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DEVELOPMENT AND EVALUATION A SIMULTANEOUS ASSAY METHOD OF METRONIDAZOLE AND DICLOFENAC POTASSIUM IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

In this study, a simple, economic and selective HPLC method was developed and validated for the simultaneous estimation of Metronidazole (MET) and Diclofenac potassium (DIC-K). Reversed-Phase chromatography (RV-HPLC) was performed on a C18 column with methanol-Buffer 70:30%(V/V) as a mobile phase at a flow rate at 1ml/min. Metronidazole and Diclofenac Potassium have a maximum absorption at 254nm. The proposed method was successfully applied for determination of both drugs in one dosage form. Statistical analysis proved the method was precise, selective, specific and accurate for simultaneous analysis of Metronidazole and Diclofenac potassium.

Keywords: Metronidazole (MET), diclofenac potassium (DIC-K),HPLC, validation.

INTRODUCTION

Metronidazole (Fig. 1a), a pro-type nitroimidazole antibiotic used particularly for anaerobic bacteria and protozoa acts by disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis (wikipedia.org, 2012; USP, 2012). The Diclofenac potassium 2(2,6 dichlorophenyl)amino benzeneacetic acid potassium salt (Fig. 1b) is a non steroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reduces pain in certain conditions such as arthritis, acute injury etc. Diclofenac works by inhibiting cyclooxygenase enzymes COX prostaglandin synthesis (wikipedia.org, 2012; USP, 2012). It was found that individually these drugs have been analyzed by many methods, RP-HPLC method has been used for determination of Diclofenac–K in combination with other drugs in one dosage form (Gowramma *et al*., 2010; Kasperek, 2008; Sunil *et al*., 2010). Many methods have been described in the literature for the determination of Metronidazole individually and in combination with other drugs in different pharmaceutical dosage forms (Rhaman *et al*., 2004; Mishal and Diana, 2005).

The combination of these two drugs is not official in any standard pharmacopoeia; hence no official method is available for the estimation of metronidazole and Diclofenac potassium in their combination dosage forms. The literature survey does not reveal any article related to the simultaneous HPLC or spectrophotometric determination of DICL-K-and MET in combination. To date there have been no published reports about the simultaneous quantitation of Metronidazole and Diclofenac potassium by HPLC. The present study describes simple, sensitive, precise, accurate, specific and repeatable HPLC method for the simultaneous estimation of Metronidazole (Fig. 1a) and Diclofenac potassium (Fig. 1b). The proposed method was validated as per ICH guidelines (ICH, 2005).

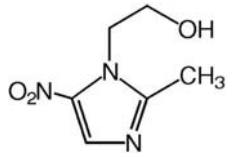

Fig. 1a. Metronidazole.

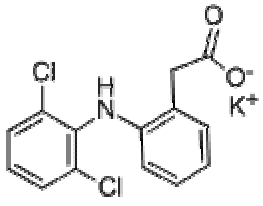

Fig. 1b. Diclofenac Potassium.

MATERIALS AND METHOD

Materials

Diclofenac potassium was provided by NPI (National Pharmaceutical Industries), Muscat, Oman and Metronidazole were obtained from Asia Pharmaceutical

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Labs, Syria. All chemicals and reagents used were of HPLC grade JT Baker, Netherland. All solvents used were of AR grade.

Instruments used were Reversed Phase RP- HPLC (Alliance) by Waters2469 with sampler programmed at 20µL capacity per injection. The instrument was controlled by using Empower software.
The wavelength used for detection was 254nm (USP, 2012).

The column used was C18 (250 mmx4.6, 5µ) obtained from Schirosorb ODS-2, Germany.

Preparation of mobile phase, buffer and standard, solution
Mobile phase used was a combination of methanol for HPLC grade and Buffer mixed in 70:30 ratio. The Buffer was prepared by mixing 0.01M phosphoric acid in equal volume with 0.01M Sod. Monophosphoric acid to obtain a buffer with pH 2.5 (pH was adjusted with phosphoric acid).

Standard stock solution containing 20µg/ml Diclofenac potassium and 20µg/ml MET was prepared using mobile phase. The stock solution was stored at 2-8^{o}C protected from light.

Optimization of HPLC method
The HPLC method was optimized and validated to develop a simultaneous assay method for DIC-K and MET. The individual standard and mixed stock solution of drugs were injected to HPLC (Fig. 2). For HPLC method optimization we used methanol and buffer in ratio70:30 at flow rate 1ml/min which gave acceptable retention time (t_R) and good resolution for Metronidazole and Diclofenac potassium drugs (Fig. 3).

Validation of the method
Validation of the optimized HPLC method was carried out with respect to the following ICH Guidelines.

Linearity and range
10.7mg of Diclofenac potassium was dissolved in 20ml of mobile phase. 2ml of this solution was diluted to 10ml resulting in a stock solution of Diclofenac potassium containing 0.107mg/ml of the drug. Six dilutions were prepared from 0.333µg/ml - 10.678µg/ml. Six dilutions for Metronidazole were also prepared ranging from 10.565µg/ ml-0.3301µg/ml. The linearity of the method was studied by injecting six concentrations of the drugs prepared in the mobile phase in five repeated injections in the LC system keeping the injection volume constant .The mobile phase was filtered through a 0.45µm membrane filter. The baseline was monitored continuously during this process. The detection wavelength was 254nm.The peak areas were plotted against the corresponding concentrations to obtain the Calibration graphs. The correlation coefficients, slope, y-intercept of the calibration curve were determined (Gowramma *et al*., 2010).

Precision
Precision was evaluated for inter-day (repeatability) and intra-day (Intermediate precision) variation. Repeatability studies were performed by analysis of three different concentrations 2.66, 5.33, 10.6µg /ml for Diclofenac potassium and 2.641, 5.28, 10.5µg/ml for metronidazole five times on the same day. The intermediate precision of the method was checked by repeating these studies on another HPLC, column on different days (Sunil *et al*., 2010; Baboota *et al*., 2007; Mahesh *et al*., 2010; Shaligrams *et al*., 2012).

Accuracy and Recovery
The accuracy method was carried out by applying the method to drug sample, Diclofenac potassium and Metronidazole on previously analyzed sample. The experiment was performed in triplicate. RSD (%), bias (%), and standard error of mean (SEM) were calculated for each concentration while the percent of recovery was found to be in the range of 101%---102% for both the drugs (Baboota *et al*., 2007; Shaligrams *et al*., 2012.

Limit of detection and limit of quantification
Limits of detection (LOD) and quantification (LOQ) represent the concentration of analyte that would yield signal – to – noise ratio of 3 for LOD and 10 for LOQ, respectively. The samples of Diclofenac potassium and Metronidazole were injected into LC system and measured signal from the samples was compared with those of blank samples (Baboota *et al*., 2007).

Robustness
To evaluate robustness of an HPLC method, few parameters were deliberately varied. The parameters included variation of HPLC system like Agilent HPLC and Water 2469 using different operators, different columns of similar type like HypersilC18, Shimadzu ODC, and at three different concentration levels 6,8,10µg/ml each for Diclofenac Potassium and Metronidazole (Gowramma *et al*., 2010; Baboota *et al*., 2007).

Specificity
The specificity of this method was ascertained by analyzing standard drug and sample. The spot for Diclofenac potassium and Metronidazole was confirmed by comparing the spectra with that of the standard. The peak purity of Diclofenac potassium and Metronidazole was assessed by comparing at three

different levels, i.e. peak start(S), peak apex (M) and peak end (E).

RESULTS AND DISCUSSION

The results of the validation of the simultaneous estimation method developed for Diclofenac Potassium and Metronidazole in the current study using Methanol: Buffer (70:30, v/v), are as discussed below:

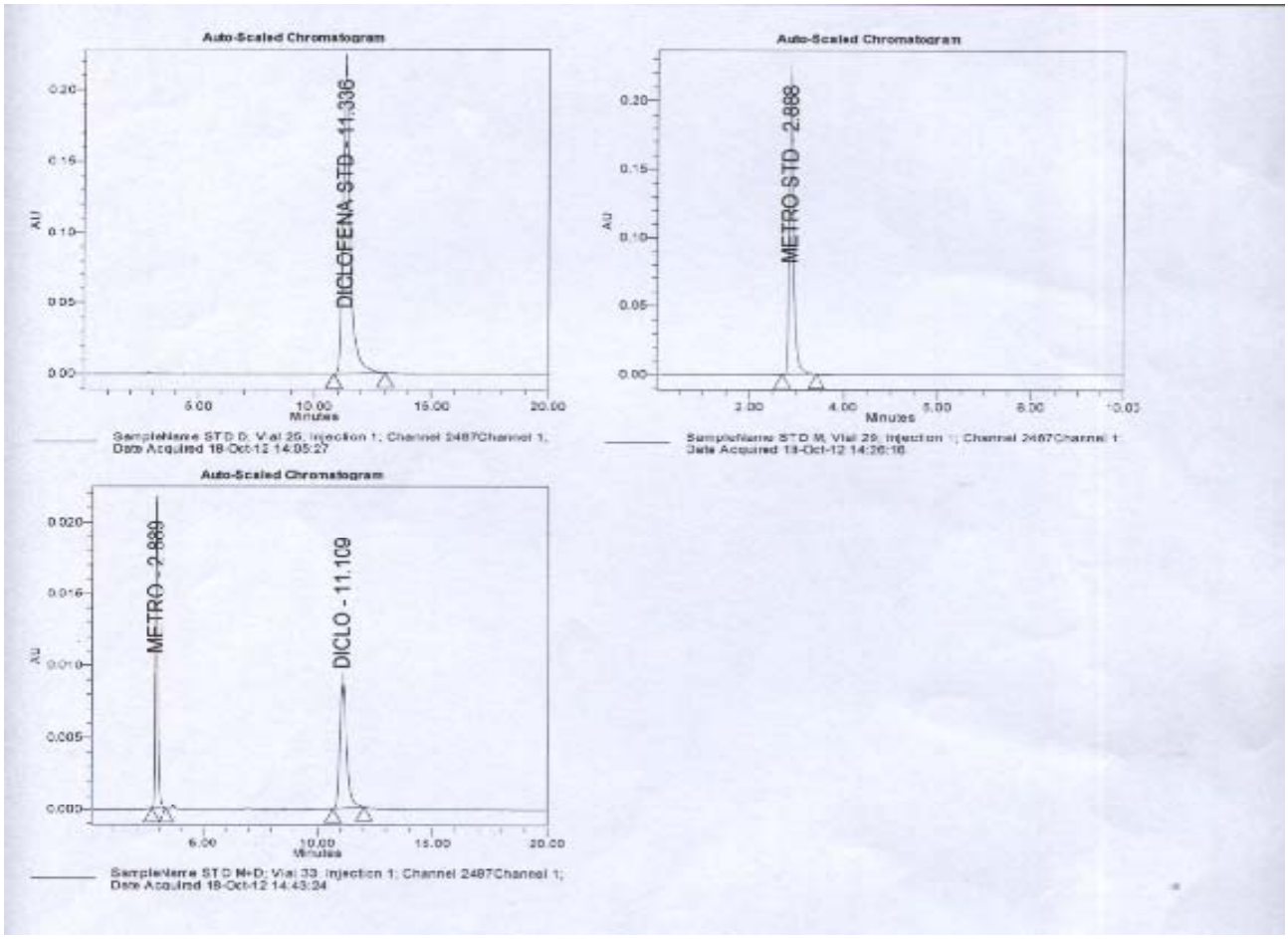
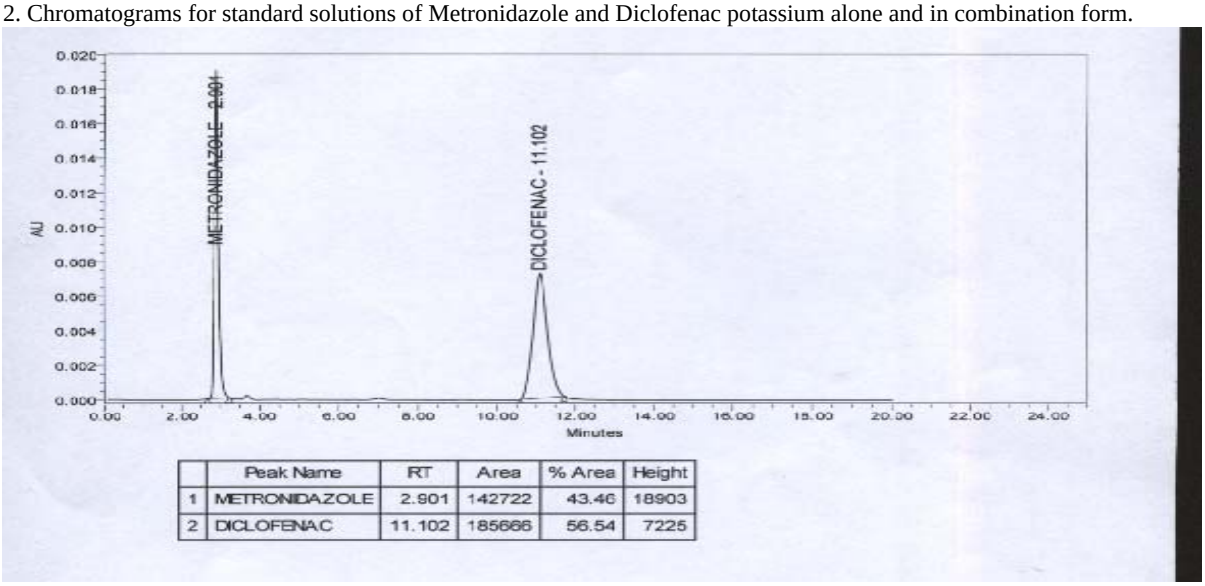


Fig. 2. Chromatograms for standard solutions of Metronidazole and Diclofenac potassium alone and in combination form.



	Peak Name	RT	Area	% Area	Height
1	METRONIDAZOLE	2.901	142722	43.46	18903
2	DICLOFENAC	11.102	185666	56.54	7225

Fig. 3. Chromatogram for both Diclofenac Potassium and Metronidazole drugs.

Linearity

The linear regression data for calibration plot are indicative of a good linear relationship between peak area and concentration of a wide range in a range of 0.33-10.67µg/ml and 0.33-10.678µg/ml for metronidazole and Diclofenac potassium respectively. The slope and intercept value for calibration was y= 9399 x (R^2=0.9996) for Diclofenac potassium and y=7097x(R^2=0.9988) for Metronidazole (Fig. 4). The percent RSD% was found to be less than 2% (Table 1). This performance shows a good correlation between response factor and concentrations of the drugs (Table 2).

Precision

Precision was evaluated by intra-day (Repeatability) and inter-day (Intermediate precision) variation, and different makes of the column. Repeatability (five replicates) was assessed independently for each of the different concentration. Results from the determination of repeatability and intermediate precision, expressed as RSD%, are listed in table 3. The low values of RSD indicated the repeatability of the method.

Recovery and Accuracy

The recovery of the method shows good recoveries of the Diclofenac potassium and Metronidazole in the range 100.41- 102.965 %. The value of recovery (%), RSD (%), Percentage bias, and SEM indicated that the method is accurate (Table 4).

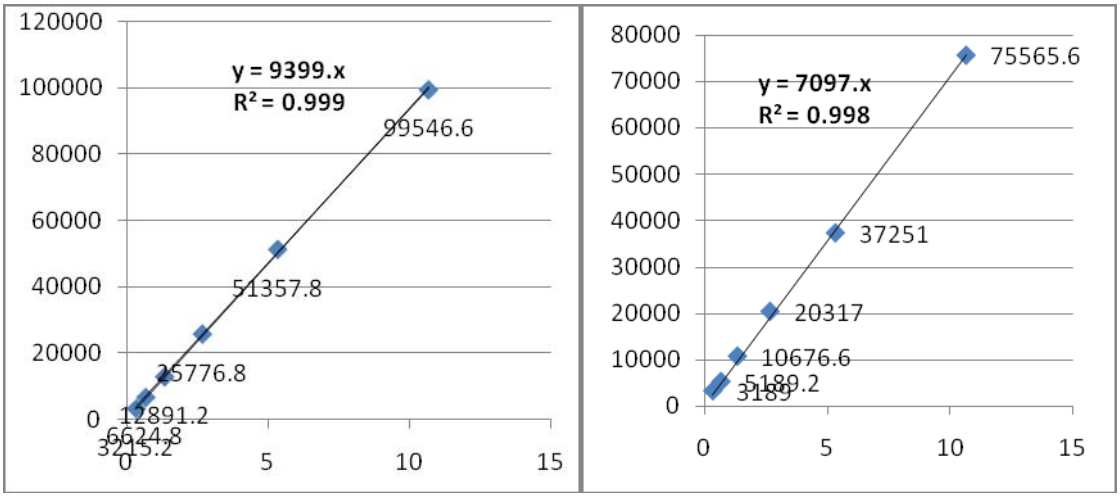


Fig. 4. Calibration curve of Diclofenac potassium and Metronidazole .

Table 1. Linear regression data for the calibration plot (n=5).

Conc.(ug/ml) DIC-K	Mean area	±SD	RSD(%)	95% confident interval	
				Low value	Upper value
2.66	25776.8	383.17	1.49	25184	25792
5.33	51357.8	395.23	0.77	50886	51779
10.676	99546.6	1284.44	1.29	98577	101116
106.6	1036417.250	9230.75	0.89	102316	1044187

Conc.(ug/ml) MET	Mean area	±SD	RSD (%)	95% confident interval	
				Low value	Upper value
2.66	20317	360.9	1.78	19924	20638
5.33	37251	460.7	1.24	36643	37658
10.676	75566	484.4	0.64	75001	76152
106.6	777373	4705.9	0.61	772088	783525

Table 2. Correlation between response factor and concentrations of the drugs.

Parameter	Diclofenac Potassium	Metronidazole
Linearity range	(0.33-10.67) µg/ml	(0.33-10.678)µg/ml
Correlation Co efficient	0.9996	0.9988
Slope	9399	70997

Table 3. Precision of the method.

Repeatability (Intra –day precision) *(n=5)*			
	Conc.µg/ml	Area under Curve AUC ±SD	RSD %
Diclofenac Potassium	2.66	25776.6 ±38.167	1.49
	5.33	51357±395.227	0.77
	10.67	99546.6±1284.4377	1.29
Metronidazole	2.641	20317±360.91	1.78
	5.28	37251± 460.72	1.24
	10.5	75565.6 ± 484.44	0.64

Intermediate precision (Inter-day)*(n=5)*			
	Conc.µg/ml	Area under Curve AUC ±SD	RSD %
Diclofenac Potassium	2	25356.1± 3993.84	1.58
	4	407547± 5917.254	1.45
	6	552918± 5660.394	1.02
Metronidazole	2	17792.33± 178.9814	1.005
	4	28872.33± 175.0467	0.61
	6	40114.67± 680.7983	1.70

Table 4. Accuracy of the method (*n=3*).

% of drug added to the analyte	Theoretical conc.µg/ml	Conc. mean	SD	Recovery	% RSD	% Bias	SEM
Diclofenac Potassium							
0	2.5	2.42	0.15272	101.76	0.64	-3.86	0.008
100	5	5.01	0.0608	101.47	1.22	+0.2	0.035
200	10	10.01	0.0666	100.41	0.67	+0.1	0.035
Metronidazole							
0	2.45	2.44	0.5	102.965	1.64	-0.4	0.030
100	4.9	4.85	0.0377	101.641	0.78	-1.04	0.028
200	9.8	9.96	0.0666	101.970	0.68	0	0.058

Limit of detection LOD and Limit of quantification LOQ

LOD and LOQ were determined by the standard deviation ($S_y/_x$) method. The LOD and LOQ was determined from the slope, S, of the calibration plot and standard deviation of the responses of the sample, by using the formula LOD=$3xS_{y/x}$ /S and LOQ=$10xS_{y/x}$/S. The LOD and LOQ were found to be 0.1345µg/ml and 0.4µg/ml for DIC-K and 0.1677µg/ml and 0,5507µg/ml for MET respectively which indicates the method can be used for detection and quantification of a quite wide range of concentrations (Table 5).

Table 5. LOD and LOQ of the method.

Drug	LOD $3xS_{y/x}$/slope	LOQ $10xS_{y/x}$/slope
Diclofenac Pot.	0.1345 µg/ml	0.4µg/ml
Metronidazole	0.1677 µg/ml	0.5507 µg/ml

Specificity

The peak purity of Metronidazole and Diclofenac potassium was assessed by comparing their respective spectra at peak start, apex and end position. It was found

that r(S, M) = 0.989071 and r (M, E) = 0.98669. A good correlation was obtained; the mobile and diluents did not show interference with the assay, indicating the specificity of the method.

Table 6. Specificity.

S (peak start)	M (Peak apex)	E (Peak end)
7.3	3.9	8.1
7	3.5	7.9
7.2	3.7	8
7.4	4	8.2
7.3	3.9	8.1

Robustness

It was observed that there were no marked changes in the chromatograms. There was no significant change in the retention time of Metronidazole and Diclofenac potassium when we changed the column and the HPLC. The low values of RSD, shown in table 7 and 8, indicated the robustness of the method.

Suitability

This included the column efficiency, resolution and peak asymmetry. The values obtained demonstrated the suitability of the system for the analysis of the drugs in combination calculated for the standard solutions (Table 9).

CONCLUSION

This HPLC method was developed and validated as per ICH guidelines which was accurate, precise and reproducible. The UV detection allowed an accurate

Table 7. Results from testing of the Robustness of the method by changing instruments (HPLC, Column).

DIC-K	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	455538	6071.0301	1.332717	9.72	0.206	2.11	1.65
	8	691007.7	12211.235	1.767163	9.94	0.092	0.93	-0.62
	10	103006.3	2900.2181	2.815573	9.75	0.258	2.65	1.61

DIC-K	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	46071.67	556.1747	1.2071	11.04	0.00493	0.04	-0.05
	8	68938.1	241.8077	0.3507	11.03	0.00057	0.005	0.003
	10	83142.33	499.1846	0.6003	10.98	0.0707	0.64	-0.39

Table 8. Results from testing of the Robustness of the method by changing instruments (HPLC, Column).

MET.	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	76173.33	555.93195	0.73	3.04	0.0015	0.05	-0.04
	8	90581.01	389.49582	0.43	3.03	0.0010	0.03	0.03
	10	105100.3	1735.0015	1.65	3.02	0.0005	0.02	0.01

MET.	Conc. µg/ml.	Area under the curve			Retention time			
		Mean AUC	±SD	RSD %	Mean t_R	±SD	RSD %	%Bias in t_R
	6	40453	545.22564	1.3	2.841	0.001	0.04	0
	8	56501.67	482.90923	0.86	2.841	0.00057	0.02	-0.023
	10	67176	829.04282	1.23	2.406	0.00577	0.24	-0.138

Table. 9. Combination calculated for the standard solutions.

Drugs	Linearity range	Regression equation	R^2	LOD	LOQ
DIC-K	0.33-10.67	Y=9399.8x	0.9996	0.1345	0.4
MET	0.33-10-.67	Y=7097.8	0.9988	0.1677	0.55

quantification of Diclofenac potassium in combination with Metronidazole. The method was established by HPLC using ODS–C18 (250mmx4.6mm,5µ) with simple mobile phase containing alcohol: buffer (70:30) at a flow rate 1ml/min using 254nm. The HPLC method was validated shown satisfactory data for all the parameters tested. Statistical analysis proves that the method is suitable for the analysis of Diclofenac potassium and Metronidazole in combination. The reported method was found capable of giving faster analysis with good resolution and found to be reliable, economical and can be successfully employed for routine simultaneous estimation of Diclofenac potassium and Metronidazole in formulations. Study of the effects of exhaustive stress conditions is in progress.

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