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Development and Validation of RP-HPLC Method for Estimation of Aceclofenac, Paracetamol and Chlorzoxazone in Bulk Drug and its Tablet Dosage Form

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Abstract:

A simple, rapid and precise Reverse Phase High Performance Liquid Chromatographic method developed for simultaneous estimation of Aceclofenac, Paracetamol and Chlorzoxazone in its combine tablet dosage form. The chromatographic separation carried out on Protecol C18 ENDURO (250mm×4.6mm, 5µm) analytical column with ambient temperature. These parathion was achieved Acetonitrile: Water (80:20v/v) flowrate1.0ml/min.at λ max 268 nm. The method was validated according to ICH guidelines with parameters like linearity, precision, accuracy, robustness, LOD & LOQ. The retention time of Aceclofenac, Paracetamol and Chlorzoxazone were 1.67 min, 2.640 min. and 3.22 min. respectively. The method was linear over the concentration range for Aceclofenac 10-50µg/ml, Paracetamol 32.5-162.5 µg/ml and Chlorzoxazone 25-125 µg/ml. the limit of detection (LOD) were 0.0570 µg/ml, 0.0086 µg/ml, and 0.2149 µg/ml and limit of quantitation (LOQ) 0.172 µg/ml, 0.4203 µg/ml,and 0.65µg/ml for Aceclofenac, Paracetamol and Chlorzoxazone respectively. The method was successfully applied for the routine analysis of drugs in pharmaceutical formulation.

Keywords: RP-HPLC, Aceclofenac, Paracetamol, Chlorzoxazone, ICH guidelines.

Introduction:

Aceclofenac [ACF] Fig.-1a chemically 2[(2-{2-[(2, 6-dichlorophenyl) amino] phenyl} acetic) oxy] acetic acid used as anti- inflammatory and analgesic for treatment of pain relief, inflammation in osteoarthritis and rheumatoid arthritis and also in dental and gynecological conditions (Shah and Kotadiya, 2022). Paracetamol [PR] Figure 1b chemically N-(4-hydroxyphenyl) acetamide used as analgesic and anti-inflammatory. Chlorzoxazone [CLZ] Fig-1c chemically 5-chloro-2, 3-dihydro-1, 3- benzoxazol-2-one used as centrally acting muscle relaxant with sedative properties (Ravisankar, *et al.*,2013). It inhibits muscle spasm by exerting an effect primarily at the level of spinal cord and subcortical areas of brain. The fixed dose combination of these three drugs used as musculoskeletal spasm along with inflammation and pain management (Kesharwani, *et al.*,2020).

Literature survey shows that there are some analytical methods reported in national, international journals, and standard pharmacopoeias. Some analytical methods are reported for few other ingredients in combination with PR and CLZ as well as PR and ACF (Ahmad, *et al.*,2016). The methods reported for estimation of PR and ACF, PR and CLZ in tablet dosage form, bulk drug formulation. Yet there is very negligible analytical methods are available for simultaneous determination of ACF, PR and CLZ in pharmaceutical dosage form (Sekar, *et al.*,2010). The present research work a simple, rapid, sensitive and specific RP-HPLC method for simultaneous estimation of Aceclofenac, Paracetamol and Chlorzoxazone in tablet dosage form (Figure 1a-c) (Gandhi, *et al.*,2010).

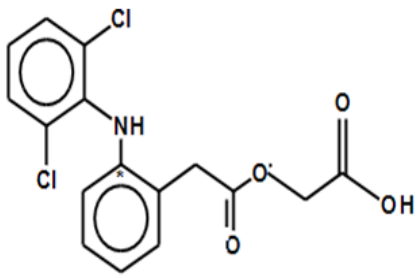

Figure 1a: Aceclofenac

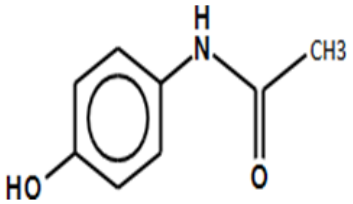

Figure 1b: Paracetamol

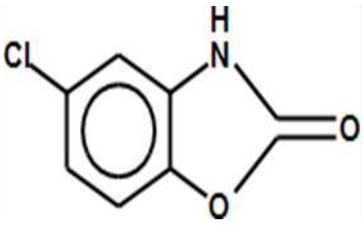

Figure 1c: Chloroxazone

Experimental:

Chemicals and reagents:

ACF, PR, CLZ obtained from Holden medical laboratories, Sinner. Acetonitrile was obtained from thermofisher scientific, India Pvt. Ltd. Pawai Mumbai. The Ultipor N_{66} Nylon 6, 6 membrane 0.2 µm filters were obtained from pall Life science, Triple distilled water were used throughout the experiments, tablets are purchase from local market containing ACF-100 mg, PR-350 mg and CLZ-250mg (Reddy and Dutt, 2014).

Instrument:

Liquid chromatography was performed on an Agilant 1260 infinity II RP-HPLC equipped with quaternary pump. Sample injector with a 20µl loop and PDA detector and Protecol C_{18} ENDURO (250mm×4.6mm, 5µm) column (Manasa and Kumar, 2012).The HPLC system was equipped with "Open Lab" software for data acquisition and quantification of peaks. UV-spectrophotometer (Shimadzu- 1800 with UV-probe software) an Digital Analytical Balance (shimadzu 220AUX) (Adhao, 2016)

Chromatographic conditions:

Chromatographic separation were achieve using Protecol C_{18} ENDURO (250mm×4.6mm, 5µm particle size) analytical column. The mobile phase consisting of mixture of ACN: H_2O (80:20 v/v.) and detected at 268 nm with a flow rate of 1.0 ml/min. the mobile phase filter by using 0.2µm membrane filter. Injection volume was20µl. The RP-HPLC system and column were kept at room temperature. Run time was 5 min. the retention time for ACF, PR and CLZ were 1.5min, 2.6min and 3.2minrespectively (Kumar, *et al.*,2010).

Preparation of mobile phase and sample solution:

Preparation of Mobile Phase:

ACN: H_2O [80:20V/V], filter through 0.2 m nylon membrane filter and degassed (Sharma, *et al.*,2020).

Preparation of Standard Solution:

Accurately weigh 10 mg of aceclofenac was transferred into a 10ml volumetric flask it was dissolved with from this solution 1ml was diluted to 10 ml to give the stock solution containing100 µg/ml of ACF (Shaikh, *et al.*,2012).

Accurately weigh 10 mg of PR was transferred into a 10 ml volumetric flask it was dissolved with from thissolution1ml was diluted to 10ml to give the stock solution containing100 µg/ml of PR. Accurately weigh 10mg of Chlorzoxazone was transferred into a10 ml volumetric flask it was dissolved with from this solution1mlwasdilutedto 10 ml to give the stock solution containing 100 µg/ml of CLZ (Ashraful Islam, *et al.*,2011).

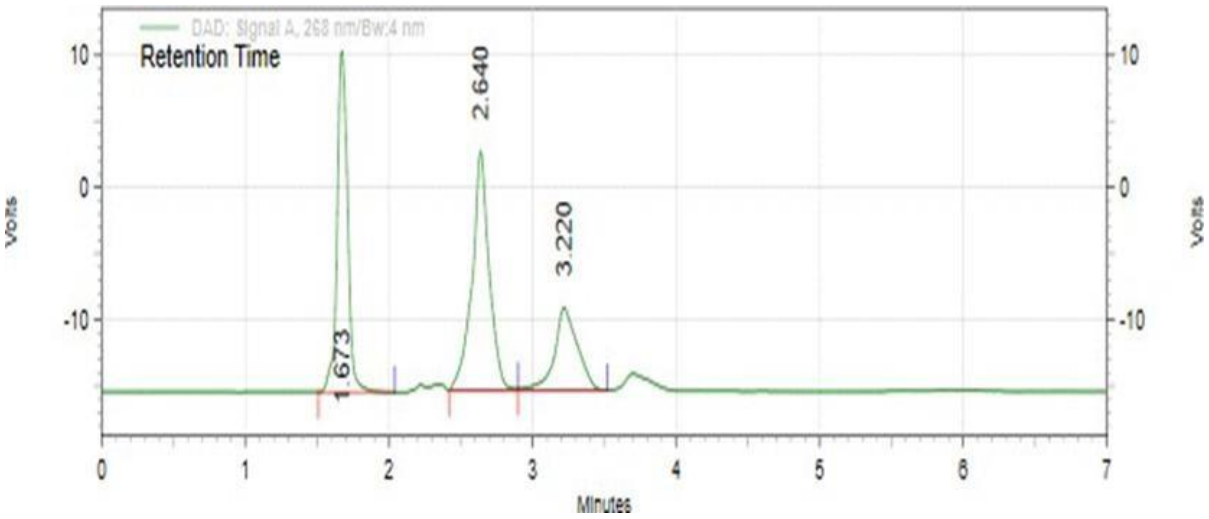

Figure 2: Chromatogram for Combination of bulk drug were ACF-1.673.min, PR- 2.640. min and CLZ- 3.220.min

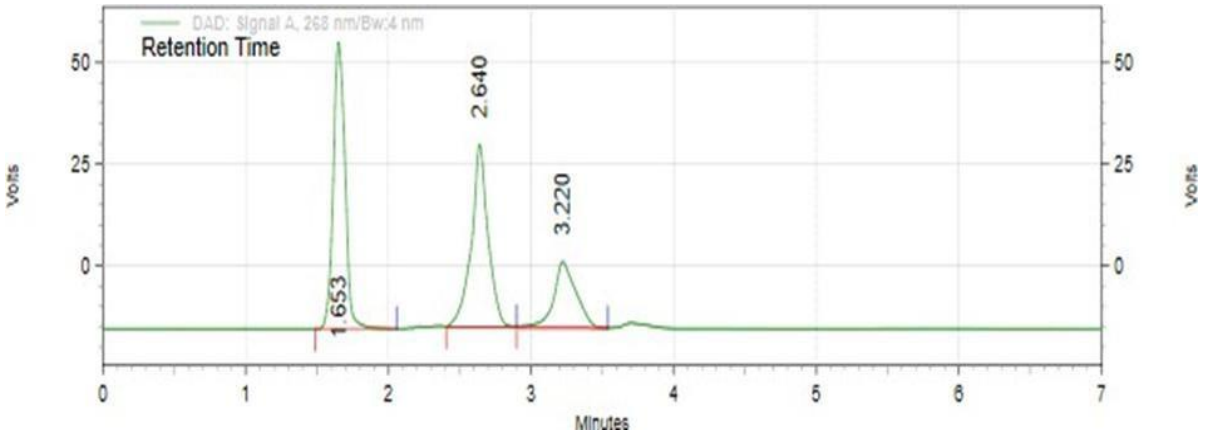

Figure 3: Chromatogram for tablet mixture were ACF 1.653.min, PR 2.640 min and CLZ- 3.220 min

Preparation of tablet Sample Solution:-

Twenty tablets weighted and finely powdered. A quantity equivalent to one tablet containing 100mg- ACF.325mg PR and 250mg CLZ was transfer in to 100ml volumetric flask. The volumetric flask containing 80 ml ACN. The solutions shake vigorously for 5 min then make up the volume with ACN and then filtered with membrane filter paper. From this solution take 10ml of solution and transfer into 100ml volumetric flask and diluted with ACN up to 100ml to get a solution containing 100µg/ml of ACF, 325µg/ml PR and 250µg/ml CLZ. From the solution respective smaller dilutions are prepared. The solution contained ACF, PR and CLZ in the proportion of 10:32.5:25. The amount of ACF, PR and CLZ per tablet was calculated by extrapolating the value of area from the calibration curve. The result of analysis of tablet formulation is reported in Table 9 (Gharge, *et al.*,2010 and Gautam, *et al.*,2015)

Validation of method:

System Suitability Parameters:

For system suitability study, six replicate injections of mixed standard solutions were injected, and the suitability parameters like area under curve, theoretical plates, resolutions, tailing factor of the peaks were calculated. The results are shown in Table 1 (Rajan, 2014).

Table 1: System Suitability Parameters.

Parameter	Aceclofenac	Paracetamol	Chlorzoxazone
Retention Time	1.67	2.64	3.22
Area under curve	341073	951501	346559
Therotical plates	1499	3203	2809
Resolution	0.00	2.3219	2.7321
Tailing factor	1.2619	1.1893	1.169

No. of replicate 6

Linearity:

For the construction of calibration curves, five serial standard dilutions were prepared over the concentration range. ACF, PR and CLZ in the range of 10-50µg/ml, 32.5-135.5µg/ml and 25-125µg/ml. The correlation coefficient (r^2) values were 0.9999, 0.9995 and 0.9988 respectively. The result was shown in Table 2 (Battu and Reddy, 2009).

Table 2: Linearity

Parameter	Correlation coefficient (R)2	Slope	Y-Intercept	Linearity range (µg/ml)
Aceclofenac	0.99929	18725	155535	10-50
Paracetamol	0.9995	87164	83401	32.5-135
Chlorzoxazone	0.9988	28510	40200	25-125

Precision:

Precision was performed by preparing three sets of standard solution of ACF, PR and CLZ and check out reproducibility of result. The result was shown in Table 3 and 4 (Navneet, et al., 2017).

Table 3: Statistical data for intraday precision:

Statistical Data	Aceclofenac	Paracetamol	Chlorzoxazone
Intraday precision			
Mean	99.88	99.83	99.65
S.D.	0.7854	1.510	0.904
R.S.D.	0.7863	1.13	0.908

Interday precision			
Mean	100.48	99.29	99.13
S.D.	0.4366	1.167	1.98
R.S.D.	0.4345	1.175	1.99

Table 4: Statistical data Day to day variation:

Statistical Data	**Aceclofenac**	**Paracetamol**	**Chlorzoxazone**
Day to day variation:			
Mean	97.01	100.4	98.08
S.D.	0.3946	1.3493	0.9624
R.S.D.	0.4068	1.343	0.9812
Analyst to analyst variation:			
Mean	97.15	99.31	97.79
S.D.	0.3551	1.0207	0.8177
R.S.D.	0.3655	1.0276	0.8324

Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (Ahmad, *et al.*,2015).

Recovery experiment should be performed by comparing the analytical result for extracted samples at three different conc. (low, high, and medium) with unextracted standard that represent 100% recovery. The result was shown in Table 5 (Aher, *et al.*,2023).

Table 5: Accuracy

Statistical Data	**Aceclofenac**	**Paracetamol**	**Chlorzoxazone**
Mean	96.4	99.38	98.06
S.D.	1.1333	0.6017	1.1421
R.S.D.	1.1756	0.6055	1.1647

Robustness:

Robustness of the method was determined by making slight changes in the chromatographic conditions as per ICH guidelines, the change in flow rate and wavelength. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed and System suitability parameters were found to be within acceptable limits. the result was shown in Table 6 and 7 (Saravanan, *et al.*,2013).

Table 6: Change in wavelength:

Statistical Data	Aceclofenac	Paracetamol	Chlorzoxazone
λmax- 267 nm			
Mean	100.24	99.91	99.67
S.D.	0.212	1.6265	0.357
R.S.D.	0.211	1.6220	0.316
λmax- 268 nm			
Mean	99.72	99.6	98.6
S.D.	0.5071	0.6928	1.2355
R.S.D.	0.5085	0.6956	1.2420
λmax- 269 nm			
Mean	98.41	99.128	99.24
S.D.	0.3434	0.3251	0.6568
R.S.D.	0.3480	0.3278	0.6569

Table 7: Change in flow rate

Statistical Data	Aceclofenac	Paracetamol	Chlorzoxazone
0.9ml/min:			
Mean	99.69	99.57	99.53
S.D.	0.1834	0.6683	0.4788
R.S.D.	0.1839	0.6711	0.4811
1.0ml/min			
Mean	99.72	99.6	98.6
S.D.	0.5071	0.6928	1.2365
R.S.D.	0.5085	0.6956	1.2420
1.1 ml/min			
Mean	100.08	98.56	98.84
S.D.	1.0539	0.6464	0.6368
R.S.D.	1.0521	0.6558	0.6442

LOD AND LOQ:-

The limit of detection (LOD) for an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value.

The quantization limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy (Sistla, *et al.*,2013).

The LOD and LOQ may be expressed as,

LOD = 3.3 x σ/S and LOQ = 10 x σ/S

Where σ = the standard deviation of the response, S = the slope of the calibration curve of analyte.

The LOD‟s for Aceclofenac, Paracetamol and Chlorzoxazone were 0.0570µg/ml, 0.0086µg/ml and 0.2149µg/ml and the LOQ‟s were 0.172µg/ml, 0.4203µg/ml and 0.65µg/ ml respectively. The result was shown in Table 8 (Dewani, *et al.*,2015).

Table 8: LOD and LOQ

Statistical Data	**Aceclofenac**	**Paracetamol**	**Chlorzoxazone**
Mean	329557	958512.6	344742.66
LOD	0.057	0.008	0.214
LOQ	0.172	0.420	0.65

Table 9: Preparation of tablet Sample Solution

Parameters	Aceclofenac	Paracetamol	Chlorzoxazone
Mean	100.06	99.65	94.65
S.D.	1.252	0.6888	0.2320
% R.S.D.	1.242	0.6918	0.2451

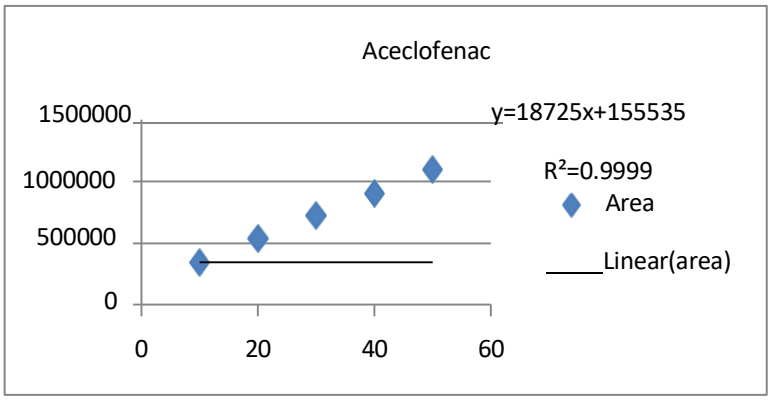

Figure 4a: Calibration curve of ACF

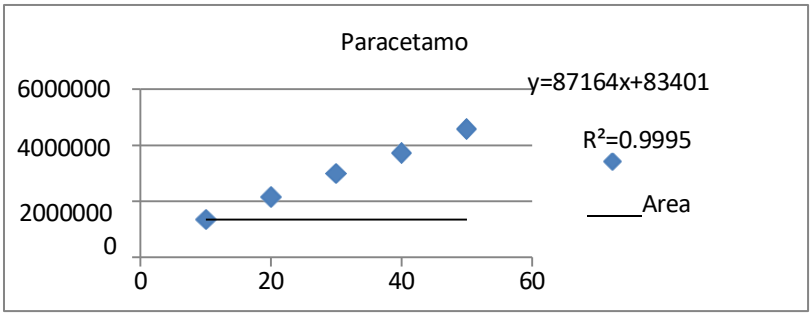

Figure 4b: Calibration Curve of PR

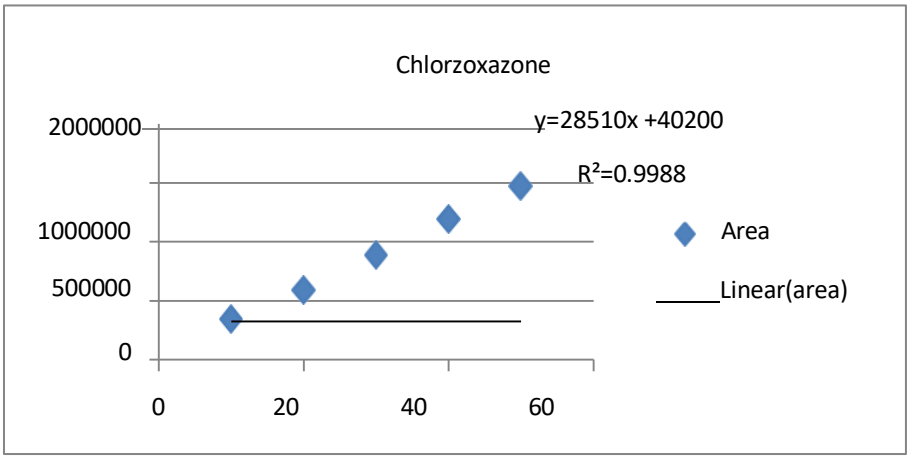

Figure 4c: Calibration Curve of CLZ

Comparison of developed method and reposted method:

The main objective of present research work to developed a simple, rapid, accurate, sensitive, reproducible and economic method for simultaneous estimation of ACF, PR and CLZ in tablet dosage form by using RP-HPLC method the developed methods compared with three previously reported methods i.e. R. Joshi, K.A. Shaikh and P.P. Desai (Table 10) (Chauhan, *et al.*,2014). Firstly, develop method compared with R. Joshi et al. reported method linearity 5-30 µg/ml, 5-50 µg/ml and 5-50 µg/ml and developed method 10-50 µg/ml, 32.5-162.5 µg/ml and 25-125 µg/ml for ACF, PR and CLZ respectively. Precision of reported method 0.5172, 0.9424 and 0.6495 and for develop method 0.4345, 1.175 and 1.99 for ACF, PR and CLZ. % recoveries of reported method fond at 100.5-100.23, 99.5-101.1 and 99.90-100.3 and for develop method 95.32-97.98, 98.74- 99.93and 96.75-98.79 for ACF, PR and CLZ respectively. Limit of detection and limit of quantization for reported method 4.8, 16.2, 14.6 and 14.5, 49.5, 0.22 and for developed method 0.0570, 0.0086, 0.2149 and 0.172, 0.4203, 0.65 for ACF, PR and CLZ (Yeola, *et al.*,2023).

K.A. Shaikh et. al. reported method gives linearity 2.5-20 µg/ml, 12.5-100 µg/ml and 12.5-100 µg/ml and for developed method 10-50 µg/ml, 32.5-162.5 µg/ml and 25-125 µg/ml for ACF, PR and CLZ. Precision for reported method 1.59, 1.21 and 1.46 and for develop

method 0.4345, 1.175 and 1.99. For ACF, PR and CLZ. Accuracy for reported method 100.4-101.0, 100.7-101.4 and 100.5-101.3 and for develops method 95.32-97.98, 98.74-99.93 and 96.75-98.75 for ACF, PR and CLZ. LOD and LOQ for reported method 0.074, 0.027, 0.067 and 0.22, 0.081, 0.201 for ACF, PR and CLZ for Develop method i.e. 0.0570, 0.0086, 0.2149 and 0.172, 0.4203, 0.65 for ACF, PR and CLZ % assay for reported method 101.2, 101.5 and 100.5 and for develop method 100.06, 99.65 and 94.45 for ACF, PR and CLZ (Sonawane, *et al.*,2023).

P.P. Desai et. al. reported method shows linearity 5-80 µg/ml, 25-400 µg/ml and 25-400 µg/ml and for develop method 10-50 µg/ml, 32.5-162.5 µg/ml and 25-50 µg/ml for ACF, PR and CLZ. precision for reported method 1.26, 1.57 and 1.74 and develop method 0.4345, 1.175 and 1.99 for ACF, PR and CLZ. accuracy for reported method 99.81-100.32, 100.05-101.04, 99.96-100.20 and for develop method 95.32-97.98, 98.74-99.93, 96.75- 98.79 for ACF, PR and CLZ. LOD and LOQ for reported method 0.66, 2.89, 2.57 and 2.02, 8.79, 7.79 for ACF, PR and CLZ. For Develop method 0.0570, 0.0086, 0.2149 and 0.172, 0.4203, 0.65 for ACF, PR and CLZ. % assay for reported method 98.63, 101.66 and 99.98 and for develop method 100.66, 99.45 and 94.75 for ACF, PR and CLZ (Sonawane, *et al.*,2023).

Table10: Comparative Study

Parameters	**Developed Method**			**Reported Method**								
				R. Joshi et. al. [58]			**K. A. Sheikh et. al. [56]**			**P.P. Desai et. al.[57]**		
	ACF	PR	CLZ	ACF	PR	CLZ	ACF	PR	CLZ	ACF	PR	CLZ
Linearity (µg/ml)	10-50	32.5-162.5	25-125	5-30	5-50	5-50	2.5-20	12.5-100	12.5-100	5-80	25-400	25-400
Precision- a) Repeatability i)intraday-	0.4345	1.175	1.99	0.517	0.942	0.649	1.59	1.21	1.46	1.26	1.57	1.74
b) Intermediate Precision Day to Day and Analyst to Analyst	0.4058	1.348	0.9812	-	-	-	-	-	-	-	-	-
	0.3655	1.0276	0.8324	-	-		-	-	-	-	-	-

Accuracy (% recovery)	1.1756 95.32- 97.98	0.6055 98.74- 99.93	1.1647 96.75- 98.79	100.5- 100.23	99.5 -101.1	99.90- 100.3	100.4- 101.0	100.7- 101.4	100.5- 101.3	99.81 - 100.3 2	100.05 - 101.04	99.96- 100.20
LOD (µg/mL)	0.0570	0.0086	0.2149	4.8	16.2	14.6	0.074	0.027	0.067	0.66	2.89	2.57
LOQ (µg/mL)	0.172	0.4203	0.65	14.5	49.0	46.5	0.22	0.081	0.201	2.02	8.78	7.79
Robustness A) change in wavelength λmax-267 λmax-268 λmax-269 change in flow rate 0.9 ml/ min 1.0 ml/ min 1.1 ml/ min	 0.211 0.5085 0.7932 0.1839 0.5085 1.0521	 1.6220 0.6956 0.3278 0.6711 0.6956 0.6558	 0.316 1.2420 0.6569 0.4811 1.240 0.6442	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -	- - - - - - -
System suitability R.T. AUC Asymmetry e)Resolution	1.5 min 341076 1499 1.2619 0.00	2.6 min 951006 3107 1.1871 2.3193	3.2 Min 3435222960 0.00 2.8123	7.605min - 4216 -	2.055Min 3491 - 15.33	5.096Min 6483 - 7.06	8.56Min 6426 - 9.10	2.85min 5069 - 0	7.72min 10798 - 9.96	3.10Min 3981 1.08 1.98	2.60 Min 1386 0.96 1.62	2.03mi n 2953 0.87 0
% Assay	100.06	99.65	94.45	99.87	99.89	99.83	101.2	101.5	100.5	98.63	101.34	99.98

										±1.33	± 4.54	±3.96

Result and discussion:

The RP-HPLC method development for simultaneous estimation of ACF, PR and CLZ from combined dosage form. RP-HPLC method development different mobile phases were tried. ACN: H2O (80:20 v/v) mobile phase gave satisfactory separation, good peak symmetry, good resolution. Chromatographic separation was achieved on Protocol C18 ENDURO (250mm×4.6mm, 5µm) column and flow rate 1.0ml/min. detection of analytes at 268nm using PDA detector. The developed method separates the analytes simultaneously i.e. ACF 1.673 min, for PR 2.640 min and for CLZ 3.220min. The excipients in the formulation did not interfere in the accurate estimation of Aceclofenac, PR and Chlorzoxazone. The method was validated according to ICH guidelines in terms of linearity, precision, accuracy, robustness, LOD and LOQ. The developed method compared with three reported methods. Form that we observe that developed method was easy to perform easily adopted and also required very less time for separation of analytes as compared to reported methods.

Conclusion:

The present study was undertaken with an object of developing suitable, sensitive and simple analytical method development and validation of RP-HPLC. In the present work application for RP-HPLC for analysis of selected drug formulation was successfully attended using HPLC Agilent 1260 infinity II) with PDA detector. The mobile phase composed of ACN: H2O (80:20 v/v) with flow rate 1.0ml/min and detection wavelength 268nm. Developed method required short run time in which ACF, PR and CLZ separated at 1.67 min, 2.64min and 3.22 min. with good resolution used were advantages and made the routine analysis. The developed method compared with other three reported methods from above comparison developed method takes less time for separation of analyte than reported method and they did not used buffers. Because of all that developed method was best for routine analysis. Among the significant advantages of this method are simplicity, selective, accuracy and precision ensuring that it is suitable for the result of recovery studies was found to be range of 98.65% to 100.06%. It indicates that there is no interference of excipients in the formulation. The method is accurate, simple, rapid, precise, reliable, sensitive, reproducible and economic and validated as per ICH guidelines.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIAL

All data and materials are available upon request.

COMPETING INTERESTS

The authors declare that they have no competing interests

FUNDING

There is no funding source for this project

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