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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF ACECLOFENAC, PARACETAMOL AND SERRATIOPEPTIDASE IN BULK AND DOSAGE FORM

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ABSTRACT

A Simultaneous estimation of Analytical method was developed and validated for the estimation of paracetamol, aceclofenac and serratiopeptidase in bulk dosage form using C18 column (250mm 4.6mm, 0.45µ) with mobile phase consisting of Acetonitrile: Water (60:40v/v) and 0.1% Glacial acetic acid with a flowrate of 1.0ml/min and UV detection at 277nm. Recovery was observed 99.1% to 99.4% for Leben laboratories PvtLtd , 98.64% to 99.1% for Leben laboratories Pvt Ltd, 98.9% to 99.5% J.C.Biotech. Hyderabadin bulk and dosage form . Accuracy of drugs was observed to be within the limits of 99.91 % to 101.32% by mean of 3 determinations. Precision and Robustness of drugs was observed to be less than 2.0 of %RSD by mean of 6 determinations. LOD and LOQ of Paracetamol, aceclofenac and serratiopeptidase were found to be 0.97µg/ml, 2.95 µg/ml, 0.33µ/ml and 0.184 µg/ml, 0.1 µ/ml, 0.33µg/ml respectively. a simple, selective, precise HPLC method can be developed for the analysis of simultaneous estimation of Paracetamol, serratiopeptidase and Aceclofenac in tablet dosage forms.

KEYWORDS

Paracetamol, Aceclofenac, Serratiopeptidase, Estimation, Precise, Analysis.

INTRODUCTION

Analytical chemistry deals with quantitative analysis of composition of substances and complex materials in various matrices by measuring a physical or chemical property of a distinctive constituent of the components of interest [1]. Analytical methods are classified into instrumental and chemical method [2]. Instrumental method involves measurement of a light absorption or emission, fluorescence, conductivity and electrode potential. Chemical method involves measurement of mass of the analyte by gravimetric or volumetric method [3].

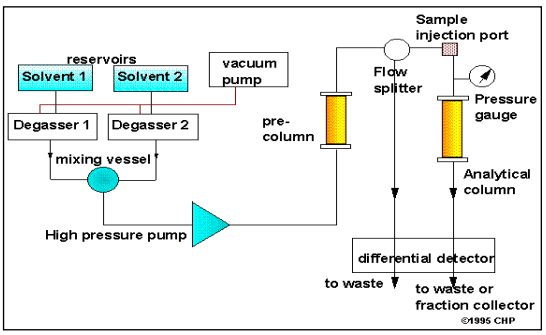
CLASSIFICATION OF ANALYTICAL METHODS:

Qualitative analysis: It deals with the identification of substances. It is concern with what elements or comoundsare present in sample.

Quantitative analysis : It provides numerical information concerning the quantity of analyte in measured amount of sample.

CHROMATOGRAPHY

chromatography from most other physical and chemical methods of separation is that two mutually immiscible phases are bought into contact; one phase is stationary and other mobile.



HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography though more troublesome than gas chromatography, has the

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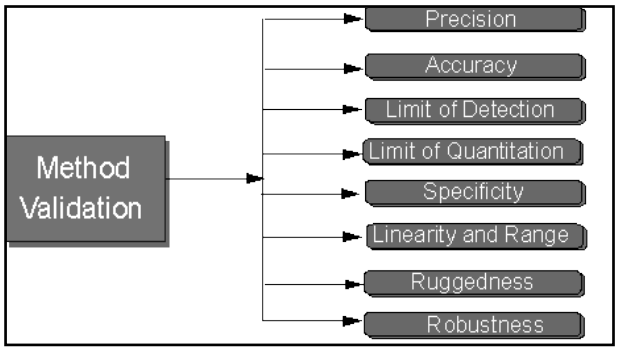
main advantage of operating at low temperature and can be used with advantage for separation of substance as proteins, nucleosides which are thermolabile.

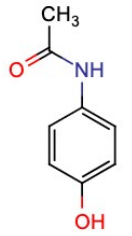
VALIDATION OF METHOD

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice [7].

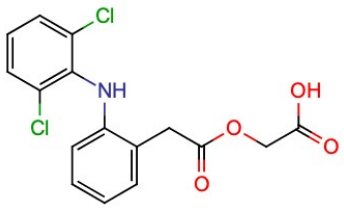
The USP has published specific guidelines for method validation for compound evaluation.USP defines eight steps for validation:



Drugs



Paracetamol



Aceclofenac

EXPERIMENTAL WORK

Chemicals used

In method development and validation of preservatives following chemicals and reagents were used.

Selection of wavelength by UV-Visible Spectrophotometry

Selection of mobile phase

Each mobile phase was vacuum degassed and filtered through 0.45µ membrane filter. The mobile phase was allowed to equilibrate until steady baseline was obtained. The standard solution containing mixture of PCM, SERA and ACF was run with different individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Acetonitrile and Water was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for PCM, SERA and ACF. Chromatograms of PCM, SERA and ACF are shown in Fig . 11, 12,13 respectively.

Instruments Name	Model number and name of manufacturer
UV visible spectrophotometer	UV 1800, Shimadzu, Japan
HPLC	Younglin (S.K.) Gradient system UV Detector, Autochro-3000 Software with 20.0 µl fixed injector, C_{18} (150mm×4.6mm, 5µm) column.
Electronic balance	AUX 120, Shimadzu, Japan
pH meter	Global Electronics, Hyderabad, India.
Sonicator	PCI Analytical, India.

Studies of Calibration plot

Optimization of Chromatographic condition

The following chromatographic conditions were established by trial and error and were kept constant throughout the analysis.

Column : C18 (150 mm× 4.6mm)
Particle size packing : 5µm
Detection wavelength : 277nm
Flow rate : 1.0 ml/min
Temperature : Ambient
Sample size : 20 µl
Mobile phase : Acetonitrile & Water (60:40) with 0.1% acetic acid

Procedure for calibration curve of Paracetamol, serratiopeptidase and aceclofenac

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. From the freshly prepared standard stock solution, pipette out 65ml paracetamol, 3ml serratiopeptidase and 20ml Aceclofenac in 200ml of volumetric flask and diluted with mobile phase. From it 1, 2, 3, 4 and 5ml of solution were pipette out in 10 ml volumetric flask and volume was made up to 10 ml with mobile phase to get final concentration 32.5, 65, 97.5, 130 and 162.5 µg/ml of paracetamol, 1.5, 3, 4.5, 6 and 7.5 µg/ml of

Ingredients	Grade	Suppliers
Paracetamol	-	Leben laboratories Pvt Ltd. Akola
Serratiopeptidase	-	J.C.Biotech. Hyderabad
Aceclofenac	-	Leben laboratories Pvt Ltd. Akola
Methanol	HPLC	Avantor Performance material India Ltd. Thane, Maharashtra
Acetonitrile	HPLC	Merck Specialities Pvt. Ltd. Shiv Sager Estate 'A' Worli, Mumbai
Water	HPLC	Merck Specialities Pvt. Ltd.Shiv Sager Estate 'A' Worli, Mumbai

serratiopeptidase and10, 20, 30, 40 and 50 µg/ml of aceclofenac. All sample were injected and peaks were recorded at 277nm as the graph plotted as concentration of drug verses peak area is depicted in fig. no. 14, 15 and 16 respectively.

Study of system suitability parameters

The system suitability is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from five replicate injections of standard solution.

Preparation of standard drug solution

i. Mixed Standard Solution of PCM, SERA and ACF:

From the freshly prepared standard stock solution, pipette out 65ml paracetamol, 3ml serratiopeptidase and 20ml Aceclofenac in 200ml of volumetric flask and diluted with mobile phase. From it pipette out 2 ml solution and made volume up to 10 ml in volumetric flask with mobile phase to get final concentration of 65µg/ml of PCM, 3µg/ml of SERA and 20 µg/ml of ACF.

Marketed Formulation	Contents	Manufacturer
Zerodolsp	Aceclofenac: 100 mg Paracetamol: 325 mg Serratiopeptidase: 15 mg	Ipca Labs Pvt.Ltd Mumbai

Procedure

The previously filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 µl standard drug solution was injected and system suitability parameters were recorded as shown in Table 7.

Application of proposed method for estimation of Paracetamol, Serratiopeptidase and Aceclofenac in marketed dosage forms

a. Preparation of Standard Solution

Accurately weighed quantity of 325 mg of PCM, 15mg SERA and 100 mg of ACF were transferred to 100 ml separate volumetric flask and dissolved in methanol. The volume was made up to the mark. The standard stock solution of PCM, SERA and ACF were mixed a properly with mobile phase to obtain a laboratory mixture containing 3250 µg/ml of PCM,150 µg/ml of SERA and 1000 µg/ml of ACF.

From the resultant solution 0.2 ml transferred in 10 ml volumetric flask and volume made up with mobile phase. To get final concentration of 65µg/ml PCM, 3µg/ml SERA and 20µg/ml ACF of standard solution.

b. Preparation of sample solution

Twenty tablets were taken and average weight was calculated .Take accurately weighed quantity equivalent to 325mg of PCM, 15 mg SERA and 100mg of ACF in 100ml volumetric flask and dissolve in methanol. The sample solution of PCM, SERA and ACF were mixed a properly with mobile phase to obtain a laboratory mixture containing 3250 µg/ml of PCM,150 µg/ml of SERA and 1000 µg/ml of ACF.

From the resultant solution, 0.2 ml was transferred in 10 ml volumetric flask and volume make up with mobile phase. To get final concentration of 65µg/ml PCM, 3µg/ml SERA and 20µg/ml ACF of standard solution.

Procedure

Equal volumes (20µl) of standard and sample solutions were injected separately after equilibrium of stationary phase. The chromatograms were recorded and response i.e.,

peak area of major peaks were measured. The content of PCM, SERA and ACF was calculated by comparing a sample peak area with that of standard peak area.

Validation

i. Accuracy

The accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method.

Preparation of standard solution of PCM, SERA and ACF:Take accurately weighed quantity equivalent to 325mg of PCM, 15 mg SERA and 100mg of ACF in 100ml volumetric flask and dissolve in methanol. The sample solution of PCM, SERA and ACF were mixed a properly with mobile phase to obtain a laboratory mixture containing 3250 µg/ml of PCM,150 µg/ml of SERA and 1000 µg/ml of ACF.

From the resultant solution 0.2 ml was transferred in 10 ml volumetric flask and volume made up with mobile phase to get final concentration of 65µg/ml PCM, 3µg/ml SERA and 20µg/ml ACF of standard solution. Then add 80, 100, and 120 % of pure drug sample that is 52, 2.4and16 µg/ml for 80%. 65, 3 and 20µg/ml for 100%.78, 3.6 and 24µg/ml for 120%,of PCM, SERA and ACF respectively.

The solution was filtered through 0.45 µ membrane filter. The amount of drug contributed by injected formulation was deduced from the total amount of respective drug estimated and the resultant quantities were assumed to be recovered from the added pure drug and the content of drug calculate. The results are shown in Table no. 11, 12 and 13.

$$\% \text{ Recovery} = A/(B+C) \times 100$$

where, A = total amount of drug estimated; B = amount of drug found on pre-analyzed basis; C = amount of pure drug added

ii. Precision

The precision of the assay method was evaluated in terms of repeatability by carrying out five independent assays of test sample preparation and the % RSD of assay was calculated. The result are shown in table no.14, 15, and 16.

iii. Robustness

The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The robustness of a method is evaluated by varying method parameters such as pH, ionic strength, temperature etc.

The studies of robustness were carried out under two different conditions:

A. Flow change

B. Wavelength change

Results are shown in table no.17 and 18.

iv. LOD and LOQ

Several approaches for determining the Limit of detection (LOD) and Limit of quantitation (LOQ) are possible. The quantitation limit is determined by

$$LOD = 3.3(\sigma / S)$$

where, σ = standard deviation of response; S = slope of calibration curve

The slope S may be estimated from the calibration curve of analyte.

The quantitation limit is determined by

$$LOQ = 10(\sigma / S)$$

where, σ = standard deviation of response; S = slope of calibration curve

The slope S may be estimated from the calibration curve of analyte. All results are shown in Table no. 19.

RESULT AND DISCUSSION

Analysis of standard drug was done by following parameter:

Melting point

Melting point of the drug were found to be

Paracetamol – 169-172^{0}C

Serratiopeptidase – 163-168^{0}C

Aceclofenac – 149-153^{0}C

Solubility

Paracetamol: Very slightly soluble in water.

Serratiopeptidase: Very soluble in water.

Aceclofenac: Freely soluble in acetone, ethanol and Miscible with water

Verification of as λ_{max}

The solution of PCM, SERA and ACF were scanned in the range of 200-400 nm in 1 cm cell against blank; from the spectrum wavelengths selected for the estimation of drugs were 252, 276

and 282nm λ_{max} of paracetamol, serratiopeptidase and aceclofenac. These are shown in Figure 8, 9 and 10 respectively.

Figure 8. UV Spectrum of Paracetamol

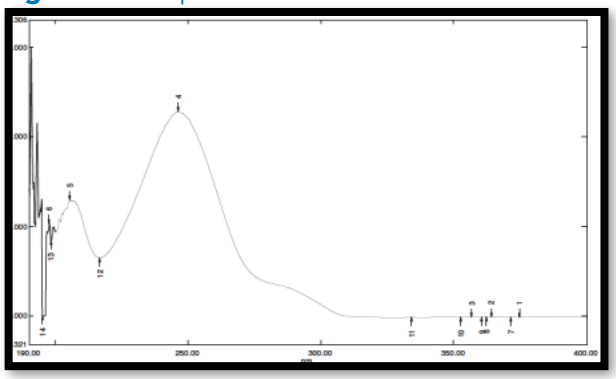
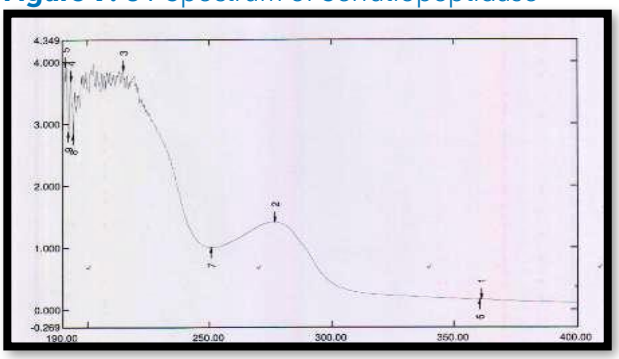
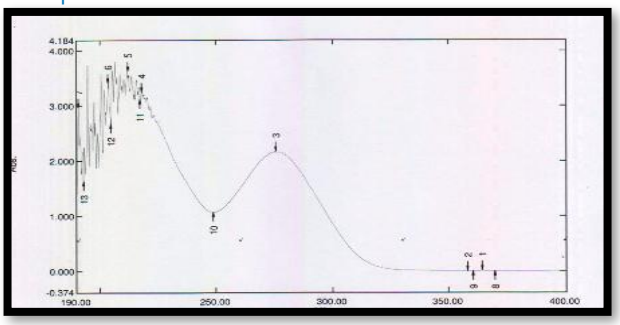


Figure 9. UV Spectrum of Serratiopeptidase

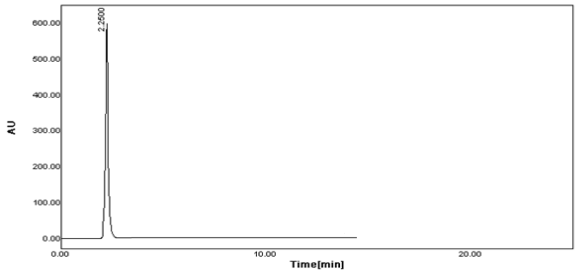


UV Spectrum of Aceclofenac



HPLC Chromatogram

Figure 11. Chromatogram for Paracetamol



From the various mobile phases tried, mobile phase containing Acetonitrile and Water (60:40 v/v) was selected since it gave sharp, well resolved peaks with symmetry within the limits and significant reproducible retention time for PCM, SERA and ACF. Chromatograms of PARA, SERA and ACF are shown in Figure 11, 12, 13 respectively.

Figure 12. Chromatogram for Serratiopeptidase

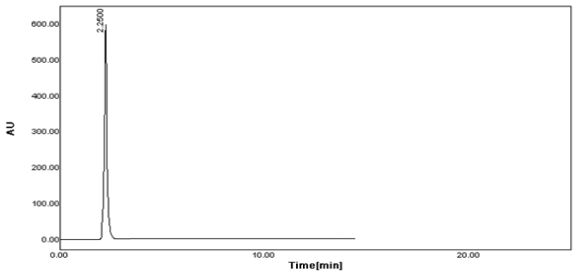
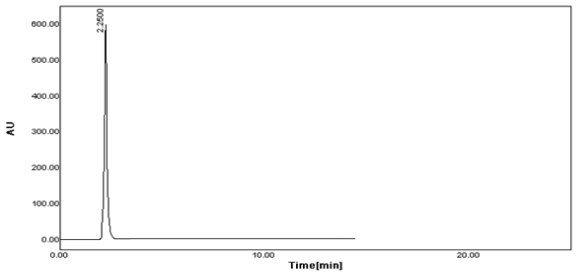


Figure 13. Chromatogram for Aceclofenac



Calibration curve

The results of calibration curves of paracetamol, serratiopeptidase and Aceclofenac are shown in Table 6 and Figures 14, 15 and 16.

Figure 14. Callibration Curve for Paracetamol

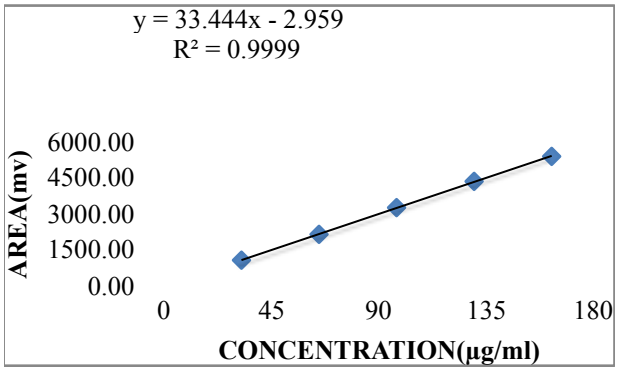


Figure 15. Callibration Curve for Serratiopeptidase

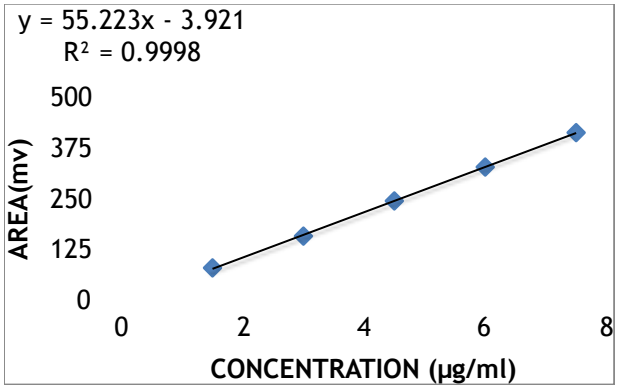


Figure 16. Callibration Curve for Aceclofenac

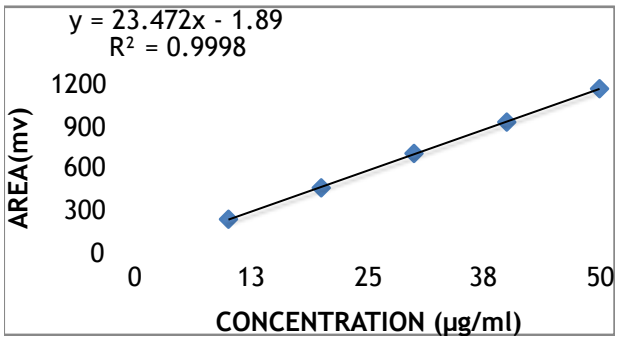


Table 6. Calibration curves

Concentration(µg/ml)			Peak area (mv)		
PCM	SERA	ACF	PCM	SERA	ACF
32.5	1.5	10	1080.60	81.25	235.61
65	3	20	2157.75	158.72	461.12
97.5	4.5	30	3272.84	244.55	708.18
130	6	40	4367.94	327.23	933.42
162.5	7.5	50	5410.22	411.17	1173.08

It was observed that, PCM, SERA and ACF obeys the Beer Lambert's law in the linearity range of 32.5-162.5, 1.5-7.5 and 10-50 µg/ml respectively. The regression coefficients were found to be 0.999 for PCM, 0.999 for SERA and 0.999 for ACF.

Study of System suitability parameter

In study of system suitability parameter, retention time, asymmetry, no. of theoretical plate, capacity factor and resolution were calculated. These factors are found within limit. System suitability parameters were recorded for lab mixture of PCM, SERA and ACF.

Estimation of Paracetamol in Marketed Formulation

Sample Concentration (µg/ml)	Standard Concentration (µg/ml)	Standard Peak Area (mv)	Sample Peak Area (mv)	% Drug estimation
65	65	2180.49	2179.36	99.94
65	65	2180.49	2178.32	99.90
65	65	2180.49	2178.56	99.90
			Mean	99.91
			±SD	0.0230
			%RSD	0.02302

Validation

i. Accuracy

The accuracy of the proposed method was evaluated by performing recovery studies. The % RSD and % recovery were within the acceptable limits in all 3 levels and % recovery was found to be 99.91 % for paracetamol, 99.65 % for serratiopeptidase and 101.32% for aceclofenac. It is evident from the results of accuracy that the proposed method is very accurate.

Estimation of Serratiopeptidase in Marketed Formulation

Sample Concentration (µg/ml)	Standard Concentration (µg/ml)	Standard Peak Area (mv)	Sample Peak Area (mv)	% Drug estimation
3	3	157.87	157.65	99.85
3	3	157.87	163.32	103.45
3	3	157.87	159.35	100.93
			Mean	101.41
			±SD	1.89
			%RSD	1.8144

Estimation of Aceclofenac in Marketed Formulation

Sample Concentration (µg/ml)	Standard Concentration (µg/ml)	Standard Peak Area (mv)	Sample Peak Area (mv)	% Drug estimation
20	20	460.37	458.59	99.61
20	20	460.37	463.74	100.73
20	20	460.37	459.36	99.78
			Mean	100.01
			±SD	0.05521
			%RSD	0.5520

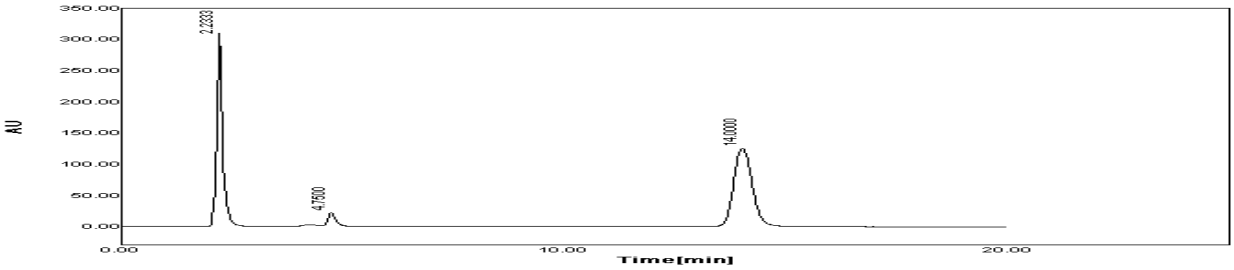
Data for recovery study of PCM

Concentration (µg/ml)	Standard Peak Area (mv)	Amount of pure drug added (µg/ml)	Sample Peak Area (mv)	% Recovery
65	2180.00	52	3920.23	99.89
65	2180.00	65	4355.32	99.91
65	2180.00	78	4791.56	99.23
			Mean	99.91
			±SD	0.02
			%RSD	0.02

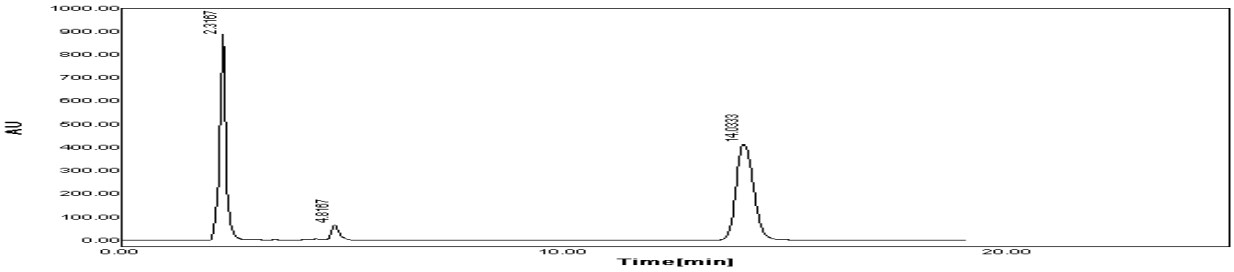
Data for recovery study of Serratiopeptidase

Concentration (µg/ml)	Standard Peak Area (mv)	Amount of pure drug added (µg/ml)	Sample Peak Area (mv)	% Recovery
3	158.81	2.4	285.60	99.80
3	158.81	3	316.52	99.30
3	158.81	3.6	348.13	99.85
			Mean	99.65
			±SD	0.30
			%RSD	0.30

Chromatogram obtained for standard PCM, SERA and ACF with retention time of 2.23, 4.7 and 14.00 minutes respectively.



Chromatogram of Tablet Marketed preparation of PCM, SERA and ACF with retention time 2.31, 4.8167and 14.03 min respectively.



Data for recovery study of Aceclofenac

Concentration (µg/ml)	Standard Peak Area (mv)	Amount of pure drug added (µg/ml)	Sample Peak Area (mv)	% Recovery
20	460.37	16	838.80	101.22
20	460.37	20	935.76	101.71
20	460.37	24	1022.65	101.05
			Mean	101.32
			±SD	0.342
			%RSD	0.337

ii. Precision

The % RSD of method precision were found to be 0.003 for paracetamol, 0.91 for serratiopeptidase and 0.072 for Aceclofenac respectively. The % RSD below 2.0 indicate high precision of proposed method.

Precision for Paracetamol

Concentration (µg/ml)	Standard Peak Area (mv)	Sample Peak Area (mv)	% Label claim
65	2179.00	2179.15	100.006
		2179.10	100.004
		2179.26	100.011
		2179.15	100.006
		2179.25	100.011
		Mean	100.007
		±SD	0.0032
		%RSD	0.003

Precision for Serratiopeptidase

Concentration (µg/ml)	Standard Peak Area (mv)	Sample Peak Area (mv)	% Label claim
3	156.811	156.65	99.89
		156.75	99.96
		156.85	102.02
		156.74	99.95
		156.81	100.00
		Mean	100.36
		±SD	0.92
		%RSD	0.91

Precision for Aceclofenac

Conc. (µg/ml)	Peak area of std.(mv)	Peak area of sample(mv)	% Label claim
20	459.37	459.12	99.94
		458.56	99.82
		459.15	99.95
		459.47	100.02
		459.23	99.96
		Mean	99.93
		±SD	0.072
		%RSD	0.072

iii. Robustness

The robustness of the method is used to determine the capacity of the intended method to

remain unaffected by changing flow rate and wavelength. The results indicated that the method is robust as the % RSD was below 2.0.

Flow change study

Flow rate (ml)	Drug	Concentration .(µg/ml)	Mean (Area)	±SD	% RSD
	PCM	65	2178.95	3.96	0.18
0.9	SER	3	163.35	2.91	1.78
	ACF	20	453.92	4.80	1.05
	PCM	65	2178.50	2.47	0.11
1.1	SER	3	163.85	0.79	0.48
	ACF	20	453.94	1.30	0.286

Wave-length (nm)	Drug	Concentration (µg/ml)	Mean (Area)	±SD	% RSD
	PCM	65	2178.17	2.81	0.12
278	SER	3	164.35	2.92	1.77
	ACF	20	457.46	1.32	0.28
	PCM	65	2177.25	2.84	0.13
276	SER	3	164.35	1.50	0.91
	ACF	20	455.45	2.72	0.59

iv. LOD and LOQ

LOQ and LOD study

Concentration (µg/ml)	Standard Peak Area (mv)	Amount of pure drug added (µg/ml)	Sample Peak Area (mv)	% Recovery
20	460.37	16	838.80	101.22
20	460.37	20	935.76	101.71
20	460.37	24	1022.65	101.05
			Mean	101.32
			±SD	0.342
			%RSD	0.337

The LOD and LOQ for Paracetamol was found to be 0.975 µg/mL and 2.95 µg/mL, forSerratiopeptidase 0.1105 µg/mL and 0.0.3350 µg/mL and for Aceclofenac 0.6091µg/mL and 1.8457 µg/mL respectively.

	PCM (µg/ml)	SERA (µg/ml)	ACF (µg/ml)
LOD	0.975	0.1105	0.6091
LOQ	2.95	0.3350	1.8457

SUMMARY

The aim of the present research study was to develop and validate HPLC method for simultaneous estimation of Paracetamol, Serratiopeptidase and Aceclofenac in solid dosage forms.

Free gift sample of standard Paracetamol, Serratiopeptidase and Aceclofenac drug sample were obtained from Leben labs Akola and J.C.BiotechHyderabad .Zerodol-sp was purchased from local market.

Selection of wavelength was done by scanning the standard Paracetamol, Serratiopeptidase and Aceclofenacs olution(10µg/ml) on shimandzu UV-visible spectrophotometer. The isobestic point was selected 277 nm for Paracetamol, Serratiopeptidase and Aceclofenac.

The method is selective and linear between concentration range of 32.5-162.5 µg/ml for Paracetamol, 1.5-7.5 µg/mL for Serratiopeptidase and 10-50 µg/mL for Aceclofenac.

The mobile phase was developed after trial and error and selected Acetonitrile:water acetic acid with pH 3.2 (60:40) was selected to get a well separated peaks of pure and marketed sample of Paracetamol, Serratiopeptidase and Aceclofenac. The retention time of Paracetamol to be 2.26 min, Serratiopeptidase to be 4.75 min and Aceclofenac to be 13.49 min. A system suitability parameter was developed on every day basis which includes number of theoretical plate, resolution ,Tailing factor, Area under curve were found within the limits.

Estimation of Paracetamol, Serratiopeptidase and Aceclofenac in tablet formulation was carried out and were found in range of 99.92% -101.65% that is Accurate, precise and with % RSD below 2.

The developed method was validated by means of accuracy, precision, linearity, robustness, LOD and LOQ as per ICH guideline and were found within limit.

Moreover, LOD and LOQ for Paracetamol was found to be 0.97µg/mL and 2.95 µg/mL, for Serratiopeptidase it was found to be 0.1 µg/ml and 0.33µg/ml, and for Aceclofenac 0.60 µg/mL and 0.184 µg/mL respectively. Thus the method is specific and sensitive. Statistical analysis proves that the method is suitable for the analysis of Paracetamol, Serratiopeptidase and Aceclofenac in a tablet dosage forms.

CONCLUSION

The results of the present study conclusively demostrated that a simple, selective, precise HPLC method can be developed for the analysis of simultaneous estimation of Paracetamol, serratiopeptidase and Aceclofenac in tablet dosage forms.

COMPETING INTERESTS

The authors declare that they have no competing interests

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