Research Article **CODEN: IJRPJK** **ISSN: 2319 – 9563**

International Journal of Research in Pharmaceutical and Nano Sciences

Journal homepage: **www.ijrpns.com**

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF DICLOFENAC SODIUM AND SERRATIOPEPTIDASE IN TABLET DOSAGE FORM

Syed Mujtaba Ahmed[*1], L. Priyadarsini Raparla[1], Mohammed Omer[1]

[*1]Department of Pharmaceutical Chemistry, Maheshwara Institute of Pharmacy, Chitkul (v), Patencheru (m), Hyderabad-502 307, Andhra Pradesh, India.

ABSTRACT

A novel, simple, sensitive and rapid Chromatographic (RP-HPLC) method has been developed for simultaneous estimation of NSAIDS (Serratiopeptidase and Diclofenac sodium) from pharmaceutical formulation. The present isocratic method was carried out on analytical column- WATERS XTERRA RP8 (4.6x150, 5 microns) with pH -3 adjusted mobile phase [Ortho Phosphoric acid buffer: methanol 70:30 (v/v)] at the flow rate 1.0 mL/min. The detection was carried out at wave length (λ max) 262 nm. The average retention time of diclofenac sodium was 3.763 min and Serratiopeptidase was 5.480 min. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability. The proposed method can be used for the estimation of these drugs in combined dosage forms.

KEY WORDS

RP-HPLC, NSAIDS, Serratiopeptidase and Diclofenac sodium.

Author for Correspondence:
Syed Mujtaba Ahmed,
Department of Pharmaceutical Chemistry,
Maheshwara Institute of Pharmacy,
Chitkul (V), Patencheru (M), Hyderabad-502 307,
Andhra Pradesh, India.

Email: mujju203@gmail.com.

INTRODUCTION

Diclofenac sodium (DCS), chemically, sodium 2-[(2,6-dichlorophenyl)-amino] Phenyl acetate (1) (Figure No.1), is a non-steroidal anti-inflammatory agent (NSAID) with antipyretic and analgesic actions. Diclofenac sodium is official in I.P., B.P. and U.S.P. Diclofenac Sodium inhibit both leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-), leading to the peripheral inhibition of prostaglandin synthesis. As prostaglandins sensitize Pain receptors, inhibition of

their synthesis is responsible for the analgesic effects of Diclofenac. Antipyretic effects may be due to action on the hypothalamus, resulting in Peripheral dilation, increased cutaneous blood flow, and subsequent heat dissipation[1].

Serratiopeptidase (SER), is a proteolytic (protein destroying) enzyme from bacteria native to the digestive system of silkworms. It is the enzyme responsible for dissolving a silkworm's cocoon. Used as an anti-inflammatory agent (2) (Figure No.2). Serratiopeptidase is official in I.P. 2010. Serratiopeptidase acts upon inflammation by thinning the fluids in the body which is collected around injured areas. This also enhances tissue repair and reduces pain. Pain is also reduced by this enzymes ability to block amines. Serratiopeptidase also has the unique ability to dissolve the dead and damaged tissue which is the byproduct of healing response without harming living tissue[2].

MATERIALS AND METHODS [3, 4]

Apparatus

Waters HPLC system connected with UV- Visible – SPD 10A Vpseries Detector and Empower-2 Software was used. Rheodyne 7725i injection with 20µL loop and analytical column- WATERS XTERRA RP8 4.6x150, 5microns are used. Serratiopeptidase and Diclofenac sodium were generously given by LARA Drugs Pvt Ltd, Hyderabad, and Telengana, India. Acetonitrile (HPLC grade) was procured from E.Merck (India) Ltd, Mumbai. Methanol and orthophosphoric acid (AR grade) were procured from Qualigens fine chemicals, Mumbai. Water (HPLC grade) was obtained from a Milli-QRO water purification system.

Preparation of Reagents and Standards

Ortho Phosphoric acid buffer

6.24g of sodium dihydrogen dehydrate and 0.68 mL of phosphoric acid in 1000mL volume of distilled water and pH is adjusted to 3 with Ortho Phosphoric acid.

Mobile Phase

580 mL of methanol and 420 mL of Ortho Phosphoric acid buffer were added in a beaker to give 1000mL. Finally pH was adjusted to 3.0 by adding Ortho Phosphoric acid.

Mobile Phase Ratio: Phosphoric acid buffer: methanol (70:30 % V/V)

Stock and Working Standard Solutions

Preparation of standard Stoke Solution

An accurately weighed quantity of 150 mg of diclofenac sodium and 30 mg of Serratiopeptidase were transferred in to a 50 mL volumetric flask. Dissolved with 50 mL of mobile phase and diluted to required volume with mobile phase, having the concentration of 0.4mg/ml of diclofenac sodium.

Preparation of Standard Stoke Solution

From the standard Stoke Solution 5 ml is pipetted out in to 100 ml volumetric flask and make up the volume with mobile phase, having the concentration of 0.02 mg/ml of Serratiopeptidase and 0.0025 mg/ml of diclofenac sodium.

Preparation of Standard solution

20 tablets were weighed and grounded to a fine powder. An amount of powder equivalent to 30 mg of Serratiopeptidase and 150mg Diclofenac sodium were weighed accurately and transferred in to 50 ml volumetric flask contains 50ml of mobile phase and sonicator for 30 min and diluted to 50 ml with mobile phase, then the solution was filtered through 0.45µm membrane filter and 5 ml of filtrate taken in to 25 ml volumetric flask and made up the volume with mobile phase. The standard stock solution is diluted to the working concentration equivalent to that of sample. 10µ of the standard and sample are injected separately and collect the generated chromatograms with peak area obtained from both test and standard.

Selection of detection wavelength

The UV spectrum of diluted solutions of various concentrations of Serratiopeptidase and Diclofenac sodium in mobile phase was recorded using UV spectrophotometer. The wavelength of maximum absorbance was observed at 262 nm. This wavelength was used for detection of Serratiopeptidase and Diclofenac sodium.

Chromatographic Conditions

The mobile phase, a mixture of Ortho Phosphoric acid buffer: methanol 70:30 (v/v) pumped at a flow

rate of 1.0 ml/min through the column WATERS XTERRA RP8 (4.6x150, 5 microns) with pH -3. The mobile phase was degassed prior to use under vacuum by filtration through a 0.22 µ membrane filter. Both drugs showed good absorbance at 262 nm, which was selected as wavelength for further analysis.

DEVELOPMENT AND VALIDATION OF HPLC METHOD

Present study was conducted to obtain a new, affordable, cost-effective and convenient method for HPLC determination of Serratiopeptidase and Diclofenac in tablet dosage form. The experiment was carried out according to the official specifications of ICH. The method was validated for the parameters like system suitability, specificity, linearity, precision, accuracy, LOD, LOQ and robustness.

System suitability

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to stabilize for 40 min. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution, tailing factor, theoretical plate count and % RSD for peak area of six replicate injections of LMS standard (% RSD NMT 2.0). The system suitability data is reported in (Table No.1).

Accuracy/Recovery

Accuracy is the degree of agreement between a measured value and accepted reference value. The accuracy of the method was tested by triplicate samples at 3 different concentrations equivalent to 50%, 100% and 150% of the active ingredient, by adding a known amount of standard to a sample with pre-determined amount of drug. The recovery results for accuracy study of LMS are presented in (Table No.2).

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 and sample solutions 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 and sample solutions were made for three 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 precision data were presented in (Table No.3).

Linearity and Range

The linearity of the method was determined at five concentration levels ranging from 300-900 µg/mL for Diclofenac and 60 to 180 µg/mL for Serratiopeptidase respectively the linearity was evaluated by linear regression analysis, using least squares method. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was y = 19288X (r^2 =0.9999) for diclofenac and y=16616X (r^2 =0.998) for Serratiopeptidase. The results shows that an excellent correlation exists between response factor and concentration of drugs within the concentration range indicated above. The calibration curves are shown in (Table No.4 and 5, Figure No.3 and 4).

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOD for diclofenac sodium and Serratiopeptidase were found to be 2.810 µg/mL and 2.2767 µg/mL, respectively.

LOD=3.3SD/Slope

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ was 9.367 µg/mL and 7.5889 µg/mL for diclofenac sodium and Serratiopeptidase, respectively in (Table No.6).

LOQ=10SD/slope

Ruggedness

The ruggedness of the method was determined by carrying out the experiment on different instruments like Shimadzu HPLC (LC-2010AHT), Agilent HPLC and Water's Breeze HPLC by different operators using different columns of similar type like Hypersil C18, Phenomenex Gemini C18 and Hichrom C18. Data is represented in (Table No.7).

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed, are rugged and robust. Data is represented in (Table No.8 and Figure No.5).

System suitability studies

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions (Table No.9). The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within ± 3 % standard deviation range during routine performance of the method.

ASSAY RESULTS

Procedure

Separately Blank, Standard and test preparation was injected in to liquid chromatogram and the areas for major peaks were recorded by using the following formula.

Calculation

Calculated the amount of Diclofenac sodium and Serratiopeptidase using the formula.

Sample area x sample dilution x purity of working standard x average weight/standard area x standard dilution x label claim.

RESULTS

The mean recovery for assay results was found to be 98 - 101% for Diclofenac sodium and Serratiopeptidase (Table No.10).

Table No.1: Optimized chromatographic conditions and system suitability parameters of proposed RP-HPLC method for Diclofenac sodium and Serratiopeptidase

S.No	Parameter	Chromatographic conditions	
1	Instrument	Waters HPLC	
2	Column	WATERS XTERRA RP8 4.6x150, 5microns	
3	Detector	UV- Visible –SPD 10A Vp series Detector	
4	Mobile phase	Phosphoric acid buffer: methanol (70:30 % V/V)	
5	Detection of wavelength	262nm	
6	Run time	12 min	
7	Volume of injection loop	10 µL	
8	Resolution	7.726	
9	Retention	**Diclofenac sodium**	**Serratiopeptidase**
		3.763	5.480
10	Number of theoretical plates	6335	7885
11	Tailing factor	1.312	1.237

Table No.2: Accuracy/Recovery studies for Diclofenac Sodium and Serratiopeptidase

DICLOFENAC SODIUM						
Spiked Level	**Sample weight**	**Sample area**	**µg/ml Added**	**µg/ml Added**	**% Recovery**	**% Mean**
50%	221.0.25	976970	296.998	297.52	100	100
50%	221.0.25	976433	296.998	297.37	100	
50%	221.0.25	976535	296.998	297.41	100	
100%	442.05	1956682	593.995	595.98	100	100
100%	442.05	1954937	593.995	595.45	100	
100%	442.05	1953955	593.995	595.12	100	
150%	663.06	2922364	890.966	890.12	100	100
150%	663.06	2921595	890.966	890.03	100	
150%	663.06	2929687	890.966	890.23	100	
SERRATIOPEPTIDASE						
Spiked Level	**Sample weight**	**Sample area**	**µg/ml Added**	**µg/ml Added**	**% Recovery**	**% Mean**
50%	221.0.25	976970	59.759	59.58	100	100
50%	221.0.25	976433	59.759	59.60	100	
50%	221.0.25	976535	59.759	59.47	100	
100%	442.05	1956682	119.518	119.32	100	100
100%	442.05	1954937	119.518	119.44	100	
100%	442.05	1953955	119.518	119.27	100	
150%	663.06	2922364	179.272	179.27	100	100
150%	663.06	2921595	179.272	179.16	100	
150%	663.06	2929687	179.272	179.23	100	

Table No.3: System Precision data for Diclofenac Sodium and Serratiopeptidase

S.No	Sample Weight	Sample Area-1	Sample Area-2	% Assay	% Assay
1	442.05	1967696	2603154	100	100
2	442.05	1943627	2595260	99	100
3	442.05	1965413	2613481	100	100
4	442.05	1948233	2598977	99	100
5	442.05	1949113	2592115	99	100
6	442.05	1955836	2598856	99	100
Avg Assay	---	---	---	99	100
STD	---	---	---	0.50	0.29
% RSD	---	---	---	0.50	0.29

Table No.4: Linearity and Range data for Diclofenac Sodium and Serratiopeptidase

S.No	Concentration of Diclofenac sodium (µg/ml)	Peak area of Diclofenac sodium (Mv)	Concentration of Serratipepdase (µg/ml)	Peak area of Serratipepdase (Mv)
1	50	96075	50	1297239
2	75	146461	75	1940458
3	100	1956723	100	2592808
4	125	2448541	125	3244625
5	150	2920200	150	3891966

Table No.5: Analytical Performance Parameters for Diclofenac Sodium and Serratiopeptidase

S.No	Parameters	Diclofenac Sodium	Serratiopeptidase
1	Linearity Range	300-900µg/ml	80-180 µg/ml
2	Correlation Coefficient	0.999	0.999
3	Slope (m)	65.93	46.22
4	Intercept	10.34	82.08

Table No.6: Limit of Detection and Limit of quantification for Diclofenac Sodium and Serratiopeptidase

S.No	Sample	LOD	LOQ
1	Diclofenac Sodium	2.810	90367
2	Serratiopeptidase	2.2767	7.5889

Table No.7: Ruggedness for Diclofenac Sodium and Serratiopeptidase

S.No	Analysts	Diclofenac Sodium	Serratiopeptidase
1	Analyst 1	849.651	4828.953
2	Analyst 2	828.034	4778.204

Table No.8: Robustness for Diclofenac Sodium and Serratiopeptidase

S.No	Effect	Retention time of Diclofenac Sodium	Retention time of Serratiopeptidase
1	Flow 1	3.151	4.587
2	Flow 2	3.151	4.587
3	Temp (23^0c)	3.760	5.503
4	Temp (30^0c)	3.752	5.366

Table No.9: System suitability studies for Diclofenac Sodium and Serratiopeptidase

S.No	Time	Area of Diclofenac Sodium	Area of Serratiopeptidase
1	Initial	845.034	4826.725
2	After 8 hours	846.390	4829.735
3	Deviation	1.356	3.010

Table No.10: Assay results for Replicate Injection

S.No	Inj.No	Area of DICLO	Area of SERRA	% of DICLO Recovered	% of SERRA Recovered
1	1	808.439	4715.886	98.55	98.11
2	2	820.048	4793.930	99.96	99.73
3	3	825.462	4771.852	100.62	99.22
4	Mean	817.983	4760.556	99.71	99.02
5	S.D	8.697344	40.22954	1.05740	0.82831
6	% R.S.D	1.06326	0.84506	1.06048	0.83651

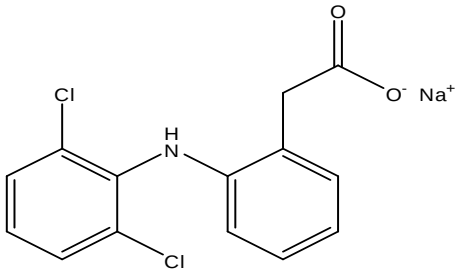

Figure No.1: Structure of Diclofenac Sodium

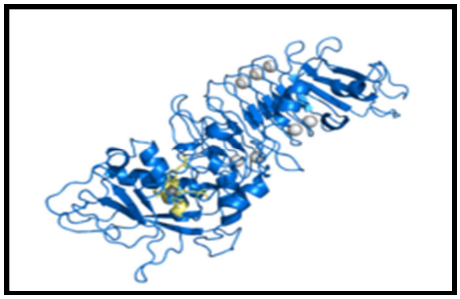

Figure No.2: Structure of Serratiopeptidase

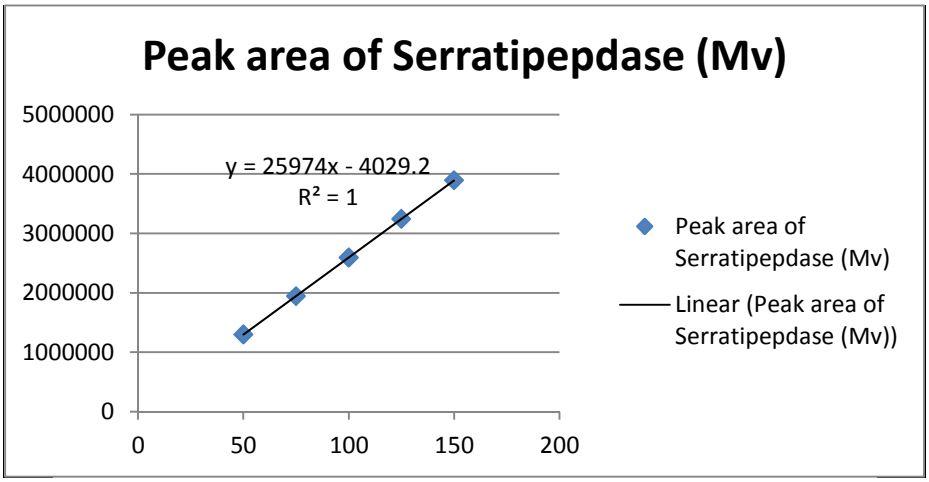

Figure No.3: Linearity and Range data for Diclofenac Sodium

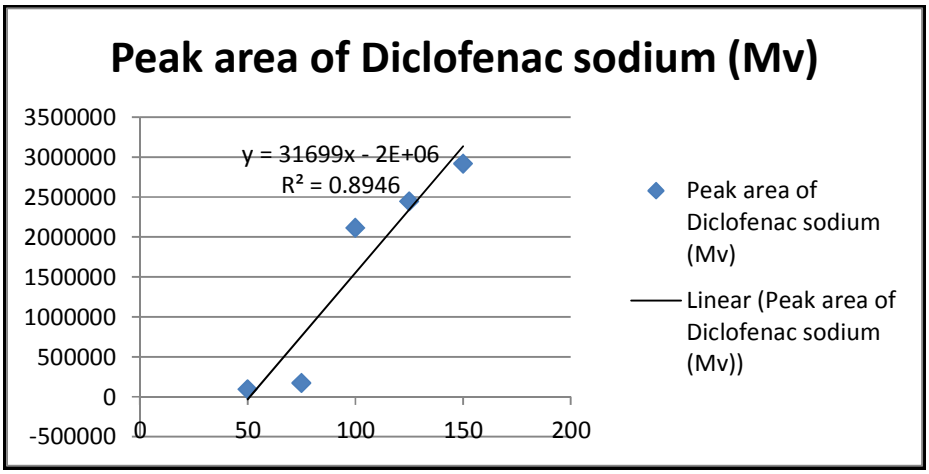

Figure No.4: Linearity and Range data for Serratiopeptidase

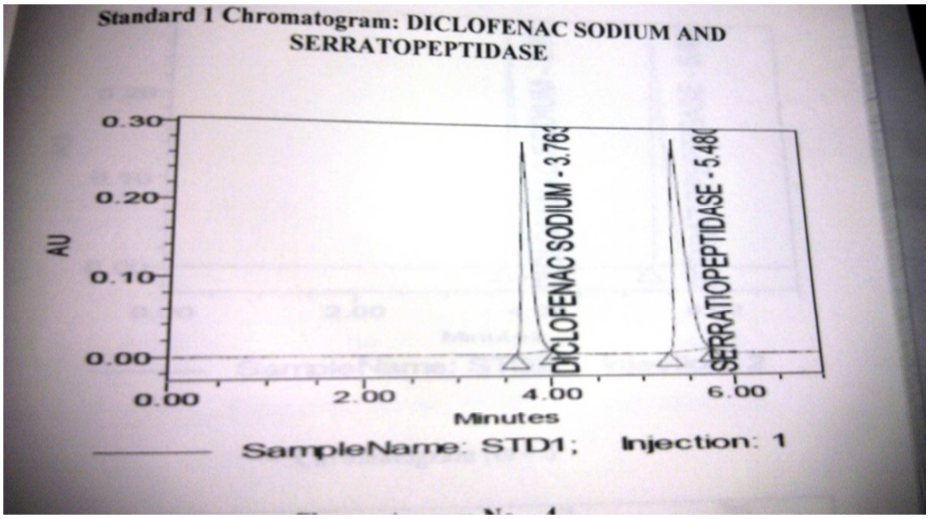

Figure No.5: HPLC Chromatogram for Diclofenac Sodium and Serratiopeptidase

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Diclofenac Sodium and Serratiopeptidase in Tablet Dosage Form was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories, biopharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

ACKNOWLEDGEMENT

The authors wish to express their sincere gratitude to Maheshwara Institute of Pharmacy, Patancheru, Hyderabad, for providing necessary facilities to carry out this research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Syed Mujtaba Ahmed *et al.* RP-HPLC Method Development and Validation for Simultaneous Estimation of Diclofenac Sodium and Serratiopeptidase in Tablet Dosage Form, *International Journal of Research in Pharmaceutical and Nano Sciences*, 4(1), 2015, 10 - 18.