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DEVELOPMENT AND VALIDATION OF A REVERSED PHASE UHPLC METHOD FOR SIMULTANEOUS DETERMINATION OF DICLOFENAC, PARACETAMOL AND CHLOROZOXAZONE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
The purpose of this present work is to develop and validate a simple, linear, accurate and precise reversed phase U-High Performance Liquid Chromatography method for the simultaneous determination of Diclofenac Paracetamol and Chlorozoxazone in tablet dosage form (fixed dose combination formulation). For this, the analysis was carried out by High Performance Liquid Chromatography using U-HPLC System (Thermo- Dionex) equipped with an UV detector and stainless steel column (25cm × 4.6mm × 5µm) packed with octadecylsilane chemically bonded to porous silica column. The mobile phase contain buffer, adjusted to pH 7.5 with 1(M) sodium hydroxide and Acetonitrile in the ratio of 60:40 v/v with a flow rate of 1.5 mL/min and detection wavelength 272 nm. The retention time of Paracetamol Diclofenac and Chlorozoxazone was found to be 1.698, 2.368 and 3.528 respectively. The method was found to be linear in the concentration range of 10-50 µg/mL for Diclofenac, 100-500 µg/mL for Paracetamol and 100-500 µg/mL for chlorozoxazone with correlation coefficient of 0.993, 0.999 and 0.994 for the three drugs respectively. The %RSD of 0.660, 0.897 and 0.790 for intra-day and 0.589, 0.856 and 0.768 for inter-day precision, respectively for of diclofenac, paracetamol and chlorozoxazone suggest the precision of the method as all these values are less than 2%. The method has shown good, consistent recoveries for of diclofenac (109.52), paracetamol (105.73) and chlorozoxazone (107.64). The method was found to be accurate, precise, specific, robust and linear for the determination of diclofenac, paracetamol and chlorozoxazone in Pharmaceutical dosage form.

KEYWORDS: UHPLC; Validation, Diclofenac, Paracetamol, Chlorozoxazone.

INTRODUCTION
Paracetamol and Diclofenac are non-steroidal anti-inflammatory drugs (NSAID) available as over the counter drug commonly used as analgesic and antipyretic in the management fever and as well provides relief from mild to moderate pain. Paracetamol (acetaminophen) has weak anti-inflammatory effects since it has poor ability to inhibit cyclooxygenase (COX) in the presence of high concentration of peroxides, as are found at sites of inflammation. Chemically Paracetamol is 4- hydroxy acetanilide. Diclofenac [[2-(2', 6'-dichlorophenyl) amino] phenyl acetoxyacetic acid] is a phenyl acetic acid derivative belongs to the group of non-steroidal anti-inflammatory drug (NSAID).[1-3] Chlorzoxazone (5-chloro-2, 3-dihydro-1, 3-benzoxazol-2- one) is a centrally acting muscle relaxant used to treat muscle spasm and the resulting pain and discomfort.[3] Chlorzoxazone may act by inhibiting calcium and potassium influx which would lead to neuronal inhibition and muscle relaxation.[4]

All conventional NSAIDs inhibit the conversion of arachidonic acid (AA) into prostaglandin H - PGH2. The stage is catalyzed by prostaglandin H synthase (PGHS), at present referred to as cyclooxygenase (COX) within which iso enzymes COX-1 (PGHS-1) and COX-2 (PGHS-2) occur. PGHS is a bi functional enzyme and possesses two different enzymatic activities: cyclooxygenase and peroxidase (POX). The conversion of AA→PGH2 involves two reactions: cyclization of AA to unstable 15-hydroxyperoxide (PGG2) with the involvement of a cyclooxygenase component and double oxidation in position 9 and 11; whereas the reduction of PGG2 molecule to its 15- hydroxy analogue, unstable structure of PGH2, takes place due to peroxidase activity of PGHS (POX). Prostaglandin H2 (PGH2) is a substrate for specific synthases, tissue-dependent isomerases catalyzing its further conversions into different endogenous regulators, namely: prostaglandins of the D (PGD2), E (PGE2), F (PGF2) series and prostacyclin (PGI2; prostacyclin is not a prostaglandin and a commonly used abbreviation is historically conditioned)

and thromboxanes (TXA2 and TXB2). They all are characterized by different biological activity and many of them have anti-inflammatory properties. Thus, the action of NSAIDs, which inhibits the stage of conversion AA→PGH2, and also the formation of the aforementioned regulators, have some favorable (anti-inflammatory, analgesic and antipyretic) and side effects (associated with the inhibition of synthesis of particular regulators in different tissues). A precise mechanism of NSAID action together with therapeutic and side effects has been presented in the recently published large study by Nowak and Dzielska-Olczak and Nowak.[5-6] Diclofenac sodium is a nonsteroidal anti-inflammatory drug designed by selection of appropriate physicochemical and steric properties. Its pharmacologic activity, specifically its effects in acute and subchronic inflammation, and its analgesic activity have been assessed in animal models. The tolerability of the compound as judged by several parameters (i.e., ratio between the acute lethal dose or the dose inducing gastrointestinal blood loss and the desired pharmacologic activity) is favorable in comparison with other nonsteroidal anti-inflammatory drugs, Diclofenac sodium acts by potent cyclo-oxygenase inhibition, reduction of arachidonic acid release, and enhancement of arachidonic acid uptake. It thereby results in a dual inhibitory effect on both the cyclo-oxygenase and lipoxygenase pathway.[7]

MATERIALS AND METHODS

Paracetamol, Diclofenac and Chlorozoxazone W/S were obtained as gift samples from Reddy's Laboratories, Hyderabad, India. Acetonitrile and Water (HPLC grade), sodium hydroxide and orthophosphoric acid were obtained from Merck Chemicals, Mumbai, India. Commercially available tablets (Diclofenac 50mg, Paracetamol 325mg and Chlorozoxazone 500mg) were procured from local market.

Chromatography instruments and conditions

The Separation was performed by High Performance Liquid Chromatography using U-HPLC System (Thermo- Dionex) equipped with an UV detector and stainless steel column (25cm × 4.6mm × 5µm) packed with octadecylsilane chemically bonded to porous silica column. The mobile phase contain buffer, adjusted to pH 7.5 with 1(M) sodium hydroxide and Acetonitrile in the ratio of 60:40 v/v with a flow rate of 1.5 mL/min and detection wavelength 272 nm. All the solutions were filtered through a 0.45 micron membrane filter paper and degassed before use.

Preparation of standard solution

Stock solutions were prepared by dissolving 10mg of Diclofenac Paracetamol and Chlorozoxazone in 10ml of the mobile phase to obtain a final concentration of 1000mcg.

Preparation of sample solution

Twenty commercially available tablet containing Diclofenac, Paracetamol and chlorozoxazone (50mg, 325mg and 500 mg respectively) were weighed. The average weight was calculated and powdered. A quantity equivalent to 50mg of diclofenac was accurately weighed and transferred to a 100ml of volumetric flask. The sample was sonicated with the mobile phase. Further dilutions were made to get a concentration equivalent to the linearity range. The results are shown in Table 1.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

To develop a method for the analysis of the drugs, preliminary tests were performed in order to select adequate and optimum conditions. Parameters such as detection wavelength, ideal mobile phase and its combination, optimum pH and concentration of the standard solutions were studied. Mobile phase consisting of buffer and Acetonitrile (60:40 v/v). Flow rate was adjusted to 1.5 mL/min and UV detection at 272 nm was selected for analysis. Retention time of Diclofenac, Paracetamol and Chlorozoxazone were found to be 2.368, 1.698 and 3.528 min respectively.

Method validationSystem suitability Six replicate of sample containing Paracetamol diclofenac and chlorozoxzone were given to evaluate equipment, analytical operations and samples suitability. Parameters calculated for system suitability were %Relative Standard deviation, Retention time, Number of theoretical plates and Tailing factor.

Linearity

Linearity for this analysis was established by least squares linear regression analysis. The calibration curves were found to be linear over the concentration range of 10-50 mg/ml for Diclofenac, 100-500 mg/ml for Paracetamol and 100-500 mg/ml for Chlorozoxazone. Further the peaks were plotted versus their respective concentrations and linear regression analysis was performed. The correlation coefficients were found to be 0.993, 0.999 and 0.994 for Diclofenac Paracetamol and Chlorozoxazone respectively. (Table 1).

Precision

The precision of the method was determined by intra-day and inter-day precision studies at 100% test concentration by measuring the sample three times on the same day at intervals of 1 hour and on three different days for intra and inter day studies respectively. Further standard deviation and relative standard deviation were calculated (Table 2).

Accuracy

The accuracy of the method was proven by recovery test. Known amounts of Diclofenac, Paracetamol and Chlorozoxazone standard (50, 100 and 150% level were added to the already analyzed sample solutions and the analysis was carried out. The method has shown good,

consistent recoveries for Diclofenac Paracetamol and Chlorozoxazone shown in Table 3.

Robustness: The robustness of the method was checked by deliberately varying the mobile phase composition, wavelength and flow rate which shows that the small changes of the method parameters do not interfere the performance of the method. All the results obtained were in accordance with the results for original conditions. The %RSD value obtained for the assay in the changed condition was less than 2% which indicates the robustness of the proposed method.

Solution stability

The %RSD of the peak areas of the test samples were less than 1% for 24 hrs which indicates that the sample was stable under proposed mobile phase condition within this period only.

LOD and LOQ

LOD for Diclofenac, Paracetamol and Chlorozoxazone were found to be 0.06 µg/mL, 0.08 µg/mL and 0.02 µg/mL respectively. LOQ for Diclofenac, Paracetamol and Chlorozoxazone were found to be 0.12 µg/mL, 0.25 µg/mL and 0.06 µg/mL respectively. Table 4.

Analysis of commercial formulations

Developed method was applied for the determination of diclofenac, paracetamol and chlorozoxazone in pharmaceutical dosage form and the results obtained were presented in Table 5. The assay value of 109.52, 105.73 and 107.64 for diclofenac, paracetamol and chlorozoxazone indicates that the method is selective for the assay of diclofenac, paracetamol and chlorozoxazone without interference from any of the excipients of the tablet dosage form. Table 5.

Table 1: Linearity and Correlation coefficient.

Parameters	Diclofenac	Paracetamol	Chlorozoxazone
Regression equation	y = 0.499x + 0.41	y = 0.250x + 0.444	y = 0.222x - 3.956
Linearity (µg/ml)	10-50	100-500	100-500
Correlation coefficient	0.993	0.999	0.994

Table 2: Precision studies.

Drug	Concentration µg/ml	Intraday Precision n=3 %RSD	Interday Precision n=3 %RSD
Diclofenac	100 µg/ml	0.660	0.589
Paracetamol	100 µg/ml	0.897	0.856
Chlorozoxazone	100 µg/ml	0.790	0.768

Table 3: Accuracy.

Drug	Amount taken	Amount added	Amount recovered	% Recovery % *RSD	% Recovery % *RSD
Diclofenac		25	24.2		
	50	50	49.2	98.17	0.653
		75	74.5		
Paracetamol		162.5	160.1		
	325	325	323.5	99.20	0.543
		487.5	486		
Chlorozoxazone		250	248.5		
	500	500	498.6	99.63	0.746
		750	748.3		

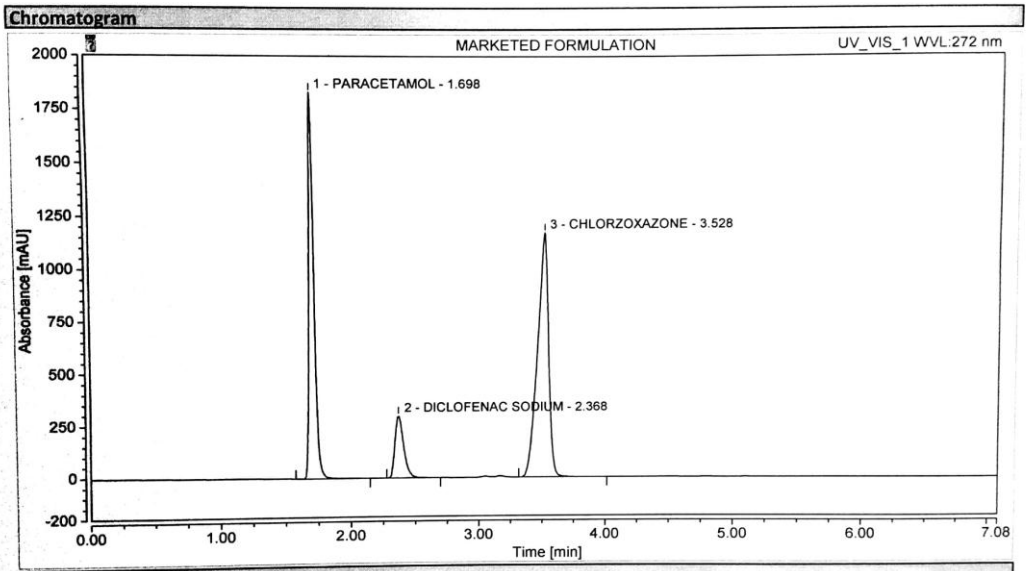
*Mean of six observations

Table 4: LOD and LOQ studies.

Validation parameters	Diclofenac	Paracetamol	Chlorozoxazone
LOD µg/ml	0.06	0.08	0.02
LOQ µg/ml	0.12	0.25	0.06

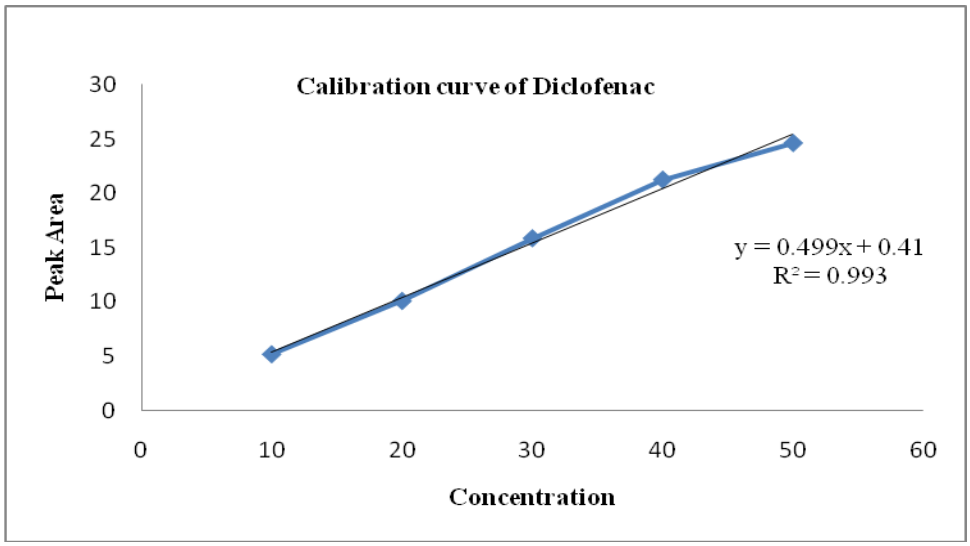
Table 5: Analysis of Formulation.

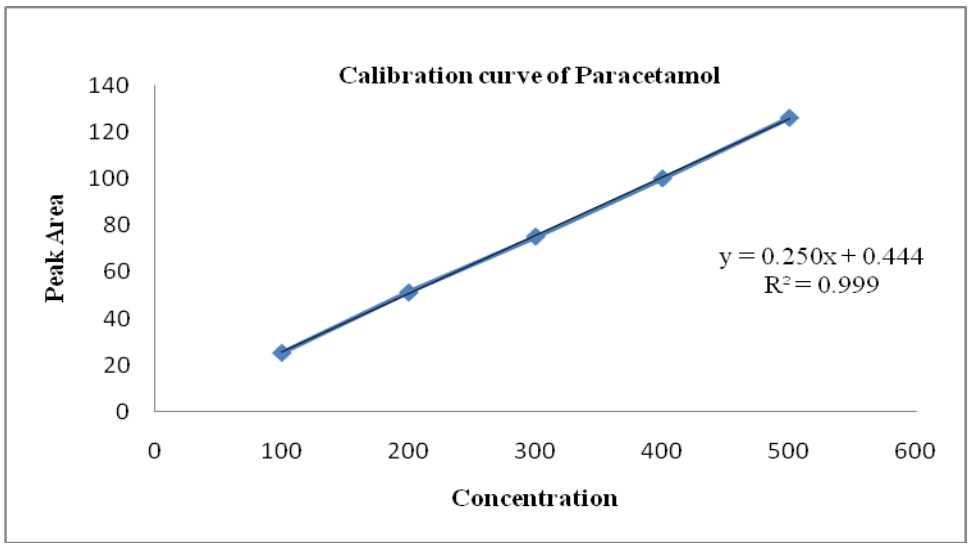
Drug	Labelled amount (mg/tablet)	Amount found (mg/tablet)	%Label claim	%*RSD
Diclofenac	50	54.76	109.52	0.50
Paracetamol	325	343.63	105.73	0.36
Chlorozoxazone	500	538.22	107.64	0.65

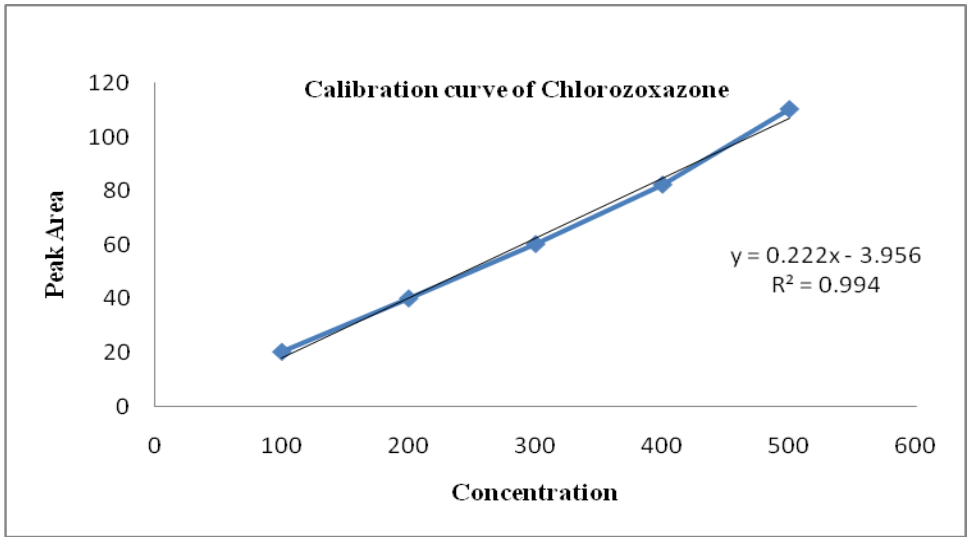


Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	PARACETAMOL	1.698	83.998	1841.379	36.99	55.40	n.a.
2	DICLOFENAC SODIUM	2.368	23.671	300.677	10.42	9.05	n.a.
3	CHLORZOXAZONE	3.528	119.397	1181.825	52.58	35.56	n.a.
Total:			227.066	3323.881	100.00	100.00	







CONCLUSION

The proposed U-HPLC method was found to be simple, precise, accurate and specific for the simultaneous estimation of Diclofenac, Paracetamol and Chlorozoxazone. The newly developed method is simple and cost effective as it uses simple mobile phase without ion-pairing reagent which was previously unreported. The separation was done in 5 minutes only. The method was validated as per ICH guidelines. All other parameters such as specificity, linearity, precision, accuracy, robustness passes the criteria set forth by ICH guidelines. It is seen that was no interference from any components of the formulation. Hence, this method can be easily used for routine quality control analysis of Diclofenac, Paracetamol and Chlorozoxazone in pure and its combined dosage form.

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