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A VALIDATED RP HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF PARACETAMOL, DICLOFENAC SODIUM AND CHLORZOXAZONE IN COMBINATION TABLET DOSAGE FORM

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ABSTRACT

A simple, precise, rapid and accurate RP-HPLC method with UV detection has been developed for the estimation of Paracetamol (PCM), Diclofenac sodium (DFS), Chlorzoxazone (CHZ) in Pharmaceutical dosage form. A WELCHROM C_{18} column (4.6mm X 250mm, 5µm), SHIMADZU LC-20AT prominence liquid chromatograph was used. An isocratic mode with mobile phase consisting of phosphate buffer (pH 6.65): acetonitrile (60:40 v/v) with a flow rate of 1 mL/min and the effluent was monitored at 280 nm . The chromatogram showed a peak of 3.20 min for PCM, 7.52 min for DFS, 8.84 min for CHZ. The detector responses for PCM, DFS, CHZ were found to be in the range of 10-50 µg/mL, 1-5 µg/mL, and 5-25 µg/mL respectively. The respective linear regression equations being Y=13.101X+7.748 for PCM, Y= 34.175X+0.3932 for DFS, Y= 15.352X-0.3408 for CHZ. The limit of detection (LOD) for PCM, DFS, CHZ were found to be 1.681 µg/mL, 0.171 µg/mL and 0.604 µg/mL. The limit of quantitation (LOQ) for PCM, DFS, CHZ were found to be 5.094 µg/mL, 0.518 µg/mL and 1.830 µg/mL respectively. The percentage Assay of PCM, DFS, CHZ was 100.891±1.946%, 101.638±1.817% and 98.492±1.354% respectively. The proposed method was validated as per ICH guidelines and successfully applied for the quantitative routine determination of PCM, DFS and CHZ in pharmaceutical dosage forms.

KEY WORDS - Paracetamol, Diclofenac sodium, Chlorzoxazone, HPLC.

1. INTRODUCTION

PCM (Fig 1(a)) also known as Acetaminophen, is commonly used for its analgesic and antipyretic effects. Its therapeutic effects are similar to salicylates, but it lacks anti-inflammatory, anti- platelet, and gastric ulcerative effects. PCM is a commonly used analgesic and antipyretic drug that is used for the relief of fever, headaches, and other minor aches and pains. It is a major ingredient in numerous cold and flu medications and many prescription analgesics. Acetaminophen, unlike other common analgesics such as aspirin and ibuprofen, has no anti-inflammatory properties or effects on platelet function, and it is not a member of the class of drugs known as non-steroidal anti-inflammatory drugs or NSAIDs. PCM is thought to act primarily in the CNS, increasing the pain threshold by inhibiting both isoforms of cyclo-oxygenase, COX-1, COX-2, and COX-3 enzymes involved in prostaglandin (PG) synthesis.

DFS (Fig 1(b)) is a non-steroidal anti-inflammatory agent (NSAID) with antipyretic and analgesic actions. It is primarily available as the sodium salt. DFS is used to treat pain, dysmenorrhea, ocular inflammation, osteoarthritis, rheumatoid arthritis and ankylosing spondylitis, The anti-inflammatory effects of DFS are believed to be due to inhibition of both leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-2), leading to the peripheral inhibition of prostaglandin synthesis.

CHZ (Fig (c)) is a centrally acting muscle relaxant used to treat muscle spasm and the resulting pain or discomfort. It acts on the spinal cord by depressing reflexes. CHZ, a synthetic compound, inhibits antigen-induced broncho spasms and, hence, is used to treat asthma and allergic rhinitis. CHZ is also a centrally-acting agent for painful musculoskeletal conditions.. CHZ inhibits degranulation of mast cells, subsequently preventing the release of histamine and slow-reacting substance of anaphylaxis (SRS-A), mediators of type I allergic reactions. CHZ also may reduce the release of inflammatory leukotrienes.

Literature survey reveals that several methods are available for the estimation of PCM, DFS, CHZ individually in biological fluids like plasma and pharmaceutical formulations. Reported methods for estimation of PCM, DFS, CHZ in dosage form are HPLC (Shinde, 1995) (Goyal and Jain, 2007) (Shaikh and Deukhile, 2008) (Joshi and Sharma, 2008) (Pawar, 2009) (Grag and Saraf, 2007) (Karthikeyan, 2009) (Ravisankar, 2013). But most of these methods are PCM, CHZ along with aceclofenac. Only two RP-HPLC methods have been reported for combination of PCM, CHZ

with Diclofenac sodium. Hence properly developed and validated analytical method is necessary for quality control of the drugs in market. The available methods are either poorly validated or uneconomical. In fact a properly validated and economical method is needed. Therefore the present research work aims to develop a simple, accurate, precise, sensitive and reproducible method for simultaneous determination of PCM, DFS, and CHZ in combined dosage form by RP-HPLC method.

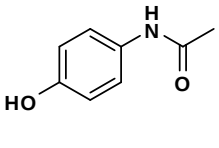

Figure 1(a): Paracetamol

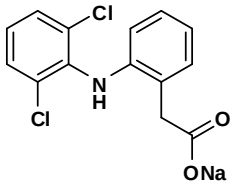

Figure 1(b): Diclofenac sodium

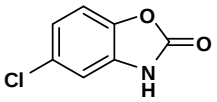

Figure 1(c): Chlorzoxazone

Figure 1: Chemical structures of the studied drugs

2. MATERIALS AND METHODS

Instruments used: HPLC was performed on an isocratic High Performance Liquid Chromatograph (Shimadzu LC-20AT Prominence Liquid Chromatograph) equipped with a LC-20AT VP pump. Sample injection was performed via a Rheodyne (USA) sample injector with a 20 µL loop and a Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} Column (4.6 mm i.d. X 250mm, 5µm particle size). The HPLC system was equipped with "Spinchrom" software for data acquisition and quantification of peaks. The pH of the solution was measured using digital pH meter (Systronics model 802). A UV spectrum of all drugs was recorded for selection of working wavelength of detection by using a UV-Visible Spectrophotometer (Systronics model 2203). In addition an electronic balance (Shimadzu TX223L), a sonicator (spectra lab, model UCB 40) was used in this study.

Materials used: PCM, DFS and CHZ are pharmaceutical grade was kindly supplied as gift sample by Hetero drugs limited, Hyderabad, Andhra Pradesh, India. All chemicals were analytical grade. Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate and phosphoric acid from S.D Fine-Chem. Ltd., Mumbai, India. Acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Limited (Mumbai, India). Commercial tablets containing PCM 500 mg, DFS 50 mg and CHZ 250 mg was purchased from local market. INTRAGESIC –MR tablets are manufactured by intra labs India private Ltd., Bangalore, India.

Preparation of solutions and Reagents:

a. Mobile phase: A freshly prepared 60:40 v/v, mixture of phosphate buffer and acetonitrile was used as mobile phase. The phosphate buffer was prepared freshly by dissolving 1.780 g of KH_2PO_4 and 0.345 g K_2HPO_4 in 600 mL of HPLC grade water. The mixture was filtered through 0.45 µm nylon membrane vacuum filter and degassed by ultrasonication.

b. Preparation of standard stock solutions: Accurately 100 mg of PCM, 50 mg of CHZ, 10 mg of DFS were weighed and transferred to a 100 ml of clean dry volumetric flask, and 60ml of mobile phase was added and sonicated to dissolve. The volume was made up to the mark with mobile phase. This is standard stock solution of PCM with concentration of 1mg/mL, DFS with concentration of 0.5mg/mL, CHZ with concentration of 0.1mg/mL.

c. Preparation of working standard solutions: From the stock solution, working standard solutions are prepared by taking 2ml of above stock solution and make up to 20 ml. Aliquots of the above solution were taken in 1ml, 2ml, 3ml, 4ml, 5ml in a 10ml volumetric flasks and made up to the mark by using mobile phase. Final dilution ranges achieved for PCM are 10-50 µg/mL, DFS are 1-5 µg/mL, and CHZ are 5-25 µg/mL respectively.

d. Preparation of sample solutions: Twenty tablets of were weighed, and then powdered. A sample of powdered tablets, equivalent to 500 mg of PCM, 50 mg of DFS and 250 mg of CHZ active ingredients, was diluted to 100 mL with mobile phase in 100 mL of volumetric flasks. The mixture was allowed to stand for one hour with intermittent sonication to ensure complete solubility of the drug and then filtered through 0.45µm nylon membrane filter. The

above filtrate was suitably diluted to give a sample mixture containing approximately 25 µg/mL of PCM, 12.5 µg/mL of CHZ and 2.5 µg/mL of DFS respectively.

e. Selection of detection wavelength: The overlain UV spectra of various diluted solutions of PCM, DFS and CHZ in mobile phase were recorded using UV spectrophotometer. The isobestic point of maximum absorbance was observed at 280 nm. This wavelength was used for detection of PCM, DFS and CHZ.

f. Construction of calibration curve: The standard stock solution of each drug was suitably diluted with the mobile phase to obtain Standard solutions of different concentrations. Replicates of each calibration standard solutions PCM 10-50 µg/mL, DFS 1-5µg/mL, CHZ 5-25µg/mL were injected using a 20µl fixed loop system and the chromatograms were recorded. Each standard solution was injected six times into the column at a flow rate of 1mL/min. Calibration curves were constructed by plotting by taking concentration of PCM, DFS and CHZ on X-axis and ratio of peak areas of standard PCM, DFS and CHZ on Y-axis and regression equations were computed for PCM, DFS and CHZ.

Validation of the proposed method: The developed method was validated as per International Conference on Harmonization (ICH) guidelines.

System suitability: System suitability tests are an integral part of chromatographic system.To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug sample solution of PCM, DFS and CHZ to check the reproducibility of the system. At first the HPLC system was stabilized for forty min. One blank followed by six replicates of a single sample solution of PCM, DFS and CHZ was injected to check the system suitability. To ascertain the systems suitability for the proposed method, a number of parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken and results were presented in Table 1.

Specificity: Specificity of the method is performed by separate injections of PCM, DFS and CHZ standard and sample. Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc., Specificity of the method was performed by comparing the chromatograms of blank, standard and sample. It was found that there is no interference due to excipients in the tablet formulation and also that there is good correlation between the retention times of standard and sample. Therefore, it was concluded that the method is specific. The specificity results are tabulated in Table 2.

Linearity: Aliquots of primary working standard solution containing PCM, DFS and CHZ were diluted such a way that the final concentrations of PCM, DFS and CHZ are in the range of 10-50 µg/mL, 1-5 µg/mL and 5-25 µg/mL respectively. The linearity graphs for the proposed assay methods were plotted over the concentration range. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed. The correlation coefficient of PCM, DFS and CHZ was found to be almost equal to 1. Calibration data and the regression data of the proposed method results are presented in Table 3 and 4 respectively.

Precision: Precision of an analytical procedure is the closeness of agreement (Degree of scatter) between a series of measurements obtained from multiple sampling of the sample homogeneous sample under the prescribed conditions. Precision of the method was performed as intra-day and inter-day precision. To study the intra-day precision, six replicate standard solutions of PCM, DFS, and CHZ were injected. The % RSD was calculated. This is within the acceptable criteria of not more than 2.0. The values of percentage of RSD obtained in intra and interday precision results are presented in Table 5.

Accuracy (Recovery studies): Accuracy of an analytical procedure is the closeness of agreement between the conventional true value or an accepted reference value and the value found. The accuracy was determined by adding a known amount of standard drug to the fixed amount of pre-analyzed tablet solution. Accuracy studies were performed for PCM, DFS and CHZ at three different levels (80%, 100% and 120%) and the mixtures were analyzed in triplicate by the proposed method. Known amount of standard PCM, DFS and CHZ 80%, 100% and 120% of predetermined sample was added to a pre quantified tablet sample. The % recovery was calculated and the results are presented in Table 6.

Robustness: The robustness of the developed method was determined by analyzing the samples under a variety of conditions of the method parameters such as variation of the pH of the buffer, flow rate, detection wavelength and mobile phase composition. It was observed that there were no significant effect on chromatographic parameters which demonstrated that the developed method was robust in nature. The complete results are shown in Table 7.

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

individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.LOD and LOQ were calculated by formula LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve. The LOD and LOQ of PCM, DFS and CHZ by proposed method are abridged in Table 8.

Assay: 20 µL of sample solution was injected into Liquid Chromatograph. The assay was repeated for six times and the amount of the drug present per tablet was determined from calibration equation. The mean % recovery was determined. The % drug content was found to be 100.891±1.946 %, 101.638 ±1.817% and 98.492±1.354 % of the labeled amount for PCM, DFS and CHZ respectively. The results of formulation analysis are given in Table No.9.

Table.1.Instrumentation optimized chromatographic conditions and system suitability parameters for proposed method.

Parameter	Chromatographic conditions		
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph		
Column	WELCHROM C_{18} Column (4.6 X 250mm, 5µm)		
Detector	SHIMADZU SPD-20A prominence UV-Vis detector		
Diluents	Buffer: Acetonitrile (60:40 v/v)		
Mobile phase	Buffer: ACN (60:40 v/v)		
Flow rate	1mL/min.		
Detection wave length	By UV at 280 nm.		
Run time	10 minutes		
Column back pressure	125-135 kgf		
Temperature	Ambient temperature(25^{o}C)		
Volume of injection loop	20 µL		
	PCM	**DFS**	**CHZ**
Retention time, min.	3.20	7.52	8.84
Theoretical plates[th.pl] (Efficiency)	10549	17623	22088
Theoretical plates/meter[t.p/m]	210978	352450	441762
Peak asymmetry	1.22	1.02	1.09
Resolution	-	24.66	5.699

3. RESULTS AND DISCUSSION

To optimize the HPLC parameters, several mobile phase compositions were tried. Decorous peak symmetry, plate count and resolution were obtained with mobile phase consisting of phosphate buffer (pH-6.65): acetonitrile in the ratio of 60:40 v/v. Optimized separation of PCM, DFS and CHZ was achieved by using a C_{18} column (250 mm X 4.6 mm i.d, 5 µm particle size). Various column types and lengths were tried regarding other chromatographic parameters. C_{18} column with a 4.6 mm inner diameter, 250 mm length and 5 micron particle size was preferred. Runtime was less than 10 min at a flow rate of 1 mL/min. The quantification was achieved with detection at 280 nm obtained from UV overlain spectra of PCM, DFS and CHZ. The injection volume was 20 µL. Under the conditions described above, separation of the mixture of PCM, DFS and CLZ was achieved with a total run-time of 10 min, with an elution window of 8 min for all the three analyses. System suitability parameters were very satisfactory (Table 1.). The proposed method was found to be specific for PCM, DFS and CHZ drugs and no interferences from placebo with PCM, DFS and CLZ peaks (Table 2.) Chromatogram of placebo is shown in Fig.2. The linear dynamic range of PCM 10-15 µg/mL, DFS 1-5µg/mL, CHZ 5-25µg/mL met all specifications with R^2> 0.999 which states that the method was linear to the concentration vs peak area responses. The regression equation obtained from linearity plot for PCM was Y=13.101X+7.748 with R^2= 0.9994, DSF was Y=34.175X+0.3932 with R^2 = 0.9994 and for CHZ was Y=15.352X-0.3408 with R^2=0.9994 which indicates this method had good linearity (Table 3 and Table 4). The calibration plot for PCM, DFS and CHZ were shown in Fig. 3, Fig. 4 and Fig. 5 respectively. The proposed method was found to be precise for the determination of PCM, DFS and CHZ. The % RSD for the proposed method was found to be less than 2.0 which indicate the proposed method is reproducible. Results of the precision study are shown in the Table 5. Recovery studies (Table 6) of the method was found to be good within the overall mean % recovery of the tablet dosage form. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, λ_{max}, mobile phase composition. The proposed method was found to be robust as there were no marked changes in the chromatograms. The Robustness results are presented in Table 7. The LOD and LOQ for the estimation of PCM was found to be 1.681, 5.094, DFS was found to be 0.171, 0.518 and for the CHZ was 0.604, 1.830

respectively. Results of LOD and LOQ are presented in Table 8. The developed method was applied to the assay of INTRAGESIC-MR tablets and results are shown in Table 9. The representative standard and sample chromatograms are shown in Fig. 6 and Fig. 7. The assay results of different injections of the sample were found to be within the proposed limits.

Table.2. Specificity study

Name of the solution	Retention time,(t_R)min.
Mobile phase	No peaks
Placebo	No peaks
Solution containing a concentration of PCM, 25 µg/mL, DFS 2.5 µg/mL, CHZ 12.5 µg/mL	Peaks at 3.20 min for PCM, 7.52 min for DFS and 8.84 min for CHZ respectively.

Table.3.Calibration data

	PCM		DFS		CHZ	
S.NO	Concentration, µg/mL	Peak area, mV.s	Concentration, µg/mL	Peak area, mV.s	Concentration, µg/mL	Peak area, mV.s
1.	0	0	0	0	0	0
2.	10	142.869	1	34.065	5	76.416
3.	20	278.117	2	68.212	10	152
4.	30	398.267	3	106.125	15	227.348
5.	40	534.068	4	136.26	20	313.187
6.	50	658.324	5	170.328	25	380.232

Table.4.Linear regression data of the proposed method

Parameter	PCM	DFS	CHZ
Detection wavelength(λ_{max})	UV at 280 nm	UV at 280 nm	UV at 280 nm
Linearity range (µg/mL)	10-50 µg/mL	1-5 µg/mL	5-25 µg/mL
Regression equation (Y = bX+ a)	Y=13.101X+7.748	Y=34.175X+0.3932	Y=15.352X-0.3408
Slope(b)	13.101	34.175	15.352
Intercept(a)	7.748	0.3932	0.3408
Standard error of slope (S_b)	0.1595	0.4234	0.1856
Standard error of intercept (S_a)	4.8301	1.282	2.8105
Standard error of estimation (S_e)	6.6737	1.7714	3.8833
Regression coefficient (R^2)	0.9994	0.9994	0.9994
% Relative standard deviation* i.e., Coefficient of variation(CV)	1.925235	1.81856	1.361184

[#]Average of 6 determinations, acceptance criteria < 2.0

Table.5.Results of Intra-day and inter-day precision study

Drug	Intra-day	Inter-day
PCM	0.775	1.299
DFS	1.226	1.437
CHZ	1.194	1.452

Acceptance criteria < 2.0.

Table.6.Recovery data of the proposed RP-HPLC method

Recovery level	PCM	DFC	CHZ
80%	99.087±0.434	99.249±0.986	100.098±1.242
100%	98.875±0.805	100.131±1.489	99.148±0.834
120%	98.847±0.475	100.713±1.278	99.008±0.925

[#]Acceptance criteria < 2.0.

Table.7.Robustness results

S.no	Parameters	Optimized	Used	Retention time (t_R)			Peak asymmetry			Remark
				PCM	DFS	CHZ	PCM	DFS	CHZ	
1	Flow rate (±0.2 mL/min)	1ml/min	0.8	3.334	7.453	8.853	1.135	1.011	1.121	Robust*
			1	3.20	7.25	8.840	1.027	1	1.02	
			1.2	3.197	7.20	8.739	1.222	1.021	1.23	
2	Detection wavelength (±5 nm)	280nm	275	3.201	7.25	8.846	1.121	1.028	1.21	Robust
			280	3.20	7.25	8.840	1.027	1	1.02	
			285	3.208	7.25	8.840	1.235	1.024	1.24	
3	Mobile phase composition (±5 %)	60:40	55:45	3.19	7.513	8.831	1.122	1.028	1.17	Robust
			60:40	3.20	7.25	8.84	1.027	1	1.02	
			65:35	3.207	7.528	8.890	1.337	1.025	1.13	

Acceptance criteria (Limits): Peak asymmetry < 1.5

Table.8.LOD and LOQ values

Name of the compound	Limit of Detection (LOD)	Limit of Quantitation (LOQ)
PCM	1.681 µg/mL	5.094 µg/mL
DFC	0.171 µg/mL	0.518 µg/mL
CHZ	0.604 µg/mL	1.830 µg/mL

Table.9.Assay results OF PCM, DFS, and CHZ formulation

S.No	Formulation details	Drug	Labeled claim(mg)	Amount found(mg)	%Assay±SD*
1	INTRAGESIC-MR tablets (Intra labs)	PCM	500	25.222	100.891±1.946
		DFC	50	2.540	101.638±1.817
		CHZ	250	12.311	98.492±1.354

*Average of 6 determinations; SD is standard deviation.

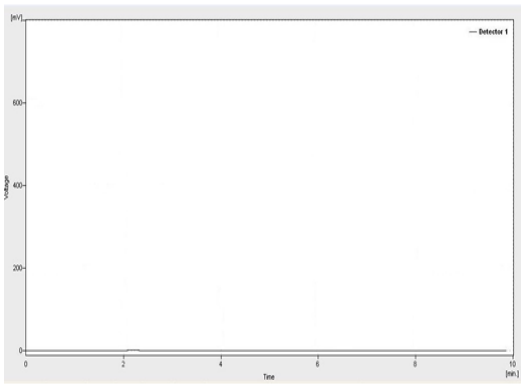

Fig. 2: Chromatogram of placebo

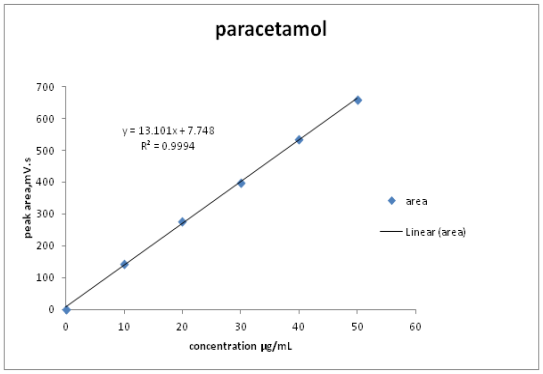

Fig. 3: calibration chart for paracetamol

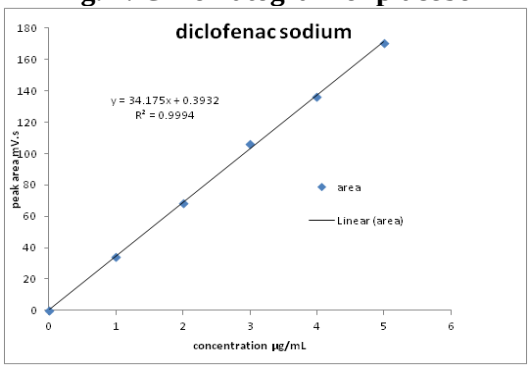

Fig. 4: Calibration chart for diclofenac sodium

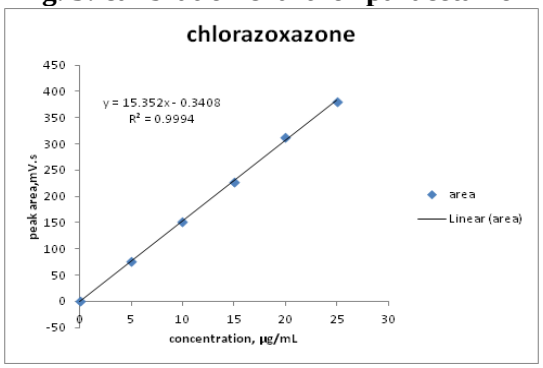

Fig. 5: calibration chart for Chlorzoxazone

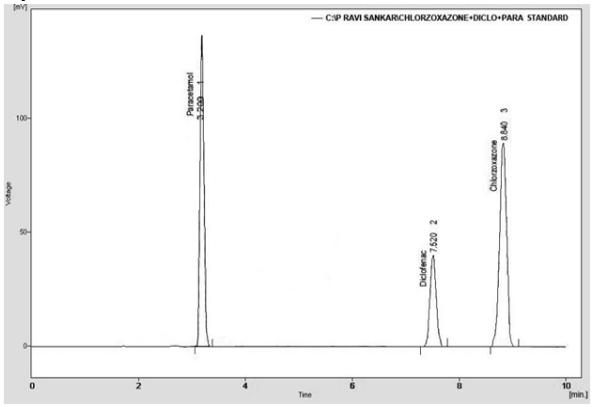

Fig. 6: Standard chromatogram of paracetamol, diclofenac sodium, chlorzoxazone mixture

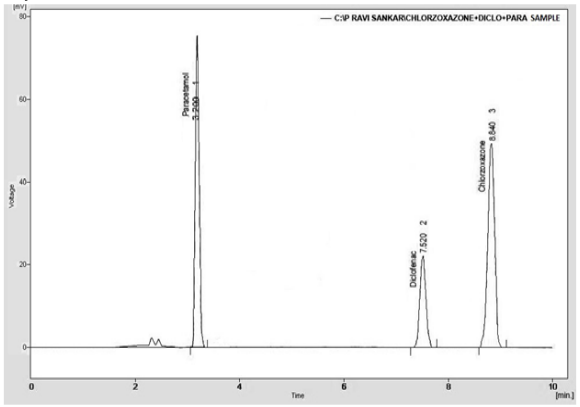

Fig. 7: Sample chromatogram of market formulation (INTRAGESIC-SR tablets)

4. CONCLUSION

Rapid separation of PCM, DFS and CHZ was successfully attained with a relatively short retention time, provides outstanding resolution, good peak shape, gives reliable and highly reproducible results on C_{18} HPLC column. Separation of PCM, DFS and CHZ mixture was achieved with a total run time of 10 minutes with an elution window of 8 minutes for all four analytes. Excellent values for precision, recovery and linearity were achieved together with low LOD and LOQ. The ease in preparation of mobile phase and economy of the components of mobile phase show explicitly the applicability of this method the best choice in routine analysis of PCM, DFS and CHZ in pharmaceutical quality control departments for routine analysis.

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