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UV/HPLC SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF DICLOFENAC SODIUM AND SERRATIOPEPTIDASE

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ABSTRACT

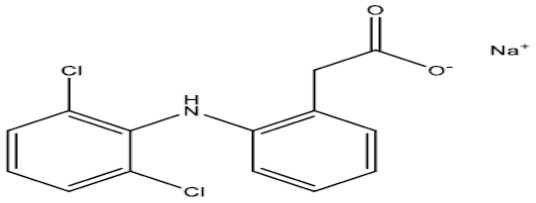
A Simultaneous estimation of UV/RP-HPLC method was developed and validated for the estimation of Diclofenac Sodium and serratiopeptidase in tablet dosage form using C18 column (150 X 4.6mm, 5µ) with mobile phase consisting of orthophosphoric acid and Methanol with a flowrate of 1.0ml/min and Absorption maxima at 260nm. Accuracy of drugs was observed to be within the limits of 97% to 102% by mean of 3 determinations 50%, 100% and 150%. Precision of drugs was observed to be less than 2.0 of %RSD by mean of 6 determinations. Linearity was observed over the concentration range 1-60 µg/ml (r2 =0.998) with regression equation y = 31265x – 9459.9 for Diclofenac sodium and Serratiopeptidase 1–60 µg/mL (r2 =0.997) with regression equation y = 153.4886x + 3130.8667. LOD and LOQ of Diclofenac Sodium and serratiopeptidase were found to be 0.016µg/ml, 0.048µg/ml, and 0.151µg/ml, 0.46µg/ml respectively. The method was validated as per ICH guidelines.

KEYWORDS: Diclofenac Sodium, Serratiopeptidase, HPLC, UV, Method development, Method validation.

INTRODUCTION

Diclofenac sodium

Diclofenac is used to reduce pain, swelling (Inflammation), and joint stiffness from arthritis. Reducing these symptoms helps you do more of your normal daily activities. Diclofenac is known as a nonsteroidal anti-inflammatory drug (NSAID). It works by blocking your body's production of certain natural substances that cause inflammation.



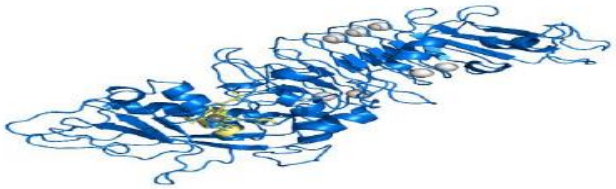
Diclofenac Sodium

Diclofenac inhibits cyclooxygenase-1 and -2, the enzymes responsible for production of prostaglandin (PG) G2 which is the precursor to other PGs. These molecules have broad activity in pain and inflammation and the inhibition of their production is the common mechanism linking each effect of diclofenac.

Serratiopeptidase

Serratiopeptidase is a proteolytic enzyme produced by enterobacterium Serratia sp. E-15, now known as Serratia marcescens ATCC 21074. This microorganism was originally isolated in the late 1960s from silkworm intestine.

Serratiopeptidase is a proteolytic enzyme that has been used for reducing inflammation, it has antiedemic, analgesic, fibrinolytic and caesinolytic properties. Serratiopeptidase is often used in oral surgery for its anti-inflammatory purpose after impaction surgery, maxillofacial trauma and infections but its use should be limited in cases of abscess due to its fibrinolytic activity



Serratiopeptidase

Experimental

Materials and Reagents

List of Instruments

S. No.	Instruments	Manufacturer
1	UV/VIS Spectrophotometer,	Shimadzu, Japan
2	Digital Weighing balance, (CY220)	Shimadzu, Japan
3	RP-HPLC instrument equipped with PDA detector	Shimadzu, Japan

4	Ultrasonicator	PCi analytics, India
5	Vortex mixer	Remi Scientific Instruments, Mumbai
6	Hot air oven	P. L. Tandon & Co, Delhi
7	Melting Point Apparatus	Remi Scientific Instruments, Mumbai
8	Infrared red spectrophotometer (FTIR)	Bruker Alpha, Berlin, Germany
9	Microcentrifuge	Remi Scientific Instruments, Mumbai
10	Vacuum pump	Suguna single phase, Chennai, India
11.	Nylon 0.22 µm membrane filter	Pall corporation, Mumbai

List of Chemicals

S. No	Materials	Source
1	Diclofenac sodium	N. S. Chemicals (NSC)
2	Serratiopeptidase	Avanscure Lifesciences Pvt. Ltd.
3	Methanol	Fisher Scientific India Pvt. Ltd.
4	Orthophosphoric acid HPLC grade	Merck, Mumbai
5	Acetonitrile HPLC grade	Merck, Mumbai

Pre-formulation studies melting point

USP technique was used to determine the melting point. The capillary fusion method was used to determine the drug's melting point. A tiny quantity of medication was put into a capillary and put inside a melting point equipment. Next, the temperature at which the medication crystals began to melt and became liquid was recorded.

Fourier transmission Infra-Red Spectroscopy

The FT-IR (Fourier Transform Infrared) spectra of a substance or drug can reveal the groups that are present. For structure investigation, FT-IR spectroscopy was employed. For the purpose of identifying any potential drug interactions with excipients, an FT-IR spectrum of a Diclofenac sodium and serratiopeptidase drug, Physical mixture of Diclofenac sodium and serratiopeptidase were recorded. The FT-IR chamber received 1-2 mg of Diclofenac sodium and serratiopeptidase drug. The region between 4000 and 400 cm-1 of the infrared spectrum was observed.

UV Spectrophotometry by Simultaneous Estimation

Determination of λ_{max} and Isobestic Point by UV- Spectrophotometer

When exposed to visible or ultraviolet light, molecules in solution can absorb light of a specific wavelength depending on the type of electronic transition associated with the absorption. This is why UV-visible spectrophotometers are commonly used to obtain specific information on the chromophoric part of the molecules in solution. Typically, the UV spectrum is plotted against wavelength to record absorption.

The λmax of drug" was determined using a double beam UV-visible spectrophotometer (Shimadzu, UV-1800, Japan). Diclofenac sodium and serratiopeptidase solutions in methanol at 4µg/ml and 12µg/ml were scanned between 200 and 400 nm. The term "isosbestic point" refers to the locations where the spectra of the two drugs cross.

HPLC method

Selection of Mobile Phase and Optimization of Chromatographic condition

➢ **Chromatographic conditions**

- **Stationary phase:** C_{18}, 150 × 4.6 mm, 5µm particle size, Phenomenex
- **Elution mode:** Isocratic mode (70:30)
- **Mobile phase:** Solvent A was orthophosphoric Acid buffer and Solvent Bwas Methanol (adjust pH 3 with OPA)
- **Detector:** UV
- **Absorption maxima:** 260 nm
- **Column Temperature:** 30 °C
- **Flow rate:** 1 ml/min.
- **Injection volume:**20 µl
- **Diluent:** Mobile phase
- **Run time:** 10minutes.

Standard Stock Solution Preparation (1000µg/ml & 3000µg/ml)

Blank: Diluent was filtered through 0.22 µ milli pore membrane filters and injected in HPLC system.

Standard solution preparation: In a 10 ml volumetric flask, an accurately weighed quantity of about 10 mg of Diclofenac sodium and 30mg of Serratiopeptidase were added. They were then mixed in 10 ml of diluents (As previously mentioned) to produce a stock solution of 1000µg/ml for diclofenac and 3000 µg/ml for Serratiopeptidase, which was then sonicated to dissolve.

Validation of HPLC metho d as per ICH guidelines

Linearity: The capacity of an analytical method to yield test findings that are exactly proportionate to the analyte concentration in the sample within a specified range is known as its linearity. The interval between the highest and lower levels of analyte that have been

shown to be determined within an appropriate degree of precision, accuracy, and linearity is known as the analytical method's range. For Diclofenac sodiumand Serratiopeptidase, the chosen linearity range was 10–60 µg/ml and 30–180 µg/ml respectively. After being filtered using a 0.22 µ filter, each dilution was then injected.

Accuracy: The method's accuracy was assessed using the standard recovery percentage. In order to conduct recovery tests sample at three different concentration levels: 50%, 100% Using this procedure.

50% Accuracy

Preparation of standard solution: Given 10mg of diclofenac and 30mg of Serratiopeptidase standard each compound was dissolved in 100ml volumetric flask and make up the volume to 100ml with diluent and concentration found 100µg/ml for diclofenac and 300µg/ml for serratiopeptidase. Further take 2ml of stock solution and dilute upto 10ml with diluent and concentration was found 20µg/ml for diclofenac and 60µg/ml for serratiopeptidase then filtered through 0.22m filter and injected in HPLC instrument. Inject duplicate injections of standard solution into HPLC.

100% Accuracy

Preparation of standard solution: Given 10mg of diclofenac and 30mg of Serratiopeptidase standard each compound was dissolved in 100ml volumetric flask and make up the volume to 100ml with diluent and concentration found 100µg/ml for diclofenac and 300µg/ml for serratiopeptidase. Further take 4ml of stock solution and dilute upto 10ml with diluent and concentration was found 40µg/ml for diclofenac and 120µg/ml for serratiopeptidase then filtered through 0.22m filter and injected in HPLC instrument. Inject duplicate injections of standard solution into HPLC.

Precision: The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intraday studies, six repeated injections of standard were made and the response factor of drug peaks and percentage RSD were calculated. In the inter-day variation studies, six repeated injections of standard were made for six consecutive days and response factor of drugs peaks and percentage RSD were calculated. From the data obtained, the developed RP-HPLC method was found to be precise. The repeatability of the injection was assessed using a 40µg/ml concentration of diclofenac and 120µg/ml of serratiopeptidase and inject six injections, and the percentage RSD was calculated.

LOD and LOQ: In accordance with ICH recommendations, the developed method's LOD and LOQ were examined. There are various methods available for figuring out the LOD and LOQ, depending on whether an instrumental or non-instrumental procedure is used. One of the techniques used here was,

LOD= 3.3σ/S and LOQ= 10σ/S

Where, σ = the standard deviation of intercept S = mean of slope in calibration curve.

Robustness: By examining the sample with a lower concentration and purposefully changing the procedure parameters, the resilience was investigated. The percentage RSD was used to indicate how the responses of the medicines changed. The method's robustness was examined using wavelength ±5 nm and flow rate variations ±0.1ml.

Preparation of standard solution: Given 10mg of diclofenac and 30mg of Serratiopeptidase standard each compound was dissolved in 100ml volumetric flask and make up the volume to 100ml with diluent and concentration found 100µg/ml for diclofenac and 300µg/ml for Serratiopeptidase. Further take 4ml of stock solution and dilute upto 10ml with diluent and concentration was found 40µg/ml for diclofenac and 120µg/ml for Serratiopeptidase then filtered through 0.22m filter and injected in HPLC instrument. Inject duplicate injections of standard solution into HPLC.

RESULT

Preformulation studies melting point determination

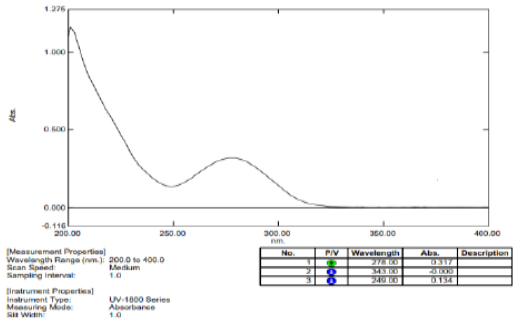
Drugs	Melting point (°C)	
	Observed	Reported
Serratiopeptidase	146.08°C ±0.576-147.04°C±0.576	146-148°C
Diclofenac	287.01°C ±0.607-288.09°C±0.607	279-289°C

The melting point of Serratiopeptidase and Diclofenac sodium was found to be 146.08°C ±0.576- 147.04°C±0.576 and 287.01°C±0.607-288.09°C±0.607 is similar to literature value i.e., 146- 148°C and 279-289°Cfor Serratiopeptidase and Diclofenac respectively indicating that there is no impurity present in the sample.

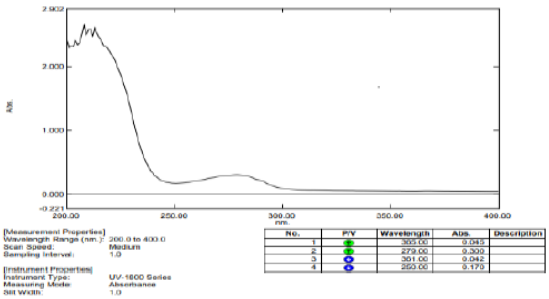
Determination of absorption maxima of Serratiopeptidase and Diclofenac

The first requirement of any Preformulation study is the development of a simple analytical method for quantitative estimation in subsequent steps. Most of drugs absorb light in UV range, UV-Visible spectrophotometer being a fairly accurate and simple method for

estimation of drug. UV absorption maximum of Serratiopeptidase and Diclofenac sodium in methanol was determined and exhibited characteristic absorption at 278 and 279 nm respectively Figure15and Figure 16 shows the UV spectrum scan of Serratiopeptidase and Diclofenac sodium in methanol respectively. It confirms the purity of drug.

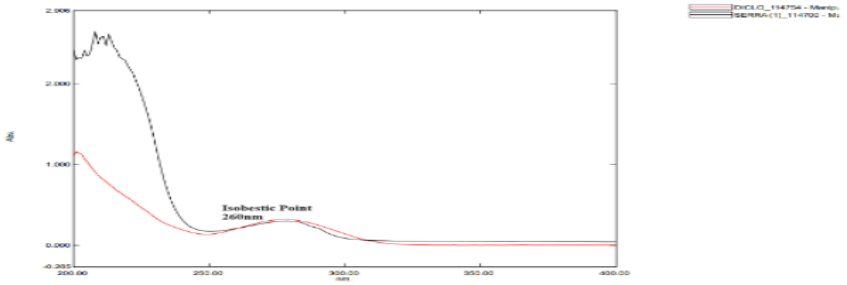


Graph of UV absorption spectra of Diclofenac



Graph of UV absorption spectra of Serratiopeptidase

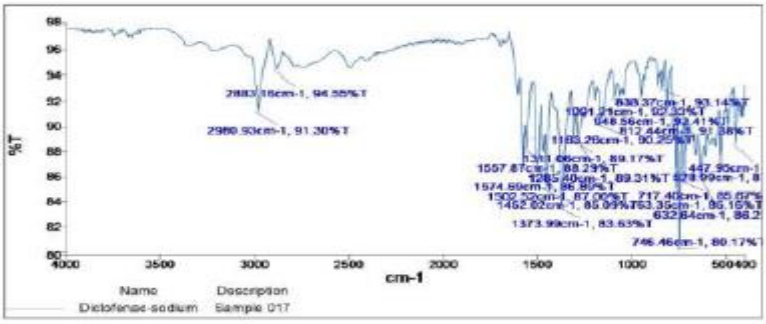
Selection of analytical wavelength



Graph of Overlay spectra of Serratiopeptidase and Diclofenac sodium

Discussion: Solutions of Diclofenac (4µg/ml) and Serratiopeptidase(12µg/ml) were prepared from working standard in methanol and spectrums were recorded between 200- 400 nm and the isosbestic point was found at 260nm.

FTIR Study of Diclofenac and Serratiopeptidase



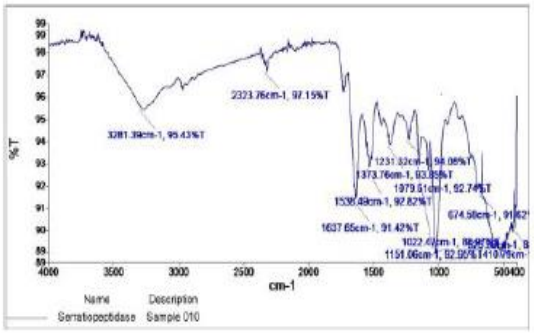
FTIR spectrum of Diclofenac

FTIR of Diclofenac sodium

Characteristics Peaks	Reported (cm^{-1})	Observed(cm^{-1})
Aromatic C-H	2890	2883.16
C-N stretching	1249	1285.40
C-H plane aromatic bending	1177	1163.26
Phenyl ring	1575	1574.69

FTIR spectrum of Diclofenac sodium peaks has Aromatic C-H at 2883.16 cm^{-1}, C-N stretching at 1285.40 cm^{-1}, 1163.26cm^{-1} due to C-H plane aromatic bending, and 1574.69 (Phenyl ring). All these vibrational peaks at different wave numbers corresponds to its functional groups, confirming the purity of the drug as per established standards.

FTIR Study of Serratiopeptidase

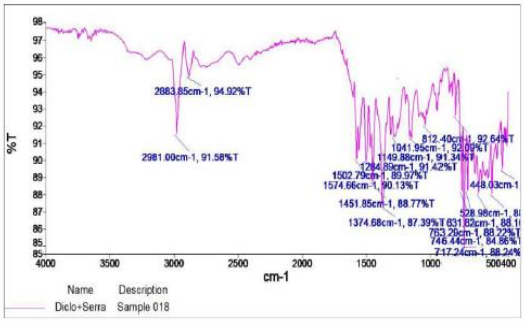


Characteristics Peaks	Reported (cm^{-1})	Observed (cm^{-1})
N-H stretching	3287.60	3281.39
C=O stretching	1649.08	1637.65
Aromatic ring	1543.19	1538.49
C-O stretching	1075.53	1079.51

FTIR spectrum of Serratiopeptidase FTIR of Serratiopeptidase

FTIR spectrum of Serratiopeptidase peaks has N-H at 3281.39 cm^{-1}, C=O at 1637 cm^{-1}, 1538.49cm^{-1} due to Aromatic ring, and 1079.51 C-O stretching. All these vibrational peaks at different wave numbers corresponds to its functional groups, confirming the purity of the drug as per established standards.

FTIR Study of Serratiopeptidase and Diclofenac



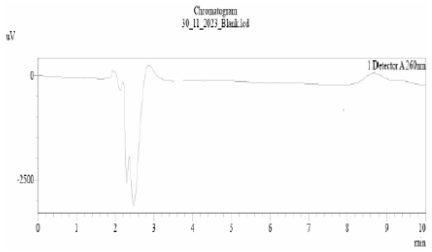
Characteristics Peaks	Reported (cm^{-1})	Observed (cm^{-1})
Aromatic C-H	2883.16	2883.85
Phenyl Ring	1574.69	1574.66
C-O stretching	1079.51	1041.95
Aromatic ring	1538.49	1502.79

FTIR of Serratiopeptidase and Diclofenac

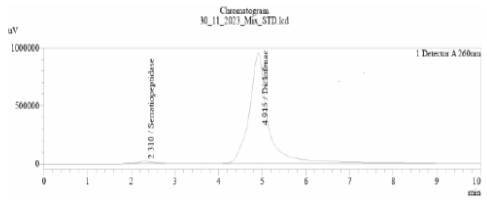
FTIR spectrum of Serratiopeptidase and Diclofenac peaks has Aromatic C-H at 2883.85 cm^{-1}, Phenyl Ringat 1574.66 cm^{-1},C-O stretchingat 1041.95cm^{-1}, and Aromatic ringat 1502.79.All these vibrational peaks at different wave numbers corresponds to its functional groups, confirming the purity of the drug as per established standards.

HPLC Method

Determination of chromatogram of Blank & Standard (Serratiopeptidase and Diclofenac) On HPLC analysis of Blank and standard solution of Serratiopeptidase (200µg/ml) and Diclofenac (100µg/ml) chromatogram was optimized & analyzed as per the proposed method.



Chromatogram of blank



(Simultaneous chromatogram of standard Diclofenac and Serratiopeptidase (100ug/ml& (200ug/ml) respectively.)

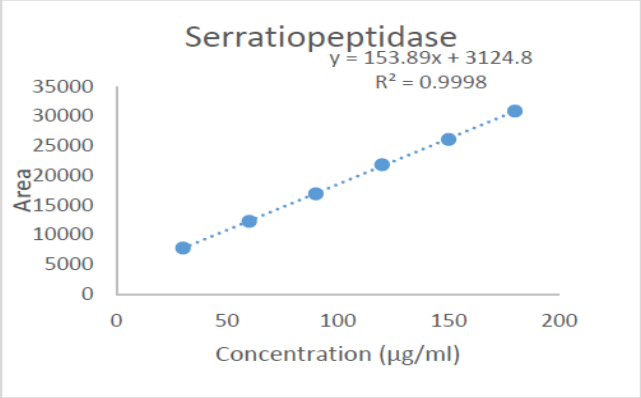
Data of Simultaneous Diclofenac and Serratiopeptidase

Sr. No.	Compound Name	Retention time	Area
1	Diclofenac	4.915	28864306
2.	Serratiopeptidase	2.310	36376

Preparation of standard curve of Serratiopeptidase by RP-HPLC

Calibration curve of Serratiopeptidase by RP-HPLC

Sr. no.	Conc.(µg/ml)	Mean
1	30	7768
2	60	12301
3	90	16911
4	120	21798
5	150	26088
6	180	30836



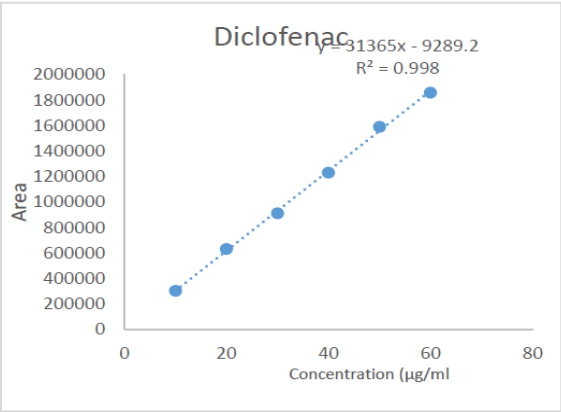
Graph of standard calibration curve of Serratiopeptidaseby RP-HPLC

Result of Statistical parameters for estimation of Serratiopeptidase

Statistical parameters	Results
Regression equation: y=mx+C	Y = 153.89x + 3124.8
Slope (m)	153.89
Intercept (C)	3124.8
Correlation coefficient (r^2)	0.999

Discussion: - The calibration curve for Serratiopeptidase was obtained by using the 30 to 180 µg/ml solution. The calibration curve as shows in graph indicated the regression equation Y = 153.89x + 3124.8 and R^2 value 0.999.

Preparation of standard curve of Diclofenac by RP-HPLC



Sr. no.	Conc.(µg/ml)	Mean
1	10	303249
2	20	632543
3	30	912526
4	40	1231307
5	50	1591900
6	60	1859449

Graph of standard calibration curve of Diclofenac by RP- HPLC

Result of Statistical parameters for estimation of Diclofenac

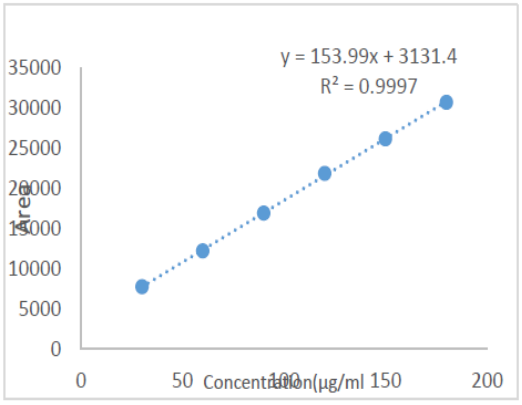
Statistical parameters	Results
Regression equation: y=mx+C	Y = 31365x - 9289.2
Slope (m)	31365
Intercept (C)	9289.2
Correlation coefficient (r^2)	0.998

Discussion: - The calibration curve for Diclofenac was obtained by using the 10 to 60 µg/ml solution. The calibration curve as shows in graph indicated the regression equation Y = 31365x - 9289.2 and R^2 value 0.998 which shows good linearity as shown in Figure 27.

Validation of HPLC method as per ICH guidelines Linearity

Linearity of Serratiopeptidase

A calibration curve was plotted over a concentration range of 30µg/ml to 180µg/ml for Serratiopeptidase. Accurately measured working stock solution of Serratiopeptidase (30µg/ml, 60µg/ml, 90 µg/ml, 120µg/ml, 150µg/ml and 180µg/ml) and all the dilutions were filtered through 0.22 µ filter and injected. The area of all solution was taken at their respective wavelength. The Linearity was constructed by plotting concentration against area where each reading.

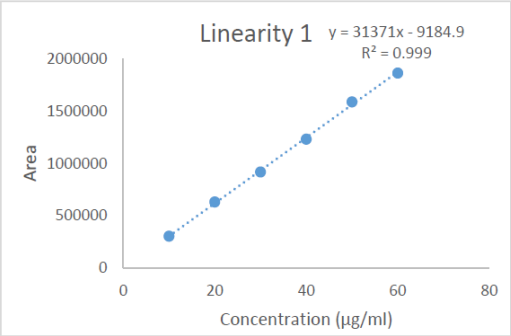


Linearity of Serratiopeptidase.

Serratiopeptidase	
conc.	**Linearity _1**
30	7759
60	12252
90	16965
120	21883
150	26193
180	30748

Linearity of diclofenac

A calibration curve was plotted over a concentration range of 10µg/ml to 60µg/ml for Diclofenac. Accurately measured working stock solution of Diclofenac (10µg/ml, 20µg/ml, 30 µg/ml, 40µg/ml, 50µg/ml and 60µg/ml) and all the dilutions were filtered through 0.22 µ filter and injected. The area of all solution was taken at their respective wavelength. The Linearity was constructed by plotting concentration against area where each reading.



Linearity of diclofenac

Diclofenac	
Conc	**Linearity**
10	303550
20	630955
30	916699
40	1232076
50	1586290
60	1863250

Accuracy

The method's accuracy was assessed using the standard recovery percentage. In order to conduct recovery tests sample at three different concentration levels: 50%, 100%, was determined.

50% Accuracy Study of Diclofenac

Accuracy				
	Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
50%	20	615172	19.91	99.55
	20	613139	19.84	99.22
	20	614178	19.88	99.39
Mean		614163		99.39
SD		1016.58		0.162
%RSD		0.166		0.163

100% Accuracy Study of Diclofenac

Accuracy				
	Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
100%	40	1246404	40.03	100.09
	40	1250899	40.18	100.45
	40	1240541	39.85	99.62
Mean		1245948		100.05
SD		5194.03		0.413
%RSD		0.417		0.414

50% Accuracy Study of Serratiopeptidase

Accuracy				
	Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
50%	60	12307	59.76	99.60
	60	12167	58.85	98.08
	60	12158	58.79	97.98
Mean		12211		98.55
SD		83.55		0.91
%RSD		0.684		0.92

100%Accuracy Study of Serratiopeptidase

Accuracy				
	Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
100%	120	21197	117.81	98.18
	120	21490	119.73	99.77
	120	21207	117.88	98.23
		21298		98.73
SD		166.3520364		0.91
%RSD		0.781		0.92

The results indicate that the recoveries are well within the acceptance range of 97% – 102%, indicating a good degree of sensitivity of the method towards detection of analytes in sample. Therefore, method is accurate, and it can be used for the estimation of drug.

Precision: Standard solution of Diclofenac and Serratiopeptidase was prepared and analyzed

as per the proposed method

Precision study of Diclofenac

Conc.	Repeatability		
	Area	Amount Recovered (µg/ml)	% recovery
40	1251622	40.20	100.50
40	1253584	40.26	100.66
40	1240024	39.83	99.58
40	1248410	40.10	100.25
40	1231578	39.56	98.91
40	1230018	39.51	98.78
Mean	1242539		99.78
SD	9138.73		0.73
%RSD	0.74		0.73

Intra-day precision study of Diclofenac

Conc.	Intra-day		
	Area	Amount Recovered (µg/ml)	% recovery
40	1246380	40.03	100.09
40	1251849	40.21	100.52
40	1240157	39.84	99.59
40	1246129	40.03	100.07
40	1236487	39.72	99.30
40	1230148	39.52	98.79
Mean	1241858		99.72
SD	5975.30		0.48
%RSD	0.48		0.48

Repeatability precision study of serratiopeptidase

Conc.	Repeatability		
	Area	Amount Recovered (µg/ml)	%recovery
120	21411	79.22	99.02
120	21249	78.51	98.14
120	21384	79.10	98.87
120	21348	78.94	98.68
120	21247	78.51	98.13
120	21301	78.74	98.42
Mean	21323		98.55
SD	76.20		0.41
%RSD	0.36		0.42

Conc.	Intra-day		
	Area	Amount Recovered (µg/ml)	% recovery
120	21237	78.46	98.08
120	21123	77.97	97.46
120	21204	78.32	97.90
120	21502	79.61	99.51
120	21234	78.45	98.06
120	21287	78.68	98.35
Mean	21265		98.23
SD	142.89		0.77
%RSD	0.67		0.79

The method was found to be precise due to low values of the %RSD.

LOD and LOQ

LOD and LOQ data

Sr. no.	Drug	LOD (µg/ml)	LOQ (µg/ml)
1	Diclofenac	0.016	0.048
2	Serratiopeptidase	0.151	0.46

The Limit of detection and limit of quantification of the method were calculated basing on standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the limit of detection and limit of quantification. The results obtained were within the limit.

Robustness

The robustness was studied by analyzing the sample of lower concentration with deliberate variation in the method parameters. The change in the responses of drugs was noted in terms of %RSD. Robustness of the method was studied by change in wavelength & change in flow rate.

Robustness data of Diclofenac with deliberate change in flow rate

Robustness_0.9ml				Robustness_1.1ml		
Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
40	1267731	40.71	101.79	1212528	38.95	97.39
40	1250612	40.17	100.42	1211500	38.92	97.31
40	1253154	40.25	100.63	1224878	39.35	98.37
Mean	1257166		100.94	1216302		97.69
SD	9238		0.736	7445		0.593
%RSD	0.735		0.729	0.612		0.607

Robustness data of Diclofenac with deliberate change in wavelength

Robustness_255nm				Robustness_265nm		
Conc	**Area**	**Amount Recovered (µg/ml)**	**% Recovery**	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
40	1264842	40.62	101.56	1224849	39.35	98.37
40	1266451	40.67	101.69	1229294	39.49	98.72
40	1256487	40.36	100.89	1210547	38.89	97.23
Mean	1262593		101.38	1221563		98.11
SD	5349		0.426	9796		0.781
%RSD	0.424		0.421	0.802		0.796

Robustness data of Serratiopeptidase with deliberate change in wavelength

Robustness_0.9ml				Robustness_1.1ml		
Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
120	21085	117.08	97.57	21273	118.31	98.59
120	21075	117.02	97.51	21590	120.38	100.32
120	21142	117.45	97.88	21315	118.58	98.82
Mean	21101		97.65	21393		99.24
SD	36.14		0.20	172.18		0.94
%RSD	0.17		0.20	0.805		0.94

Robustness data of Serratiopeptidase with deliberate change in wavelength

Robustness_255nm				Robustness_265nm		
Conc	**Area**	**Amount Recovered (µg/ml)**	**% recovery**	**Area**	**Amount Recovered (µg/ml)**	**% recovery**
120	21213	117.92	98.26	21456	119.50	99.59
120	21271	118.30	98.58	21220	117.96	98.30
120	21318	118.60	98.84	21115	117.28	97.73
Mean	21267		98.56	21264		98.54
SD	52.6		0.29	174.64		0.950
%RSD	0.247		0.29	0.821		0.964

The Percentage RSD should not be more than 2. The %RSD obtained for change of flow rate was found to be below 2, which was within the acceptance criteria. Hence the method was robust.

CONCLUSION

The objective of the present experimental investigation reported in this thesis is to improve analytical method development and validation of the developed method as per the ICH guidelines. It was concluded that the proposed new RP-HPLC method developed for the quantitative determination of Diclofenac and Serratiopeptidase in bulk was simple, selective, sensitive, accurate, precise and rapid. The method was proved to be superior to most of the reported methods.

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