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DEVELOPMENT AND VALIDATION OF HPLC-PDA METHOD FOR SIMULTANEOUS ESTIMATION OF FAMOTIDINE, PARACETAMOL, CHLORZOXAZONE AND DICLOFENAC POTASSIUM IN COMBINED SOLID DOSAGE FORM

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ABSTRACT

The present work describes development and validation of HPLC-PDA method for simultaneous estimation of Famotidine (FMO), Paracetamol(PAR), Chlorzoxazone (CZX) & Diclofenac potassium (DCL) in bulk powder and tablet formulation. In this method, chromatographic separation was achieved using C18 Phenomenex kinetex column (250 × 4.6mm id, particle size 5µm) as stationary phase with mobile phase comprising of 10mM sodium phosphate (Dibasic) buffer pH 2.82 adjusted with orthophosphoric acid : Acetonitrile (50:50 %v/v) with flow rate of 1.2ml/min in isocratic mode and eluents were monitored at 268nm. Linearity of method was studied & it was observed that the method was found to be linear in concentration range of 50-150% of labelled claim of each drug. The developed method was validated by different validation parameters. Accuracy of method was assessed by Multi level Recovery Studies by standard addition method at 80%,100% and120% resulted in recoveries of standard upto 100%±% RSD <2. The method was found to be precise for intraday, interday studies; the %RSD values were found to be <2. The method was found to be robust as there were no significant alterations on system suitability parameters by deliberately changing the parameters like pH of mobile phase, detection wavelength, flow rate, organic phase composition of mobile phase. The study concluded the validated HPLC- PDA method for analysis of complex mixture consisting of FMO, PAR, CZX and DCL in bulk and tablet dosage form.

Key words: Linearity; Recovery; Mobile phase; Precision, Accuracy

INTRODUCTION [1][2][3][4]

Famotidine (FMO) chemically is 3-[[2-(diaminomethylideneamino)-1,3thiazol-4-yl]methylsulfanyl]-N'-sulfamoylpropanimidamide. FMO is H_2 receptor antagonist and used in treatment of Zollinger Ellison syndrome and for prevention of aspiration pneumonia. FMO is H_2 blocker which binds tightly to H_2 receptors & exhibits longer duration of action. PAR is analgesic, antipyretic. Chemically, it is N-(4-hydroxyphenyl) acetamide. CZX is centrally acting skeletal muscle relaxant. Chemically, Chlorzoxazone (CZX) is 5-chloro-3H-1,3 benzoxazol-2-one. CZX is centrally acting skeletal muscle relaxant. CZX selectively depress spinal & supraspinal polysynaptic reflexes involved in the regulation of muscle tone without significantly affecting monosynaptically mediated stretch reflex. Diclofenac potassium (DCL)ischemically Potassium;2[(2,6dichloroanilino)phenyl]acetate. DCL is analgesic, antipyretic, anti-inflammatory drug. DCL inhibits PG synthesis & is COX-2 selective. It is poor inhibitor of PG synthesis in peripheral tissues & but more active on COX in the brain. The fixed dose combination of these four drugs is effective in treatment of pain, swelling, joint stiffness, heartburn, upset stomach, indigestion, acidity, gastric ulcers, headache, toothache, muscle ache, dental pain, muscle spasm and menstrual cramps. The Tablet 1 formulation consists of variable amount of labelled claim for all four drugs according to their pharmacological doses. Literature survey revealed that there are UV spectrophotometric, RP-HPLC, RP-UPLC, HPLC-PDA, HPLC-DAD, HPTLC, LCMS, Capillary zone electrophoresis, voltametry methods for simultaneous estimation of all these drugs in alone or in combination with other drugs. FMO is found to be estimated by spectroscopic [5,6], RP-HPLC[7], RP-UPLC[8], HPLC-PDA[9] methods in combination with other drugs. PAR is analysed by UV spectrophotometry [10-17], HPLC [18-23], LCMS [24], capillary zone electrophoresis [25], voltammetry [26] methods alone or in combination with other drugs. DCL is quantified by spectroscopy [27], HPLC [28-30], HPLC-DAD [31] and HPTLC [32]. CZX is found to be determined by spectroscopy [33,34], RP-HPLC[35-38], TLC-Densitometric[39], chemometric method in plasma[40]. FMO, PAR, DCL simultaneously estimated by both HPLC [41] and HPTLC method. Recently a HPTLC method have been reported for selected drug combination. However, no reports are published indicating HPLC method for selected combination. As the fixed dose combination consists of complex mixture of four drugs; it pose the problem for separation of these drugs. HPLC being a rapid, economic and routinely applicable analytical technique, we proposed to develop a rapid, economic, nontedious and simple HPLC method for the selected drug combination. [Table 2,3] (Figure 1,2).

Table 1: System suitability parameters

Sr No.	**Mean ± %RSD (n=5)**	**Eluted peaks**			
		FMO (1)	**PAR(2)**	**CZX(3)**	**DCL(4)**
1	**t_r (min) ± %RSD**	1.96±0.67	2.24±0.92	3.54±1.17	9.04±1.12
2	**k´ ±% RSD**	3.26±0.89	3.865±1.15	6.69±1.35	18.658±1.18

3	**N ± % RSD**	4503±1.04	18863.6±0.51	15578±1.85	7892.4±1.35
4	**Peak area ± % RSD**	210589.17±0.034	3817033.83±0.37	1461496±1.95	659589±0.48
Eluted Peak Pairs					
		1&2	**2 & 3**	**3 & 4**	
1	**Rs ± % RSD**	8.48 ±1.83	5.32±1.14	1.98±1.85	
2	**α ± % RSD**	1.26±0.45	1.98±1.36	1.95±1.062	
3	**asymmetry**	5.29±1.66	7.82±0.91	1.87±0.67	

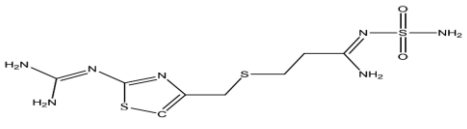
Note: tr – retention time, k´– Capacity factor, N – Plate number, Tf – Peak asymmetry factor, Rs – Resolution, α – Selectivity (Separation factor).

Table 2: Linearity parameters

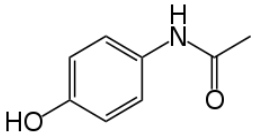
Sl No	Linearity parameter	FMO	PAR	CZX	DCL
1	Range (µg/ml)	5-15µg/ml	162.5-487.5 µg/ml	125-375 µg/ml	25-75µg/ml
	Regression Equation	y=20531x+4918	y=38240x+46355	y=5798x+23579	y=13335x+6214
2	Slope(m)	20531	38240	5798	13335
3	Intercept(c)	4918	46355	23579	6214
4	Regression coefficient (r2)	0.998	0.999	0.999	0.999
5	LOD (µg/ml)	0.00085	0.04846	0.07565	0.00658
6	LOQ (µg/ml)	0.00259	0.14686	0.22925	0.01993

Table 3: Data for Intraday and Interday Precision (n=6)

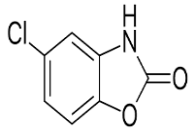
Sr No			Measured mean concentration ± %RSD		%amount ±%RSD	%amount ±%RSD
	% Concentration level	Concentration µg/ml	Intra-day precision (n=6)	Inter-day precision (n=6)	Intra-dayPrecision (n=6)	Inter-day precision (n=6)
1			**FMO**		**FMO**	
	50	5	4.99±0.50	4.97±0.81	99.73±0.45	99.34±0.76
	80	8	7.93±0.93	7.94±0.52	99.4±0.95	99.31±0.56
	150	15	14.87±0.88	14.95±0.80	99.1±0.89	99.67±0.80
2			**PAR**		**PAR**	
	50	162.5	**161.38±0.59**	**161.53±0.91**	**99.32±0.73**	**99.4±0.90**
	80	260	259.45±0.54	258.45±0.98	99.79±0.54	99.4±0.98
3	150	487.5	484.38±0.79	486.89±0.24	99.36±0.79	99.87±0.24
			CZX		**CZX**	
	50	125	124.53±0.37	123.95±1.07	99.63±0.37	99.16±1.06
	80	200	198.71±0.68	198.5±0.96	99.36±0.68	99.25±0.96
	150	375	372.4±0.58	372±1.08	99.31±0.57	99.2±1.08
			DCL		**DCL**	
4	50	25	24.79±0.99	24.77±0.26	99.16±1.00	99.06±0.25
	80	40	39.62±0.83	39.55±0.91	99.06±0.83	98.88±0.90
	150	75	74.46±0.99	74.42±0.66	99.28±0.99	99.22±0.66



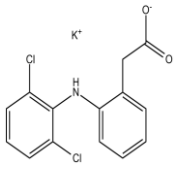
Famotidine (FMO)



Paracetamol(PAR)



Chlorzoxasone (CZX)



Diclofenac potassium (DCL)

Figure 1: Chemical structures of drugs Famotidine (FMO), Paracetamol(PAR), Chlorzoxazone (CZX), Diclofenac potassium (DCL).

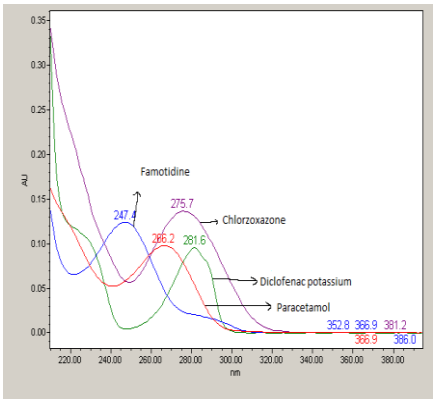


Figure 2: Overlain spectrum of Famotidine (FMO), Paracetamol (PAR), Chlorzoxazone (CZX), Diclofenac potassium (DCL).

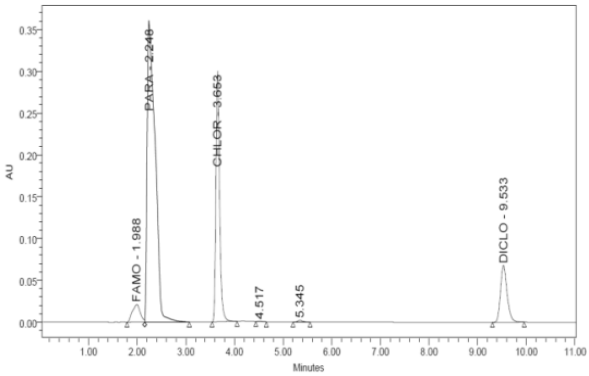


Figure 3: Chromatogram Famotidine (FMO), Paracetamol (PAR), Chlorzoxazone (CZX), Diclofenac potassium (DCL).

EXPERIMENTAL

Instrumentation

HPLC-PDA analysis was carried out using WATERS liquid chromatography equipped with a model 600 solvent pump, 996 photodiode array detectors and 515 autosampler. The software used was Empower version 2 (Waters Spa, Milford, USA) for data acquisition & recording chromatograms. Effective resolution of all the analytes was obtained on Phenomenex Kinetex C_{18} (250×4.6mm id, particle size 5µm) column as a stationary phase.

The other instruments used were Digital Weighing Balance Citizen Model CY 104, Ultrasonic Bath Sonicator Model PCI 1.5L 50H, and Digital pH meter Model EQ 610.

Chemicals & Reagents

Working standards of FMO, PAR, CZX, DCL were obtained as gift samples from Cadila Healthcare, Ahemedabad, Ria International Lic, Madurai, Triveni Interchem,Vapi, and Leben Pharmaceuticals, Akola. The marketed formulation FASTRAN MR manufactured by Horizon pharmaceuticals containing fixed dose combination of FMO (10mg), PAR(325mg), CZX(250mg), DCL(50mg) was purchased from local market. All the chemicals used for HPLC-PDA analysis were of HPLC grade purchased from E. Merck, Mumbai.

Preparation of standard stock solution & sample solution

Preparation of standard stock solution

Accurately weighed quantities of all the pure drugs(Working standards) were taken for each drug FMO(10mg), PAR(325mg), CZX(250mg), DCL(50mg) and transferred to 100ml volumetric flasks separately to give standard stock solutions of 100µg/ml for FMO, 3250µg/ml for PAR, 2500 µg/ml for CZX and 500 µg/ml for DCL in methanol.

Preparation of mixed standard solution

Aliquot portions of stock solutions of all the drugs were taken and diluted to mark with mobile phase to get final concentration of 10µg/ml for FMO, 325 µg/ml for PAR , 250 µg/ml for CZX and 50 µg/ml for DCL. The mixed standard solution was subjected to HPLC-PDA analysis with developed chromatographic conditions and chromatogram was obtained with complete resolution of all the four analytes with sharp peaks [Figure 3]

Preparation of sample solution

About 20 tablets (FASTRAN MR) containing complex mixture of all the four drugs were weighed accurately and average weight of tablet was determined. The tablets were crushed and accurately weighed quantity of tablet powder equivalent to FMO (10mg), PAR (325mg), CZX (250mg) and DCL (50mg) was transferred to 100ml volumetric flask & dissolved in methanol. The solution was sonicated for 15 min & filtered through 0.45 µ membrane filter. An aliquot was taken and further diluted with mobile phase to get final concentration of 10µg/ml for FMO, 325 µg/ml for PAR, 250 µg/ml for CZX and 50µg/ml for DCL. The sample solution was subjected to HPLC-PDA analysis with developed chromatographic conditions. The results obtained were found to be within acceptable limit, %RSD<2 (Table 4).

Table 4: Results of assay of marketed formulation

Sl No.	Brand Name Marketed tablet formulation	Name of drug	Label claim (mg)	Amount found(mg) ± % RSD	% amount obtained ±%RSD
1		FMO	10	9.96±1.03	99.59±1.03

2		PAR	325	321.8±1.29	99.02±1.29
3	FASTRAN MR	CZX	250	248.48±1.83	99.39±1.83
4		DCL	50	49.93±0.73	99.86±0.73

Chromatographic conditions

For mobile phase is to be developed; different trials were carried out by differing the aqueous phase and organic phase compositions with varying pH. An attempt was made to develop the simple mobile phase with isocratic approach for chromatographic analysis. So, the developed mobile phase consisting of 10mM sodium phosphate (Dibasic) pH 2.82 adjusted with orthophosphoric acid : Acetonitrile (50:50 % v/v). The mobile phase was filtered through 0.45 micron membrane filter & degassed before use. The eluents were resolved on reverse phase C_{18} Phenomenex kinetex column (250 × 4.6mm id, particle size 5µm) as stationary phase. The detection wavelength kept was 268nm with flow rate of 1.2ml/min and runtime kept was 11 min.

RESULTS & DISCUSSION

Method development & optimization of chromatographic conditions

Use of different varying length C_8 & C_{18} columns were tried as stationary phase, finally, reverse phase C_{18} Phenomenex kinetex column (250 × 4.6mm id, particle size 5µm) was selected as it showed the good resolution of all the four analytes. For separation of all four analytes in mixture; composition and pH of mobile phase was varied. Several binary or ternary compositions of organic solvents such as methanol, acetonitrile with aqueous solvents water and buffer with varying pH were tried. Finally, isocratic mobile phase comprised of 10 mM sodium phosphate (Dibasic) buffer pH 2.82 adjusted with orthophosphoric acid : Acetonitrile (50:50 % v/v) showed good resolution and therefore was selected. It was further optimized by varying flow rate in order to obtain sharp peaks and comparatively shorter run time. The optimized mobile phase was run in an isocratic mode with flow rate and runtime of 1.2ml/min and 11 min respectively. The eluents were monitored at 268nm as detection wavelength. The injection volume was 20 µl with analysis was carried out ambient temperature. The mean retention time of all the four analytes were found to be 1.96±0.67, 2.24±0.92, 3.54±1.17, 9.04±1.12 for FMO, PAR, CZX and DCL respectively. Individual peak of each analyte was identified by injecting each analyte individually with same chromatographic conditions. The resolution obtained for two consecutive peaks of analytes; was observed >1.5 indicating an adequate degree of resolution. System suitability parameters were recorded for optimized chromatographic conditions. It was observed that there was no any effect on separation and sharpness of peaks due to changes in pH.

Method Validation

Specificity studies

Specificity studies concluded that there was no interference of placebo (diluents i.e. mobile phase) and of excipients present in formulation (Figure 4: Overlain chromatogram)

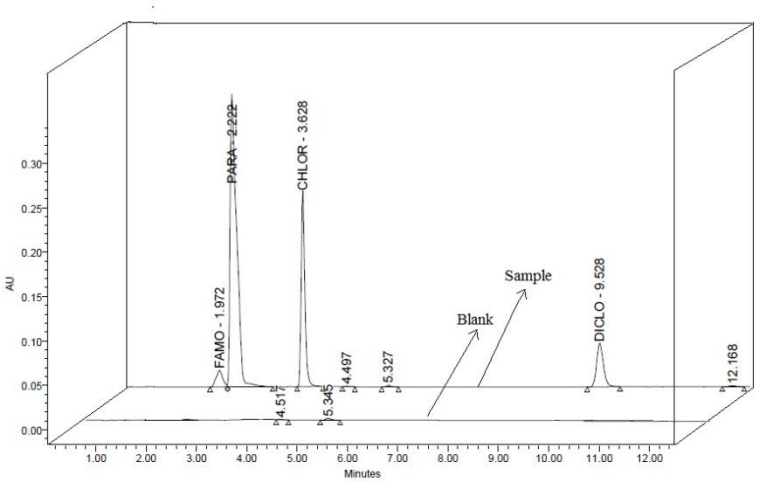


Figure 4: Overlain Chromatogram of resolved analytes Famotidine (FMO), Paracetamol (PAR), Chlorzoxazone (CZX), Diclofenac potassium (DCL) with blank.

Linearity & Range

The standard solution of each analyte was prepared and diluted with mobile phase to get final concentrations ranging from 50-150% of labelled claim of each analyte. The solution of each analyte was injected five times and mean value of peak area was considered for plotting the graph of peak area Vs concentration of analyte in µg/ml. The linear regression equations were found to be y=20531x+4918, y=38240x+46355, y=5798x+23579, y=13335x+6214 for FMO, PAR, CZX and DCL respectively. The values for correlation coefficient for all the four drugs were found to be nearly 0.999 indicating the acceptable degree of linearity. The method was found to be linear in concentration range of 5µg/ml-15µg/ml for FMO, 162.5µg/ml-487.5µg/ml for PAR, 125µg/ml-375µg/ml for CZX and 25µg/ml-75µg/ml for DCL.

Accuracy studies

To determine the accuracy of method, *Multilevel Recovery Studies* by standard addition method was carried out. The accuracy was expressed in terms of % recovery of standard analyte spiked to preanalysed tablet powder sample. The standard analytes were spiked to preanalysed tablet powder sample at three different level 80%, 100% and120% of labelled claim and injected in developed chromatographic conditions in six times. The mean recovery values of FMO, PAR, CZX and DCL were found to be excellent for all three levels of recovery studies (Table 5).

Table 5: Recovery data for accuracy studies

Sr No	Recovery Level	Standard added	Amount added (mg)	Mean Recovery mg at each level (n=6)	Mean % Recovery at each level	Mean % Recovery ± % RSD
1	**80**	FMO	8	8.054±	100.68± 0.13	100.016±0.96

				0.13		
		PAR	260	260.91±1.04	100.35± 1.04	99.78±0.49
		CZX	200	199.83±0.77	99.92± 0.77	99.67±0.24
		DCL	40	39.97±0.56	99.92± 0.55	99.9±0.10
2	**100**	FMO	10	9.93± 0.80	99.32±0.80	
		PAR	325	322.55±0.98	99.25±0.98	
		CZX	250	248.63± 1.30	99.45± 1.30	
		DCL	50	49.9±1.34	99.79±1.34	
3	**120**	FMO	12	12.01±1.42	100.048±1.43	
		PAR	390	388.03±1.25	99.5±1.25	
		CZX	300	298.92± 1.40	99.64±1.40	
		DCL	60	59.99±1.77	99.99±1.78	

Precision

The closeness of replicate analytical results obtained from analysis of the same homogeneous sample. Precision was determined through the estimate of % relative standard deviation (RSD) at three concentration levels**;** 50%, 80%, 150%. Intraday precision was carried out by analyzing the standard samples on same day using the optimized chromatographic conditions. Interday precision was carried out by analyzing standard samples on three consecutive days. The %RSD values for intraday, interday precision were found to be within acceptable limit (RSD ±<2, as given in table no 3).

LOD & LOQ of method

Limit of detection refers the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

Limit of quantitation refers to the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

LOD & LOQ were determined to mark sensitivity of method. It was determined by injecting the series of known concentration of analytes. The LOD of FMO, PAR, CZX and DCL were found to be 0.00085 µg/ml, 0.04846 µg/ml, 0.07565 µg/ml, 0.006577 µg/ml respectively. The LOQ of FMO, PAR, CZX and DCL were found to be 0.002591 µg/ml, 0.146855 µg/ml, 0.22925 µg/ml, 0.01993 µg/ml respectively.

Robustness studies

Robustness of method is ability of method to remain unaffected by small, deliberate changes in chromatographic parameters. So, changes were made deliberately in pH of mobile phase, in organic phase composition of mobile phase, in flow rate, in detection

wavelength. Only one parameter was changed deliberately at once and remaining parameters were kept unchanged; so as to obtain the accurate results. Firstly, change in pH ± 0.1unit was changed deliberately and standard analyte solutions were run; the obtained results were unaffected by pH change. The method was found to be so rugged that the mobile phase with varying pH from 2.57-3.12 was tried; still results obtained were acceptable.

Secondly, change in organic phase composition (acetonitrile) ±10% was made; there was no remarkable change in resolution and retention time of analytes. Thirdly, flow rate was altered to ±10%. (i.e. 1.1ml/min & 1.3ml/min). Flow rate of 1.1ml/min resulted into increased runtime by 2.0min (from 11min to 13min). Flow rate of 1.3ml/min resulted into decreased runtime by 2 min(from 11min to 9.0 min). During deliberate change in flow rate to increased level, it was observed and concluded that the method can be made shorter i.e. within nine min by keeping the flow rate 1.3ml/min. Fourthly, the change in detection wavelength ±5nm was carried out and system suitability parameters were recorded. All the robustness studies were carried out using mixed standard analyte solutions.

Conclusion

In this study a simple, precise, reliable HPLC-PDA method had been developed and validated for separation of complex multidrug formulation. No RPHPLC, HPLC-PDA analytical method had been attempted for this formulation yet. It was concluded that the developed method is rapid, advantageous because, all the four analytes were successfully resolved & quantified using a reverse phase Phenomenex kinetex C_{18} (250×4.6 mm id, particle size 5µm) column as stationary phase with mobile phase comprised of 10 mM sodium phosphate (Dibasic) buffer pH 2.82 adjusted with orthophosphoric acid : Acetonitrile (50:50 % v/v) in relatively shorter runtime. The % amount drug recovered for standard analytes showed that there was no interference of excipients in the formulation. The RSD % <2 showed the high degree of precision. The proposed method was found to be robust with respect to flow rate, pH of mobile phase, organic phase composition of mobile phase, detecting wavelength. The method was found to be simple,accurate, rapid, precise, reliable, economic, reproducible. Hence, the method can be used for routine analysis of complex mixture of multidrug formulation in bulk powder and tablet formulation.

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