

## Development and Validation of a New Chromatographic Method for the Simultaneous Estimation of Serratiopeptidase, Aceclofenac and Paracetamol by RP-HPLC

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### Abstract

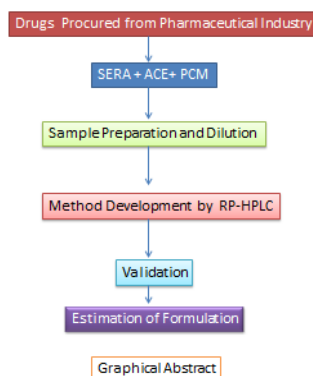
**Background:** Method development, validation is an important parameter for the simultaneous estimation of Serratiopeptidase (SERA), Aceclofenac (ACE) and Paracetamol (PCM) by RP-HPLC, is supposed to be a costly and tedious process. The present study revealed using cheap and cost effective solvent system for the simultaneous estimation of Serratiopeptidase (SERA), Aceclofenac (ACE) and Paracetamol (PCM).

**Objective:** Development and validation of a new chromatographic method for the simultaneous estimation of Serratiopeptidase (SERA), Aceclofenac (ACE) and Paracetamol (PCM) by RP-HPLC of marketed formulations.

**Methods:** Simultaneous determination of SERA, ACE and PCM were carried out by RP-HPLC at the wavelength 327 nm, flow rate 0.4 mL/min, and the mobile phase used was water: methanol in the ratio (50:50 v/v). Further validation parameters such as system suitability, linearity, accuracy, precision, specificity, LOD, LOQ and robustness were taken into account to carry out the validation of the method.

**Results:** Absorbance maxima for the simultaneous determination were selected by the UV spectrophotometer and that was found to be 327 nm in methanol and water. During the process of RP-HPLC, the linearity was obtained in the concentration range of 2-10 µg/mL for SERA, 100-500 µg/mL for ACE and 20-100 µg/mL for PCM. Correlation coefficient (r) for SERA, ACE and PCM in methanol and water was found to be 0.9817, 0.991 and 0.9949 respectively.

**Conclusion:** The RP-HPLC method was simple, accurate, precise, and rapid and can be used for the simultaneous determination of SERA, ACE and PCM in bulk and pharmaceutical dosage form. The method is also economical as RP-HPLC grade water methanol in a ratio of (50:50) was used to achieve all the validation parameters.



**Keywords:** Aceclofenac; Correlation coefficient; Paracetamol; Pharmaceutical dosage; RP-HPLC; Simultaneous estimation; Serratiopeptidase; Validation

### Introduction

#### Paracetamol

Paracetamol (acetaminophen) is one of the most popular over-the-counter analgesic and antipyretic drugs. Paracetamol is available in different dosage forms: tablet, capsules, drops, elixirs, suspensions and suppositories. Paracetamol and its combined dosage form with the other drugs have been mentioned in many pharmacopeias [1,2].

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Paracetamol (PCM) (4-hydroxyacetanilide) is a non-steroidal anti-inflammatory drug and inhibits isoforms of cyclooxygenase, COX-1, COX-2 and COX-3 enzymes involved in prostaglandin (PG) synthesis. It is centrally and peripherally acting non-steroidal anti-inflammatory drug (Figure 1).

### Serratiopeptidase

Serratiopeptidase (SERA) is Proteolytic enzyme, secreted from enterobacterium *Serratia* sp. E-15. Serratiopeptidase is found in the intestine of silkworm and allow the emerging moth to dissolve its cocoon. The culture of *Serratia* E-15 bacteria produce Serratiopeptidase enzyme by the process of purification [3]. Serratiopeptidase hydrolyses histamine, bradykinin and serotonin, responsible for the oedematous status, thus producing anti-inflammatory effect [4,5]. Serratiopeptidase reduces swelling, improves microcirculation and expectoration of sputum, etc.

### Aceclofenac

Aceclofenac (ACE), 2-[(2, 6-dichlorophenyl) amino] phenyl] acetyl] oxyacetic acid is used as anti-inflammatory drug. It is official in B.P. [6] and I.P. [7]. Aceclofenac is a non-steroidal anti-inflammatory drug (NSAID). Aceclofenac has higher anti-inflammatory action than conventional NSAIDs. It is a cytokine inhibitor. Aceclofenac works by blocking the action of a substance in the body called cyclooxygenase. Cyclooxygenase is involved in the production of prostaglandin (chemical in the body) which causes pain, swelling and inflammation [2]. A tablet dosage from containing all the three, (ACE 50 mg, SERA 5 mg and PCM 250 mg), is commercially available in the market and used as anti-inflammatory, analgesic and antipyretic agents. ACE, SERA and PCM are officially available in IP. Literature reviews revealed that UV Spectroscopy method have been reported for the determination of ACE, SERA and PCM individually in pharmaceutical dosage form [8]. Many UV [9,10] and HPLC [11-14] based methods have been reported for determination of these drugs alone as well as in combination with other drugs in pharmaceutical dosage form. Adidala et. al. has developed a simultaneous method for determination of these three drugs in relatively more expensive solvents such as methanol, acetonitrile and formic acids using RP- HPLC. Therefore, the present work was aimed to develop and validate an economical RP- HPLC method using simple methanol and water (50: 50) as mobile phase in isocratic flow for simultaneous determination of ACE, SERA and PCM in pharmaceutical dosage forms (Figure 2).

## Materials and Methods

### Chemical and reagents

ACE, SERA and PCM were procured from pharmaceutical industry Baddi, solan, HP, India. Commercial tablets of above combination used for analysis were procured from local pharmacy. HPLC grade methanol and water were procured from SD Fine-Chem Ltd, Mumbai.

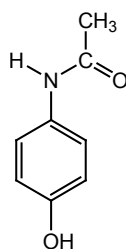


Figure 1: Structure of Paracetamol.

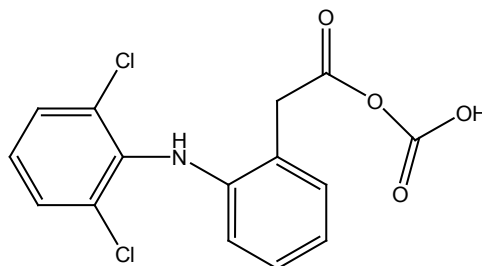


Figure 2: Structure of Aceclofenac.

### Instruments

Agilent RP-HPLC 1200 series was used for the analysis. The system consists of binary pump, auto sampler and DAD detector manufactured by Germany manufacturer. The method was carried out on C18 Column (4.6 × 150 mm × 5 μm) as a stationary phase. Software EZChrome Elite was used throughout the experiment.

### Preparation of mobile phase

The mobile phase comprised of water and methanol in the ratio (50:50). The container of solvent reservoir was rinsed first from the acid, base and from the solvent to be used. Vials were rinsed from the distilled water, HPLC grade water and methanol. The mobile phase was filtered through 0.42 μm nylon filter paper along with the ultrasonication for 20 minutes.

### Preparation of standard stock solution

Standard Stock solutions of SERA, ACE and PCM were prepared separately by dissolving 20 mg of drug in 20 mL of methanol and volume was made up to 40 mL to get the concentration of 500 μg/mL of solution of each drug. Preparation of standard stock solution of SERA, ACE and PCM were made by accurately mixing 1 mL of serratiopeptidase, 10 mL of aceclofenac and 50 mL of paracetamol from the stock solutions to get the concentration as described in the marketed formulations. Hence, dilutions were made accordingly to prepare a calibration graph.

### Preparation of sample solution

The above prepared standard solution was used to prepare the further aliquots of SERA (2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL, 10 μg/mL), ACE (20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, 100 μg/mL) and PCM (100 μg/mL, 200 μg/mL, 300 μg/mL, 400 μg/mL, 500 μg/mL). Filter out the samples from 0.22 μm nylon syringe filter for the further process.

### Selection of analytical wavelength

Selection of wavelength was done on the basis of scanning a fixed concentration in the range of 200-800 nm for SERA, ACE and PCM. Wavelength of 327 nm was recorded using UV visible spectrophotometer. At the 327 nm SERA showed maximum absorbance while, ACE and PCM showed optimum absorbance, thus the elution exhibited accurate response at 327 nm using UV detector [15].

### Optimized RP-HPLC chromatographic parameters

For optimization of the chromatographic conditions, parameters of HPLC such as flow rate, mobile phase ratio, injection volume, temperature, and wavelength were nearly fixed in a set number of experiments [16]. By making changes in all the above parameters,

the best one was selected at which elution response was significant, accurate and recorded a sharp symmetric peak as shown in Table 2.

### Formulation analysis

Accurately 230 mg of the powdered formulation was weighed dissolved in 10 mL of the solvent and subsequently ultrasonicated. From this a, mid conc. of 20 µg/mL was prepared and filtered out by 0.22 µm siring filter. The injection volume (10 µL) was taken and three peaks were distinctly observed at the wavelength 327 nm with flow rate 0.4 mL/minute [17,18].

### Validation

Validation was performed as per International Council for Harmonization (ICH) guidelines. The following parameters should be considered as major and significant criteria for the method development and validation.

**Linearity:** Linearity was determined for paracetamol, aceclofenac and serratiopeptidase separately by plotting a calibration curve (Between peak area and their respective concentration). Evaluation of drug was performed with UV detector at 327 nm. The peak areas were recorded for all the peaks. Hence, standard calibration curves were plotted between peak area and concentrations [19,20].

**System suitability:** System suitability should be based on the criteria and parameters collected as a group that will be able to define the performance of the system.

**Precision:** Performed system precision by multiple injections of a homogeneous standard solution to indicate the performance of the HPLC instrument under the chromatographic condition and day tested [21]. The relative standard deviation shall be not more than 2.0 %. (ICH. Q2B [22]).

**LOD and LOQ:** Using average standard deviation of precision and slope of a straight-line coefficient, the values of LOD and LOQ were determined.

Parameters	Optimized conditions
Pump	Binary Pump system
Detector	Photo Diode array(DAD)
Sampler	Auto Sampler
Column	C <sub>18</sub> column (4.6mm × 250mm, 5µm)
Mobile phase	Water : Methanol (50:50)
Stationary Phase	C18 column
Flow rate	0.4 mL/min
Run time	14 min
Vol. of injection	10µL
Detection Wavelength	327 nm

**Table 1:** Optimized HPLC parameter for method.

SERA			ACE			PCM		
R <sub>t</sub> (min)	Conc. (µg/ml)	Area	R <sub>t</sub> (min)	Conc. (µg/ml)	Area (mAU)	R <sub>t</sub> (min)	Conc. (µg/ml)	Area (mAU)
6.000	2	938	9.507	100	24544	7.807	20	52205
5.997	4	1200	9.547	200	33977	7.833	40	83955
5.947	6	1563	9.553	300	40046	7.847	60	110216
5.947	8	1812	9.573	400	46406	7.853	80	134120
6.347	10	1960	9.58	500	53436	7.867	100	156397
Slope		132.8			341.07			258.55
Intercept		697.8			19018			19019
r <sup>2</sup>		0.9817			0.991			0.9949

**Table 2:** Calibration curve reading.

**Robustness:** The robustness study was done by making small changes in the optimized method & parameters.

**Accuracy (Recovery study):** % Recovery studies were carried out at three different levels of 50%, 100%, and 150% of standard solution in triplicate. Serratiopeptidase, Aceclofenac and Paracetamol were taken as samples [23].

### Results

#### Optimized RP-HPLC chromatographic parameters

The chromatographic parameters had optimized for RP-HPLC and were shown in Table 1 and Figure 3.

#### Linearity

To determine the linearity or calibration curve of PCM, ACE and SERA the samples solution of various concentration ranges were prepared as shown in Table 2. 10 µL of each concentration was injected into the RP-HPLC system separately (Figure 4).

#### Regression equation and correlation coefficient

It (y and R<sup>2</sup>) were determined and illustrated in Figure 5A, 5B and 5C by RP-HPLC of SERA, ACE and PCM.

#### System suitability

The System suitability should be based on the criteria and parameters collected as a group that was able to define the performance of the system Table 3.

#### Limit of detection (LOD) and Limit of quantification (LOQ)

A system precision was conducted by disposing multiple injections of a homogeneous standard solution which indicates the performance of the HPLC instrument under the chromatographic condition Tables 4 and 5.

#### Precision

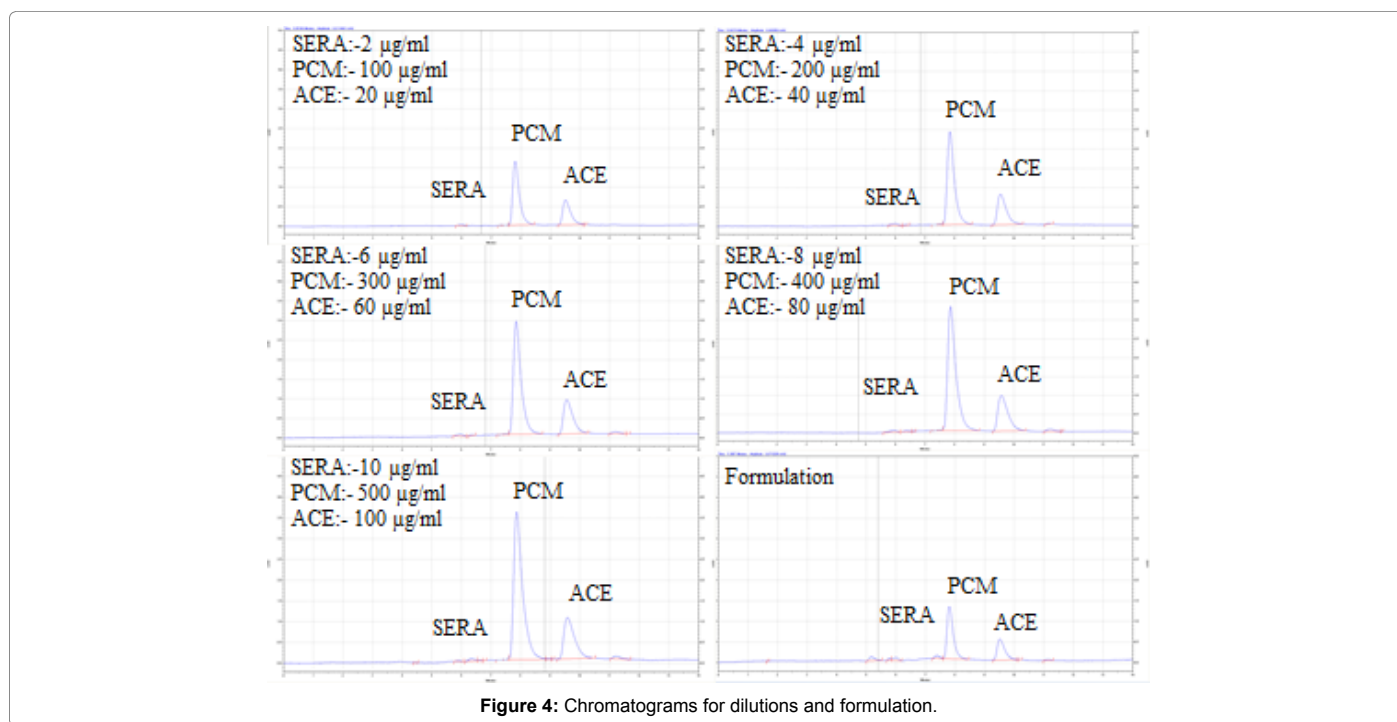
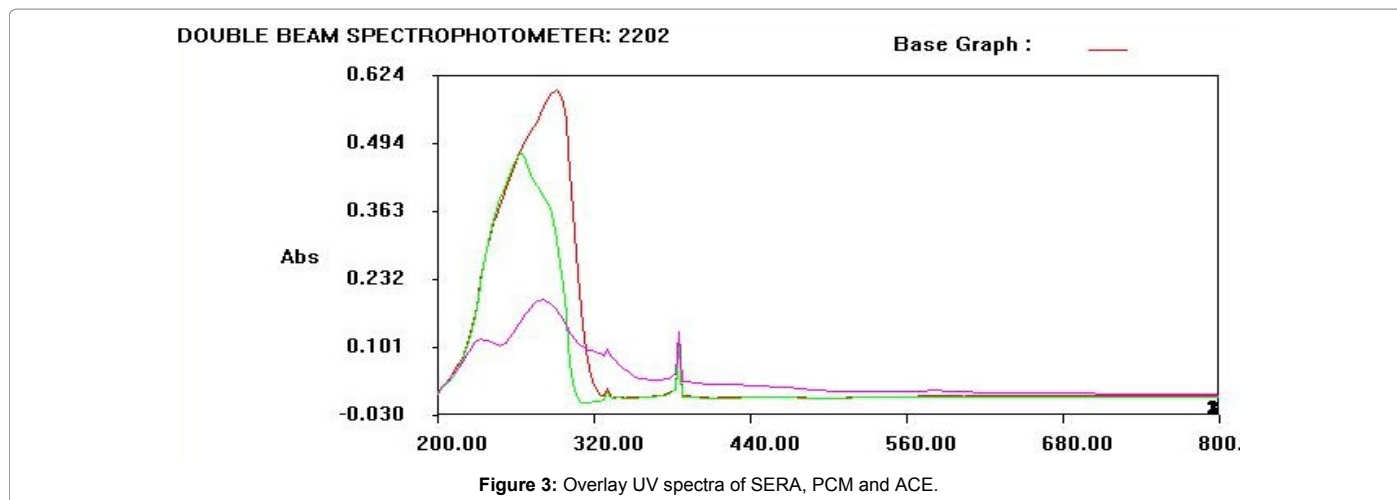
Precision of the performance of the RP-HPLC was done under similar chromatographic condition for day 1 and day 2 the results were shown in Table 6.

#### Robustness

The robustness study was done by making small changes in the optimized method & parameters and no significant affect was recorded, even though, 5 mL change was brought about in mobile phase ratio. There was no significant impact observed on the retention time.

#### Accuracy (% Recovery study)

% Recovery studies were carried out at three different levels of 50%,



SERA		ACE		PCM	
Injection No.	Peak area	Injection No.	Peak area	Injection No.	Peak area
1	1563	1	40046	1	110216
2	1582	2	39532	2	110032
3	1528	3	39922	3	110276
4	1539	4	40115	4	110283
5	1574	5	40150	5	110380
6	1532	6	40128	6	110254
Average	1553	Average	39982.2	Average	110240
Std. Dev.	22.9957	Std. Dev.	235.553	Std. Dev.	115.569
% RSD	1.41661	% RSD	0.58914	%RSD	0.10484

Table 3: Reading of system suitability.

Parameters	SERA	PCM	ACE
Beer's Law Limit (µg/mL)	2-10	100-500	20-100
LOD (µg/mL)	0.5714	1.475	2.279
LOQ (µg/mL)	1.7316	4.4698	6.906
Regression equation	$y = 132.8x + 697.8$	$y = 258.55x + 29814$	$y = 341.07x + 19018$
Correlation coefficient (R <sup>2</sup> )	0.9817	0.9949	0.991
Accuracy (% Mean Recovery)	99.403	102.403	103.32
Precision(%RSD)	Day 1	0.10484	0.58914
	Day 2	1.4824	0.09832

Table 4: Optical characteristics of the RP-HPLC method.

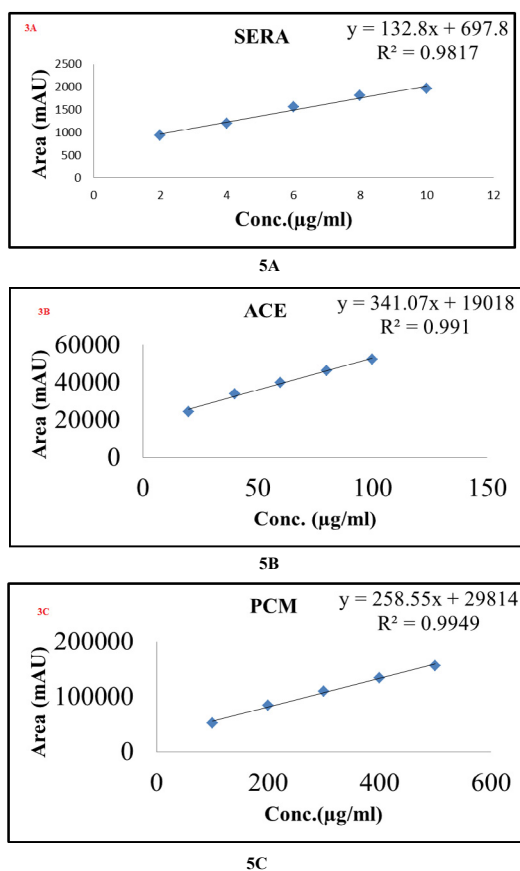


Figure 5: Calibration curve of SERA (5A), ACE (5B) and PCM (5C) Determined by RP-HPLC.

S. No.	SERA	PCM	ACE
1	1563	110216	40046
2	1582	110032	39532
3	1528	110276	39922
4	1539	110283	40115
5	1574	110380	40150
6	1532	110254	40128
AVG	1553	110240	39982.2
Std. Dev.	22.9957	115.569	235.553
%RSD	1.41661	0.10484	0.58914
Slope	132.8	258.55	341.07
LOD(µg/mL)	0.5714	1.475	2.279
LOQ(µg/mL)	1.7316	4.4698	6.906

Table 5: LOD and LOQ Limits.

100%, and 150% of standard solution in triplicate in each level. The results are reported in terms of % recovery, RSD as shown in Table 7.

## Discussion

### Optimized RP-HPLC chromatographic parameters

To develop a cheap, precise, accurate and suitable RP-HPLC method for the simultaneous determination and estimation of PCM, ACE and SERA in marketed formulation tablet dosage form. The different mobile phases e.g. methanol and water in different proportions were used and finally methanol: water (50:50 v/v) selected

SAMPLE	Day 1			Day 2		
	SERA	PCM	ACE	SERA	PCM	ACE
1	1563	110216	40046	1645	110246	41146
2	1582	110032	39532	1628	110382	41232
3	1528	110276	39922	1683	110528	41182
4	1539	110283	40115	1639	110305	41126
5	1574	110380	40150	1684	110483	41243
6	1532	110254	40128	1637	110327	41328
AVG	1553	110240.2	39982.17	1652.667	110378.5	41209.5
Std	22.99565	115.5689	235.5525	24.50034	108.5297	74.06956
%RSD	1.416613	0.104835	0.58914	1.4824	0.09832	0.17973

Table 6: Reading of precision parameter.

as an appropriate proportion, which give significant retention time, acceptable peak parameters and suitable absorbance maxima (Figure 5) for PCM, ACE and SERA.

### Calibration curve

Linearity or calibration curve of PCM, ACE and SERA: the sample solutions of various concentration ranges were prepared and the correlation coefficient ( $R^2$ ) of PCM, ACE and SERA were determined as 0.9949, 0.991 and 0.9817 respectively, shown in Table 2 which were much closed to required parameter for validation.

### System suitability

The system suitability should be based on criteria and parameters

Drug	Conc. (µg/mL)	Standard added(µg/mL)	Area Before Spiking	Area after Spiking	% Recovery
SERA	6	4	1563	1341	96.68
	6	6	1582	1539	105.56
	6	8	1528	1590	95.97
	Mean				99.403
	Std				5.343
	%RSD				5.38
ACE	60	40	40046	36864	104.65
	60	60	40115	40128	103.15
	60	80	40150	43406	102.15
	Mean				103.32
	Std				1.26
	%RSD				1.22
PCM	300	200	110216	94635	100.28
	300	300	110276	110380	103.89
	300	400	110254	123058	103.04
	Mean				102.403
	Std				1.89
	%RSD				1.845

Table 7: Recovery studies.

collected as a group that will be able to define the performance of the system. The average peak areas of six injections were determined for SERA (1553), ACE (39982.2) and PCM (110240). The standard deviations were found for SERA (22.9957), ACE (235.553) and PCM (115.569). The % Regression standard deviations (%RSD) were determined for SERA (1.41661), ACE (0.58914) and PCM (0.10484). All the parameters significantly describe the excellent system suitability (Table 3).

### Limit of detection (LOD) and Limit of quantification (LOQ)

A system precision was conducted by disposing multiple injections of a homogeneous standard solution which indicates the performance of the HPLC instrument under the chromatographic condition Table 4. The relative standard deviation shall not be more than 2.0 %. (ICH. Q2B). Limit of detection (LOD) for PCM, ACE and SERA were found to be 1.475 µg/mL, 2.279 µg/mL and 0.5714 µg/mL respectively and Limit of Quantification (LOQ) for PCM, ACE and SERA also were found to be 4.4698 µg/ml, 6.906 µg/mL and 1.7316 µg/mL respectively as shown in Table 5. It has been concluded from the data, the lowest value of LOD and LOQ could be used for the determination of same drugs.

### Precision

A system precision was performed by subjecting injections of a homologous standard solution to indicate the performance of the HPLC instrument under the chromatographic condition and day tested for day 1 and day 2 as shown in Table 6 and %RSD were found less than 2.

### Robustness

The robustness study was done by making small changes in the optimized method and parameters e.g. 5 mL change was brought about in mobile phase ratio. There was no significant impact observed on the retention time and tailing factor so we can use this method for further study as a robust method.

### Accuracy (% Recovery study)

% Recovery studies were carried out at three different levels of 50%,

100%, and 150% of standard solution in triplicate. Serratiopeptidase, Aceclofenac and Paracetamol present in the sample solution were determined by adjusting the responses into the regression equation for Serratiopeptidase, Aceclofenac and Paracetamol at specific concentration levels. All The results were reported in terms of % recovery, RSD which found within the critical parameter.

### Conclusion

The present study describes proposed RP-HPLC method for the simultaneous estimation of PCM, ACE and SERA in tablet dosage form are cheap, accurate, precise, linear, robust, simple and rapid. Acceptable regression values, RSD % and standard deviations are achieved which make it versatile and valuable for simultaneous determination of three drugs in tablet formulation. According to ICH guidelines of validation precision and accuracy have been within acceptable range. So, a large number of samples can be analyzed in short period of time. The results of this validated RP-HPLC method could be conveniently adopted for quality control analysis of PCM, ACE and SERA, simultaneously from tablet dosage forms.

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