flow rate change - robustness: (2) Flow rate - 1.0 mL**

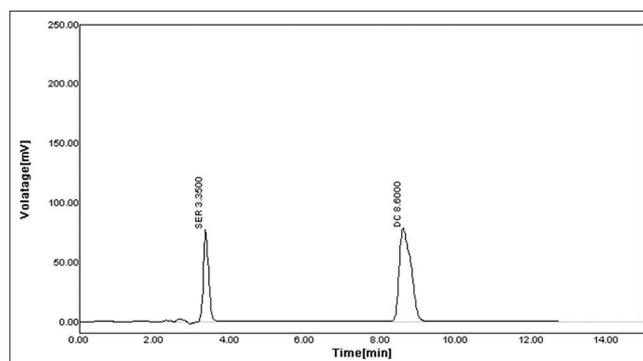**Fig. 8: Flow rate change - robustness: (3) Flow rate - 1.1 mL**

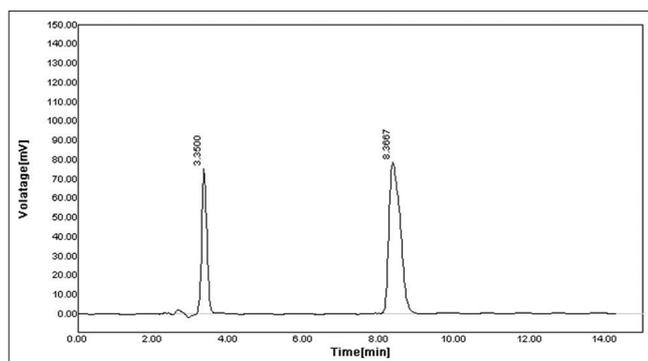**Fig. 12: Wavelength change: At 269 nm**

Fig. 9: Composition change: 9% acetonitrile+42% MeOH+49% buffer
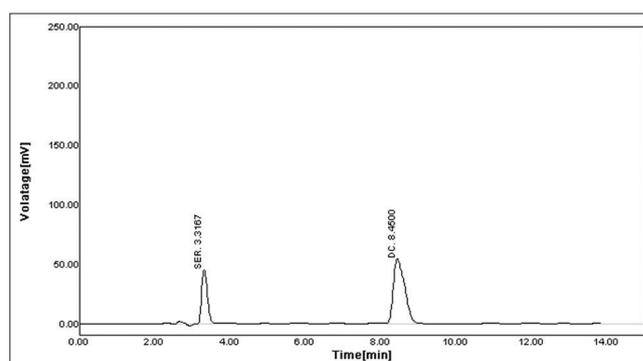
Fig. 13: Wavelength change: At 270 nm
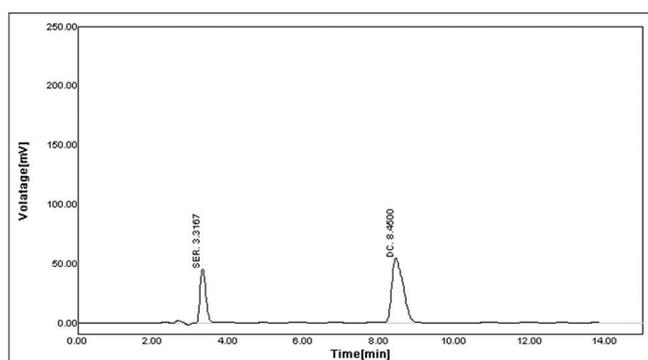
Fig. 10: Composition change - 10% acetonitrile +40% MeOH+ 50% buffer
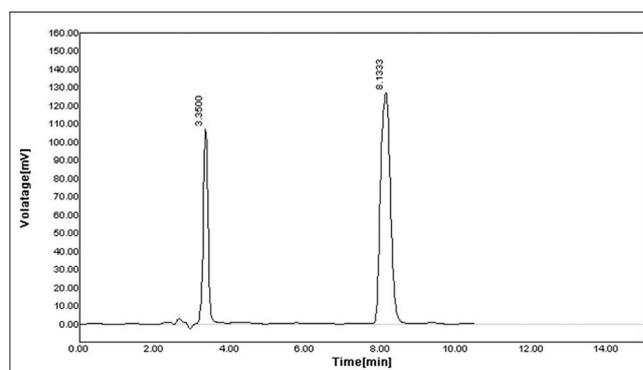
Fig. 14: Wavelength change: At 271 nm. The method was found to be robust as there was no significant change in retention times

Preparation of mix working standard solution of SER and DC

Working standard solution was prepared by diluting 0.1 mL of stock solution SER and 0.5 mL of stock solution DC with diluents in 10 mL of volumetric flask up to the mark with mobile phase. To get 10 μg/mL for SER and 50 μg/mL for DC.

Preparation of tablet mixture

Tablet mixtures were prepared by pipetting out 0.1 mL from stock solution in 100 mL volumetric flask and diluted it with mobile phase upto the mark.

Preparation of mix sample solution of SER and DC

Working sample solutions were prepared by taking aliquots as mentioned in Table 1 from stock solution in 10 mL of volumetric flask and volume was made up to the mark with diluent to get 5–50 10 μg/mL concentrations of SER and DC (Table 1).

Fig. 11: Composition change - 11% acetonitrile +38% MeOH+ 51% buffer

Table 1: Preparation of mix working standard solution of SER and DC

Concentration (μg/mL)		Volume (mL)	
SER	DC	SER	DC
5	5	0.05	0.05
10	10	0.1	0.1
15	15	0.15	0.15
20	20	0.2	0.2
25	25	0.25	0.25
30	30	0.3	0.3
35	35	0.35	0.35
40	40	0.4	0.4
45	45	0.45	0.45
50	50	0.5	0.5

Table 2: Analysis of pure mixed standards of DC and SER

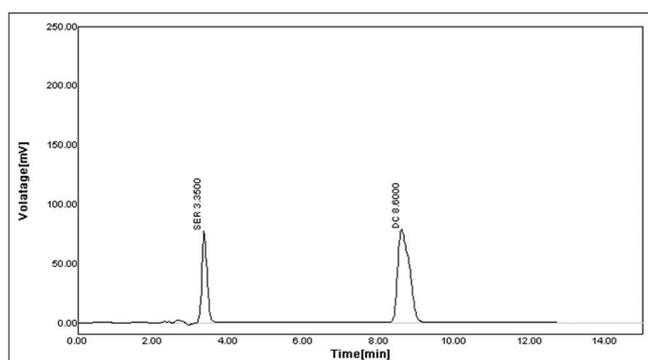| Concentration of drugs taken (μg/mL)		Concentration of drugs found (μg/mL)		* % drug found	
SER	DC	SER	DC	SER	DC
10	50	9.22	48.34	92.2	96.68

*Average of five determination. Buffer (pH-7) with OPA: methanol: ACN (5:4:1 v/v/v) with Flow rate: 1.0 ml/min was selected, ACN: Acetonitrile

Table 3: Standard calibration data for DC

DC sodium	
Concentration (μg/mL)	Peak area*
5	85.05
10	130.60
15	261.2
20	391.7
25	520.6037
30	651.2037
35	781.8037
40	912.3937
45	1043.0037
50	1188.1166

DC: Diclofenac

Table 4: Standard calibration data for SER

SER	
Concentration (μg/mL)	Peak area*
5	535.54
10	831.2185
15	1366.75
20	1901.74
25	2437.29
30	2972.80
35	3508.37
40	4043.90
45	4579.45
50	5114.45

SER: Serratiopeptidase

Selection of mobile phase
Mixed solution of SER and DC was prepared and injected into the HPLC system. The solution was analyzed using buffer:methanol:ACN.

Selection of flow rate
Chromatogram of a mixed solution of SER (10 μg/mL) and DC (50 μg/mL) was studied at different flow rate such as 0.9, 1, and 1.1 mL/min.

Table 5: Statistical data for SER and DC by chromatographic method

Parameters	SER	DC
Linearity range (μg/mL)	5–50	5–50
Correlation coefficient	0.998	0.999
Slope	103.1	20.77
Intercept	98.05	5.409
LOD (μg/mL)	0.215	0.542

Linearity range for both SER and DC was found to be 5–50 μg/mL, SER: Serratiopeptidase, DC: Diclofenac, LOD: Limit of detection, LOQ: Limit of quantitation

Table 6: Repeatability data (statistical evaluation) bulk mix

Drugs	*% Mean	*SD	*% RSD
SER	92.32	0.0454	0.0491
DC	94.12	0.6401	0.6800

*Average of five determinations, SER: Serratiopeptidase, DC: Diclofenac

Table 7: Repeatability data (statistical evaluation) tablet mix

Drugs	*% Mean	*SD	*% RSD
SER	92.9	0.096	0.103
DC	94.04	0.640	0.680

*Average of five determinations. The % RSD is <2 for SER and DC which indicate that the method is precise, SER: Serratiopeptidase, DC: Diclofenac

Selection of analytical wavelength
The standard solutions of SER (10 μg/mL) and DC (50 μg/mL) in mobile phase were scanned separately in the UV region of 200–400 nm, and the overlain spectra were recorded.

Chromatographic separation
Standard or sample solution of 20 μL was injected into the column. The chromatogram was run for 15 min with mobile phase, phosphate buffer pH 7 with OPA:methanol:ACN (5:4:1 v/v/v), which was previously sonicated for 10 min and detection done at wavelength 270 nm. The chromatogram was stopped after the separation was achieved completely. Data related to peak suc as area, height, retention time, and resolution were recorded using software.

Calibration curve for SER and DC
Aliquots of mix working standard solution of SER (5–50 μg/mL) and DC (5–50 μg/mL) were injected into the system with stated chromatographic conditions as described under table. The graph of the area of peak obtained versus respective concentration was plotted. The mean area and its standard deviation (SD) were calculated.

Validation of proposed method [5-15]
Precision

Repeatability
For repeatability, the separate concentration of 10 μg/mL for SER and 50 μg/mL for DC was analyzed 5 times on the same day and % relative SD (% RSD) were calculated.

Intraday precision
Separate concentration of 10 μg/mL for SER and 50 μg/mL for DC was analyzed 5 times on the same day and % RSD was calculated.

Interday precision
Separate concentration of 10 μg/mL for SER and 50 μg/mL for DC was analyzed 5 times on the different days and % RSD was calculated.

Table 8: Inter- and intra-day precision

Drugs	Inter-day			Intraday		
	%Mean	SD	%RSD	%Mean	SD	%RSD
SER (BULK)	92.42	0.0516	0.0558	93.04	0.0472	0.0507
SER (TABLET)	92.38	0.0511	0.0553	92.82	0.0481	0.0518
DC (BULK)	94.12	0.6401	0.6800	94.12	0.6422	0.6823
DC (TABLET)	93.86	0.6386	0.6803	93.87	0.6386	0.6803

*Average of five determinations. The % RSD is <2 for SER & DC which indicate that the method is precise, SER: Serratiopeptidase, DC: Diclofenac

Table 9: Statistical validation of recovery studies

Level of % recovery	% Mean recovery*		SD		% RSD	
	SER	DC	SER	DC	SER	DC
80	92.74	98.92	0.0589	0.0152	0.063	0.0153
100	98.1	99.51	0.635	0.0115	0.647	0.0115
120	98.12	98.56	0.230	0.0057	0.234	0.0057

*Average of three at each level of recovery. The % recovery is within limit (98.0–102.0%), so the method is accurate, SER: Serratiopeptidase, DC: Diclofenac

Accuracy

Procedure

Preparation of sample solution for SER
Recovery studies were carried out by pipetting out 0.1 mL from tablet stock solution in 10 mL volumetric flask and in that 0.8 mL (80%), 1.0 mL (100%), and 1.1 mL (120%) bulk mixtures of DC and SER were added, and area was measured at the selected maximum wavelength.

The accuracy of the method was performed by conducting the standard edition method (80%, 100%, and 120% level) of tablet. The percentage recoveries were calculated and reported with SD and % RSD.

Specificity

The specificity of the HPLC method was ascertained by analyzing standard drug and sample solutions. The retention times of SER and DC in the sample solution were confirmed by comparing with that of the respective standards. The chromatogram of tablet sample showed only two peaks at retention time of 3.3833 and 8.1667 min for SER and DC, respectively, indicating that there is no interference of the excipients in the tablet formulation.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were estimated from the set of three calibration curves used to determine method linearity. The LOD and LOQ were calculated as follows,

$$LOD = \frac{3.3\ \sigma}{S}, \quad LOQ = \frac{10\ \sigma}{S}$$

Where σ = SD of replication, S = slope of calibration curve

System suitability

Combined standard solution of SER (10 μg/mL) and DC (50 μg/mL) was prepared and analyzed 3 times. Chromatograms were studied for different parameters such as tailing factor, resolution, and theoretical plates to see that whether they comply with the recommended limit or out of recommended limit.

Analysis of tablet dosage form

An accurately weighed powder sample equivalent to 50 mg of DC and 10 mg of SER was transferred to 10 mL volumetric flask; 10 mL of phosphate buffer pH-7.0 was added, and the flask was sonicated for 5 min. Solution was filtered through 0.45 μ membrane filter paper.

From the prepared solution, 0.1 mL of the filtrate was transferred to 100 mL volumetric flask, and volume was made with mobile phase to get final concentration 50 μg/mL for DC and 10 μg/mL for SER. After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the tablet sample solution was injected using 20 μL syringe filter. Chromatographic detection was carried out at 270 nm. Chromatogram was obtained, and peak areas were recorded. The procedure was repeated 5 times for the analysis of homogeneous sample.

RESULTS AND DISCUSSION

Selection of mobile phase

Buffer (pH-7) with OPA:methanol:ACN (5:4:1 v/v/v) was selected as mobile phase with flow rate 1.0 ml/min. The chromatogram is shown in Fig. 3. The proposed method was applied on 5 mixtures of SER and DC. The concentrations obtained are shown in Table 2.

Validation of proposed method

Linearity

The linearity range for SER and DC was found to be 5–50 μg/mL.

The linearity graphs and correlation coefficients are shown in Figs. 4 and 5 for DC and SER . The data for calibration is given in Tables 3 and 4. The statistical data for linearity studies is given in Table 5.

Precision

The statistical data for repeatability studies for bulk mixture and tablet mixture are given in Tables 6 and 7.

Intra- and inter-day precision

Intraday precision was determined by analyzing SER and DC for 3 times in the same day. Interday precision was determined by analyzing SER and DC daily for different days. The results for inter-day and intra-day precision studies are shown in Table 8.

Accuracy

Recovery studies were carried out by applying the method of standard drug addiction at 80%, 100%, and 120% levels. The solutions for recovery studies at 80%, 100%, and 120% level were prepared. The solutions were filtered through a 0.45-μm membrane filter paper and analyzed by RP-HPLC method. At each level of recovery, three determinations were performed. The results of the same are shown in Table 9. The proposed method was found to be accurate.

Robustness

Robustness of the proposed method was studied by changing flow rate of mobile phase (0.9 ml/min, 1.0 ml/min and 1.1 ml/min), changing the composition of the mobile phase (9% acetonitrile+42% MeOH+49% buffer, 10% acetonitrile 40% MeOH+50% buffer and 11% acetonitrile+38% MeOH+51% buffer) and by changing the detection wavelength (269 nm, 270 nm and 271 nm). The chromatograms with changed flow rates are shown in Figs. 6-8. The chromatograms with changed mobile phase compositions are shown in Figs. 9-11. The chromatograms with changed detection wavelengths are shown in Figs. 12-14. The statistical data for retention times and tailing factors for

Table 10: Flow rate change

Flow rate	Retention time		Tailing factor		Area		%Content	
	SER	DC	SER	DC	SER	DC	SER	DC
0.9	3.4333	8.2167	0.7333	1.2333	1150.2328	2389.3639	101.5	101.88
1.0	3.3167	8.4500	1.3889	1.4286	831.2185	1988.1166	93.20	96.31
1.1	3.4167	8.2833	0.7667	1.2000	1035.22	2133.2555	101.36	99.89

SER: Serratiopeptidase, DC: Diclofenac

Table 11: Composition change

Composition change	Retention time		Tailing factor		Area		%Content	
	SER	DC	SER	DC	SER	DC	SER	DC
42% MeOH+9% ACN+49% BUFFER	3.3500	8.3667	1.1000	1.4615	756.57	1646.64	73.34	77.57
CAN (10%)+MeOH (40%)+BUFFER (50%) PH 7.0	3.3167	8.4500	1.3889	1.4286	831.2185	1988.1166	92.5	96.62
38% MeOH+11% ACN+51% BUFFER	3.3500	8.6000	1.1667	1.3929	786.3861	1643.9059	76.23	79.97

SER: Serratiopeptidase, DC: Diclofenac, ACN: Acetonitrile

Table 12: Wavelength change

Wavelength change	Retention time		Tailing factor		Area		%Content	
	SER	DC	SER	DC	SER	DC	SER	DC
269	3.3500	8.6000	1.1663	1.3919	776.3875	1643.8069	75.26	76.97
270	3.3167	8.4500	1.3889	1.4286	831.2185	1988.1166	93.5	96.62
271	3.3500	8.1333	1.0000	1.0625	1276.552	2303.7380	109.76	107.87

SER: Serratiopeptidase, DC: Diclofenac

Table 13: Details of chromatogram of DC and SER in tablet mix

Name	Rt (min)	Area	Tailing factor	Theoretical plate
SER	3.3833	1031.4727	0.9444	3570.5
DC	8.1667	2135.55	1.0625	3328.5

SER: Serratiopeptidase, DC: Diclofenac

Table 14: Assay results of marketed dosage forms

Label claim (mg/tab)		Amount found (mg/tab)		% of label claim	
SER	DC	SER	DC	SER	DC
10	50	9.31	46.42	93.1	92.84
10	50	9.23	47.50	92.3	95
10	50	9.24	46.64	92.4	93.28
10	50	9.22	47.55	92.2	95.1
10	50	9.31	47.87	93.1	95.74

SER: Serratiopeptidase, DC: Diclofenac

Table 15: Validation parameters

Parameters	SER	DC
Linearity range (µg/mL)	5–50	5–50
Correlation coefficient	0.998	0.999
LOD (µg/mL)	0.215	0.542
LOQ (µg/mL)	0.653	1.643
Precision: bulk Intraday (% RSD)	0.0507	0.6823
Interday (% RSD)	0.0558	0.6800
Precision: Tablet intraday (% RSD)	0.0518	0.6803
Interday (% RSD)	0.0553	0.6803
Tailing factor	1.3889	1.4286
Mean % recovery bulk	92.32	94.12
Mean % recovery tablet	92.9	94.04
Theoretical plates	1814.8	2945.0

SER: Serratiopeptidase, DC: Diclofenac, LOD: Limit of detection, LOQ: Limit of quantitation
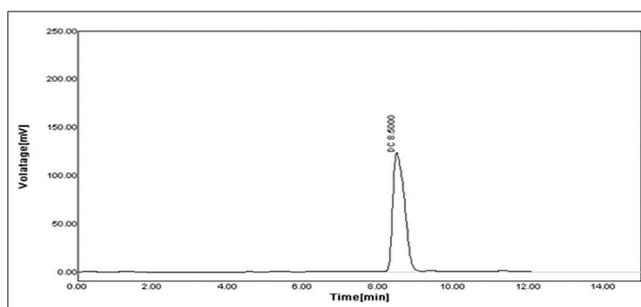

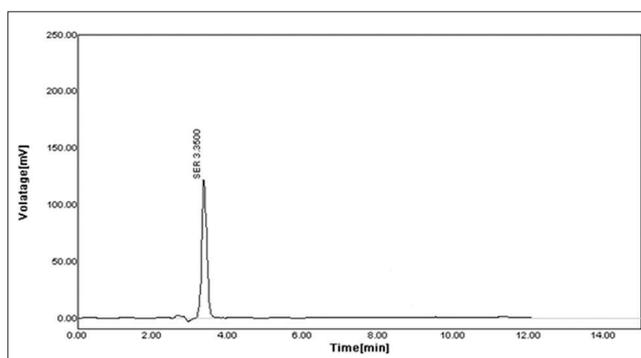
Fig. 15: Chromatogram of diclofenac

Fig. 16: Chromatogram of serratiopeptidase

the flow rate change, mobile phase composition change and detection wavelength change are shown in Tables 10-12. The retention time is not affected significantly. Thus, the proposed method was found to be robust.

Specificity

By comparing the chromatogram of diluent, standard solution, and test preparation solution, it was observed that there was no any interference of excipients with the peak of SER and DC, as shown in Figs. 15-17.
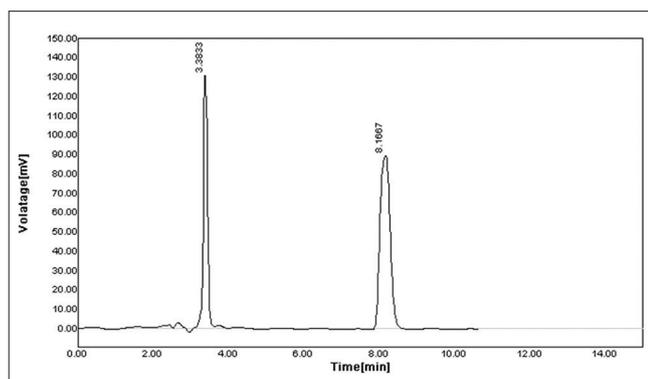
Fig. 17: Chromatogram of tablet mixture serratiopeptidase (10 μg/mL) and diclofenac (50 μg/mL)
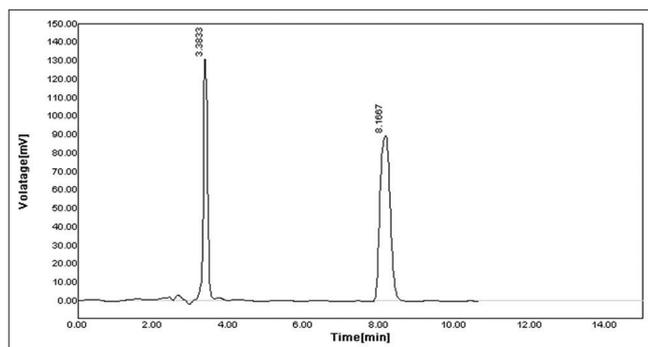

Fig. 18: Chromatogram of tablet mixture

System suitability data
The system suitability data is shown in Table 13.

Application of the developed method to tablet formulation
The proposed validated method was successfully applied to the determination of SER and DC in its tablet dosage form (Fig. 18).

Summary of validation parameters
Summary of the validation parameters studied is given in Table 15. The proposed method was validated as per ICH Q2R1 guidelines.

DISCUSSION

The literature survey gives few reported RP-HPLC methods for simultaneous estimation of DC sodium and SER in bulk and tablet dosage form. This work has made an attempt to develop a new RP-HPLC method for the estimation of DC sodium and SER in bulk and tablet dosage form. This work reports in detail, a more rigorously studied RP-HPLC method.

CONCLUSION

The proposed work concludes that the developed RP-HPLC method is simple, accurate, rapid, sensitive, precise, and economic. Hence, this method can be employed successfully for the estimation of SER and DC sodium in both bulk and tablet dosage forms.

AUTHORS' CONTRIBUTION

Experimental design, guidance, supervision, and review work for the research were done by Dr. Anagha Joshi M., Principal, SCES's Indira College of Pharmacy, Tathawade, Pune. Experimental work, interpretation of result, and writing of this manuscript were done by Manasi Kulkarni B., PhD Research Scholar, Punnaiyah Ramajayam Institute of Science and Technology, PRIST Deemed to be University, Thanjavur, Tamilnadu and Assistant Professor, SCES's Indira College of Pharmacy, Tathawade, Pune. All authors read and approve the final manuscript.

CONFLICTS OF INTEREST

The authors do not report any conflicts of interest.

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