

Analytical Method Development and Validation of Simultaneous Estimation of Paracetamol, Aceclofenac and Serratiopeptidase by RP-HPLC

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Abstract: In this study, high performance chromatographic method have been developed and validated for the estimation of Paracetamol (PC), Aceclofenac (AF), and Serratio peptidase (SP) in combined tablet dosage form. The chromatography was carried out on a phenomenex C18 (Luna) coloumn (250mmX 4.6mm, 5 μ m) with a mobile phase consisting of buffer containing 1gm heptanesulfonic acid in 1lit water (adjusted to pH 2.5 with ortho phosphoric acid) acetonitrile (90:10 v/v) at a flow rate of 1.0 mL/min and ultraviolet detection at 226 nm. The retention time of PC, AF and SP were 3.119, 7.196 and 13.560 minutes respectively. Validation of the proposed method was carried out according to International Conference on Harmonisation (ICH) guidelines. Linearity range was obtained for PC, AF and SP over the concentration range of 2.1-31.5, 0.66-9.90 and 0.1-1.5 μ g/mL and the r² values were 0.9995, 0.9998 and 0.9992 respectively. The calculate limit of detection (LOD) values were 0.21, 0.066, and 0.01 μ g/mL and limit of quantitation (LOQ) values were 0.42, 0.132 and 0.02 μ g/mL for PC, AF, and SP correspondingly.

Keywords: Paracetamol (PC), Aceclofenac (AF), Serratio Peptidase (SP), RP-HPLC

1. Introduction

Paracetamol is chemically N-(4-hydroxy phenyl) acetamide (Figure-1) with molecular formula C₈H₉NO₂. It is a centrally and peripherally acting non-opioid analgesic and antipyretic. Many methods have been described in the literature for the estimation of paracetamol with other drugs individually and in combination. [1-11] Paracetamol is available in different dosage forms: tablet, capsules, drops, elixirs, suspensions and suppositories. Dosage forms of paracetamol and its combinations with other drugs have been listed in various pharmacopoeias. [12, 13]

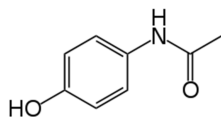


Figure 1. Chemical Structure of Paracetamol.

Aceclofenac (Figure-2) is a non-steroidal anti-inflammatory drug [14] (NSAID) analog of Diclofenac. It is a cytokine inhibitor. Aceclofenac is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis with the recommended dose of 100 mg twice daily. [15,16] The drug works by inhibiting the action of cyclooxygenase (COX) that is involved in the production of prostaglandins (PG) which is accountable for pain, swelling, inflammation and fever. [15] [17-19]

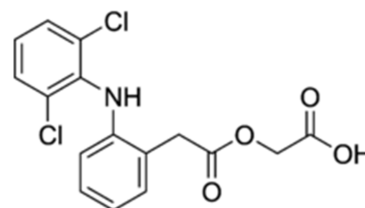


Figure 2. Chemical Structure of Aceclofenac.

Serratiopeptidase (Figure-3) (Serratia E-15 protease, also known as serralyisin, Serratiopeptidase, Serratia peptidase, serration peptidase or serrapeptidase) is a proteolytic enzyme (protease) produced by enterobacterium *Serratia* sp. E-15. Serratiopeptidase is present in the silkworm intestine and allow the emerging moth to dissolve its cocoon. Serratiopeptidase is produced by purification from culture of *Serratia* E – 15 bacteria. [20-22]

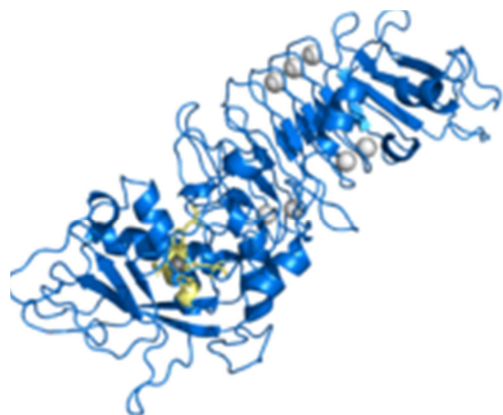


Figure 3. Chemical Structure of Serratiopeptidase.

In Most of researchers are reported different HPLC methods they are use many different substances have been used for buffering in HPLC mobile phase, but no reports on the combined dosage form of Paracetamol, Aceclofenac and Serratiopeptidase. In this paper, we reported the development and validation of accurate HPLC method for analysis bulk and formulations of Paracetamol, Aceclofenac and Serratiopeptidase.

2. Material and Methods

2.1. Chemicals

Acetonitrile (HPLC grade), Heptane-1-sulphonic acid (AR grade), Ortho phosphoric acid (HPLC grade) were purchased from Merck (India) Ltd. Worli, Mumbai, India. All active pharmaceutical ingredients (APIs) PC, AF and SP as reference standards were obtained from ZydusCadila, Ahmedabad, India (99.7-99.9% purity).

2.2. Equipment

Waters alliance HPLC quaternary system with PDA detector (2996) was used for method development and validation.

2.3. Method Development and Optimization of Chromatographic Conditions for Separation

The chromatographic condition was optimized by using different columns, mobile phase composition, pH (2.0, 2.5, and 3.0), wavelength (221, 226, and 231), flowrate (0.9, 1.0, and 1.1), column temperature (ambient to 45°C) and injection volume (10, 20, 30, and 50µL).

2.4. Sample Preparation

2.4.1. Chemical Form of the APIs

PC is a white powder. AF white to off-white crystalline powder. SP yellow colour pallets.

2.4.2. Preparation of PC, AF and SP Stock Solutions

Stock solution was prepared by weighing 210mg of Paracetamol, 66mg of Aceclofenac and 10mg of Serratiopeptidase standards in a 100ml volumetric flask, dissolving in mobile phase, and diluting to volume with the same mobile phase up to 100 mL and retained as stock solution. Further di- lutions were made with mobile phase.

2.4.3. Preparation of PC, AF and SP Standard Dilutions

One milliliter from the stock solutions of PC, AF, and SP were transferred into a 100 ml volumetric flask separately. To attain the final concentrations of 21 µg/mL, 6.6 µg/mL and 1µg/mL of PC, AF and SP respectively.

2.4.4. Preparation of Mixed Standard Solutions

From the aforementioned standard stock solution, mixed standard solution was prepared by dissolving appropriate concentration of the stocks in the mobile phase and used for the estimation of individual drugs from the combination.

2.4.5. Preparation of the Sample Solution

The label claim of the dosage form includes 325mg of Paracetamol, 100mg of Aceclofenac and 15mg of Serratiopeptidase.

Twenty tablets of PC, AF and SP available as combination dosage forms were weighed and powdered. An amount of the powder equivalent to 52.2 was weighed accurately and mixed with the mobile phase in a 100-mL volumetric flask, sonicated for 20 minutes and filtered through 0.45µ nylon syringe filter to remove insoluble matter. Five milliliter of the filtrate was then diluted to 50mL with mobile phase in volumetric flask.

2.4.6. Method for the Estimation

With the optimized chromatographic conditions, a steady baseline was recorded. After stabilization of the baseline for about 30 minutes, successive aliquots of the standard solution of the same concentration were injected and chromatogram was recorded until the reproducibility of the peak areas was satisfactory. This procedure was repeated using the sample solution so that duplicate injection of the sample solution was bracketed by injection of the standard solution.

The response factor of the standard peak and sample peak was obtained and the amount of each drug in the sample was determined. This procedure was repeated six times.

The concentration of each drug in the multicomponent dosage form was calculated using the formula (1):

$$\text{Concentration of drug} = \frac{\text{Response factor of the sample}}{\text{Response factor of the standard}} \times \text{Concentration of standard}$$

2.5. Validation of the Method

The developed method was validated for as per ICH Q2 (R1) guidelines for various parameters such as accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantitation (LOQ), Forced degradation and stability.

2.5.1. Accuracy

The accuracy of the RP-HPLC method was evaluated by selecting three different concentrations lower quantitation limit (LQC), medium quantitation limit (MQC), and higher quantitation limit (HQC). In each concentration, a minimum of six injections were given and the amount of the drugs present, percentage recovery, and related standard deviation were calculated. The percentage recovery was calculated using the formula (2):

$$\text{Percentage recovery} = \frac{[b - a]}{c} \times 100$$

Where 'a' is the amount of the sample drug, b is the amount of the sample drug and the standard drug and c is the amount of standard drug added.

2.5.2. Precision

The precision of the developed method was studied by performing intraday and intraday variations. Intraday variations were studied by consecutively injecting the standard and sample solutions for six times on the same day. Intraday variations were studied by estimating the drugs present in the multicomponent dosage forms on three different days. Six injections of standard and sample solutions were made every day. The amount of each drug, percentage content, standard deviation, and percentage coefficient of variation were calculated.

2.5.3. Linearity and Range

The six series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

2.5.4. LOD and LOQ

The LOD and LOQ of PC, AF and SP were determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions in accordance with 3.3 s/n and 10 s/n criteria, respectively, where s/n indicates signal-to-noise ratio.

2.5.5. Robustness

For demonstrating the robustness of the method, slight variations in the optimized conditions were done and the standard solution was injected. The variations made were $\pm 5\%$ in the ratio of acetonitrile in the mobile phase, ± 0.2 unit in the pH of the buffer, ± 0.2 mL/min in the flow rate and ± 5 nm in the wavelength. The separation factor, retention time and peak asymmetry were calculated.

2.5.6. Forced Degradation

Forced degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principal peaks shall pass.

2.5.7. Stability

The mobile phase, standard solution, and the sample solution were subjected to long-term (3 days) stability studies. The stability of these solutions was studied by storing the standard solution for 3 days and observing for changes in the separation, retention, and asymmetry of the peaks, which were then compared with the pattern of the chromatogram of freshly prepared solution.

3. Results and Discussion

Proper selection of the HPLC method depends on the nature of the sample (ionic or ionizable or neutral molecule), its molecular weight, and solubility. The drugs selected for the current study are polar in nature; hence, RP-HPLC was selected for its separation because of its separation because of its simplicity and suitability.

3.1. Optimization of the Chromatographic Condition

Method optimization for the simultaneous estimation of the combination of PC, AF and SP in multicomponent dosage forms was carried out.

3.1.1. Column selection

Experiments with different columns were conducted to achieve best separation of analyte peak with other blank and placebo peaks. It was found that the peak shape, retention time, tailing factor, and column efficiency were good with Phenomenex C18 column (250x4.6 mm, 5mm) with C18 guard column (4mm 3mm 5mm).

3.1.2. Mobile Phase Composition

On the basis of the solubility study, Heptane-1-Sulphonic acid was decided as the buffer preparation to be used. A mixture of 1.0 gm Heptane-1-Sulphonic acid and the organic solvents in different proportions were tested, as variation in the mobile phase composition led to substantial changes in the chromatographic performance. Decreasing the organic modifier content resulted in decrease in the retention time of the analyte but had no effect on analyte response. When experiments were performed with methanol instead of acetonitrile as organic modifier in the mobile phase, late elution of analyte with peak tailing and increased column pressure were observed. Hence, acetonitrile was selected as an organic modifier. Many trials on the composition of buffer and organic solvents were made to decide the ultimate composition of the mobile phase as buffer: acetonitrile (90:10).

Based on the peak shape, peak symmetry, and retention time, the flow rate of 1 mL/min, and ambient column temperature were also optimized.

3.1.3. Detection Wavelength

The sensitivity of a HPLC method with UV detection depends on the proper selection of detection wavelength, which can be determined by recording overlaid UV spectra. In the current study, solutions containing 21 µg/ml of PC, 6.6 µg/ml of AF, 1 µg/ml of SP were prepared in mobile phase and scanned under 200-400 nm of UV region to record the overlaid UV spectra.

3.1.4. pH of the Buffer

P^H plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling ionization characteristics. The pK_a values for PC, AF and SP were 5, 6.2 and 7.4 respectively. Heptane-1-Sulphonic acid buffer was selected based on the solubility studies. Various trials on pH were made to determine the optimized pH at which the APIs are separated well. At pH 2.5, peak shape, peak tailing and theoretical plate count were found to be satisfactory; hence, 2.5 were decided as the pH of the buffer. A tolerable limit of pH 2.5 ± 0.1 was optimized using a pH meter.

In order to determine the adequate solution and reproducibility of the proposed method, suitability parameters including retention time, plate number, and tailing factor were investigated and were found to be 3.119 min, 3274, 1.29 for PC; 7.19, 6003, 1.27 for AF; and 13.56, 7450, 1.10 for SP, respectively. Which indicates the method suitability?

The optimized chromatographic conditions are mobile phase buffer: Acetonitrile 90:10 % v/v, pH 2.5, 226 nm as detection wavelength, 1.0 ml/min flow rate, ambient column temperature, 5 µl injection volume.

3.2. Validation of Method

3.2.1. Specificity

The specificity of the existing method of analysis by HPLC is shown in Figure 4; the complete and clear separation of PC, AF and SP was observed without any interference in retention time.

3.2.2. Accuracy

The accuracy of the method was determined by recovery experiments. Recovery studies were carried out with six injections and three different concentrations. The percent recovery, mean and relative standard deviation (%RSD) was calculated and presented in Table 1. APIs with concentration 10.5, 21 and 31.5 µg/mL of PC; 3.3, 6.6 and 9.9 µg/mL of AF; and 0.5, 1 and 1.5 µg/mL of SP were prepared. The test solution was injected three times for each spike level and the assay was performed as per the test method. Analysis of the results has shown that the percentage recovery values were close to 100% and also the RSD values were less than ±2%. The accuracy and reliability of the developed method was established.

3.2.3. Precision

The precision of the method was demonstrated by interday and intraday variation studies at various concentrations. 21 µg/ml for PC, 6.6 µg/ml for AF and 1 µg/ml for SP and their data summarized in Table 2. The lower RSD% values (<2.00) indicate good precision of the developed method.

3.2.4. Linearity and Range

Six series of standard solutions were selected for assessing linearity range (2.1-31.5 µg/mL for PC, 0.66-9.9 µg/mL for AF, and 0.1-1.5 µg/mL for SP). The calibration curve was plotted using response factor versus concentration of the standard solution. From the calibration curve, the slope and intercept were calculated. The data obtained from the linearity range are depicted in the graph and the results show the Y intercept as 73481x+61756 for PC, 289991x+9548.1 for AF and 741443x+17240 for SP with higher correlation coefficient value 0.9995, 0.9998, and 0.9992 for PC, AF and SP respectively.

3.2.5. LOD and LOQ

The LOD and LOQ of the compounds were determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions. The LOD values were found to be 0.21, 0.066 and 0.01 µg/mL for PC, AF and SP respectively.

The LOQ values were found to be 0.42, 0.132 and 0.02 µg/mL for PC, AF and SP respectively.

3.2.6. Robustness

The robustness was evaluated by making slight variations in the optimized conditions such as flow rate, pH of mobile phase, column temperature, wavelength, and percentage of organic solvent. The mixed standard solution was injected in five replicates and %RSD of assay was calculated for each condition. The results obtained (Table 3) as a cause of small deliberate variations in the method parameters has proven that the analytical method is robust.

3.2.7. Forced Degradation

Forced degradation study was observed that upon treatment of PC, AF and SP with different strengths of base (0.05 N and 0.5 N NaOH), acid (0.05 N, 0.5 N and 1 N HCl) and hydrogen peroxide and Thermal and Photolytic (20%) the degradation was observed in (Table 4). The PC, AF and SP stable under the applied stress conditions like Thermal, acid and alkaline and oxidative degradation states.

3.2.8. Stability

The stability of the drug solutions was assessed by maintaining the solution at room temperature for 3 days and observing for changes in the chromatographic pattern as well as the content of the solution on comparison with the freshly prepared solution. The results were expressed in terms of percent deviation between actual and stored recovery (Table 5).

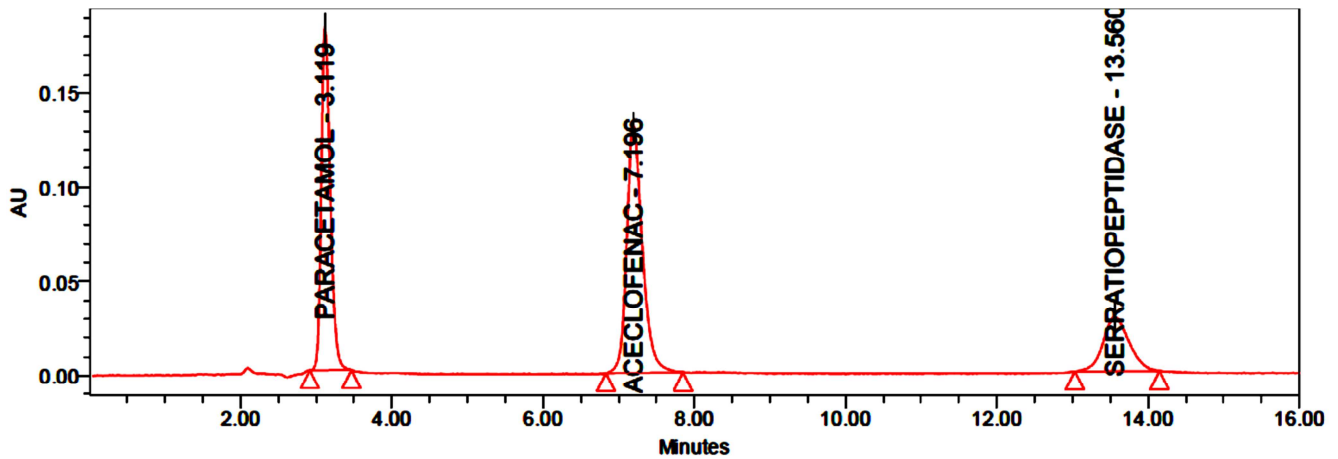


Figure 4. The representative chromatogram. The simultaneous estimation of PC, AC and SP, the peaks at the retention time of 3.119, 7.186 and 13.58 minutes respectively.

Table 1. Accuracy (recovery, %) studies expressed in concentration (mg/ml) of Paracetamol at three different concentration.

Studyno.	Paracetamol		
	10.5	21	31.5
1.	100.2	100.0	100.6
2.	100.1	100.2	99.8
3.	99.9	100.6	100.4
4.	100.3	100.4	100.9
5.	100.6	99.8	100.1
6.	100.5	100.3	100.3
Avg	100.2	100.217	100.35
%RSD	0.259	0.289	0.379

Table 2. Accuracy (recovery, %) studies expressed in concentration (mg/ml) of Aceclofenac at three different concentration.

Studyno.	Aceclofenac		
	3.3	6.6	9.9
1.	100.1	99.5	100.6
2.	99.4	100.8	100.2
3.	100.5	100.2	100.1

Studyno.	Aceclofenac		
	3.3	6.6	9.9
4.	98.8	100.9	99.2
5.	99.2	99.4	100.4
6.	100.6	101.2	100.5
Avg	99.767	100.333	100.16
%RSD	0.742	0.757	0.509

Table 3. Accuracy (recovery, %) studies expressed in concentration (mg/ml) of Serratiopeptidase at three different concentration.

Studyno.	Serratiopeptidase		
	0.5	1	1.5
1.	99.5	99.5	100.1
2.	100.6	100.8	100.2
3.	100.8	100.2	100.6
4.	100.1	100.9	100.4
5.	99.4	99.4	99.1
6.	98.5	101.2	99.6
Avg	99.817	100.333	100.00
%RSD	0.862	0.757	0.550

Table 4. Intraday and interday assay precision analysis data of the proposed method.

	Actual concentration ($\mu\text{g/mL}$)	Measured concentration (RSD(%))	Intraday Interday
Paracetamol	21	0.82	0.66
Aceclofenac	6.6	0.99	0.92
Serratiopeptidase	1.0	0.47	0.65

Table 5. Robustness study of the proposed method.

Factor	Level	PC (Assay, %) RSD(n=3)	AF (Assay, %) RSD(n=3)	SP (Assay, %) RSD(n=3)
Flow rate (ml/min)	0.9	99.8, 0.89	100.5, 0.45	99.6, 0.48
	1.0	100.2, 0.45	100.6, 0.85	99.9, 0.88
	1.2	100.1, 0.55	100.1, 0.77	100.5, 0.55
Percentage organic solution	5	100.6, 0.44	99.5, 0.14	100.1, 0.99
	10	100.7, 0.58	100.2, 1.02	99.6, 0.88
	15	99.8, 0.77	100.5, 0.56	99.4, 0.68
pH of mobile phase	2.3	98.8, 0.42	100.1, 0.45	100.8, 0.57
	2.5	100.1, 0.57	99.8, 0.44	100.5, 0.79
	2.7	100.6, 0.52	100.5, 0.68	100.6, 0.44
Wave length	221	100.1, 0.95	100.2, 0.77	99.9, 0.89
	226	100.2, 0.68	100.1, 0.58	100.1, 0.52
	231	100.5, 1.06	100.8, 0.45	100.5, 0.98

Table 6. Results of force degradation studies.

Stress Condition/duration/solution	Degradation
Acid degradation (0.5 N HCl, 1 hr)	28%
Alkaline degradation (0.5 N NaOH, 1 hr)	23%
Oxidative degradation (30 % H ₂ O ₂ , 80°C for 10min)	26%
Reduction Degradation (10% Sod. Bisul, 1hr)	23%
Thermal degradation (Solid sample, 80°C, 3hr)	23%
Photolytic Degradation (sample expose sun light 6 hr)	28%
Hydralysis Degradation	25%

Table 7. Solution stability studies.

Studyno.	(Percentage deviation between actual and stored recovery)		
	PC	AF	SP
1	0.56	0.28	0.46
2	0.52	0.16	0.35
3	1.28	0.35	0.62
4	1.18	1.18	0.92
5	1.02	0.27	0.55
6	0.99	0.09	0.53
Average	0.92	0.38	0.57

4. Conclusion

A convenient and rapid simultaneous RP-HPLC method has been developed for the estimation of PC, AF and SP. Best separation was achieved on a Phenomenex C18 (250mm x 4.6mm internal diameter, 5 μ) with C18 guard column, 1gm Heptane-1-Sulphonic acid (adjusted to pH 2.5): acetonitrile (90:10v/v) at a flow rate of 1.0mL/min as mobile phase and 226 nm as detection wavelength. The method was validated in terms of accuracy, precision, specificity, linearity, robustness, and solution stability according to ICH guidelines. The proposed method is simple, fast, accurate, and precise for the simultaneous quantification of PC, AF and SP in bulk drugs and finished products as well as for routine analysis in quality control.

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