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Simultaneous assay of Mupirocin and Metronidazole in formulations using Reverse Phase-High Performance Liquid Chromatography

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Abstract: A new reverse phase-high performance liquid chromatographic method for the assay of mupirocin and metronidazole in formulation has been developed and validated as per ICH guidelines. The present study was carried on Water's X-bridge C-18 column (4.6 x150mm, 5µ particle size) with mobile phase containing a mixture phosphate buffer (pH 2.5) and acetonitrile in the ratio of 70:30, %v/v at a flow rate of 1.0ml/min with UV detection at 220nm in ambient column temperature. The retention times for mupirocin and metronidazole were found to be 2.153 and 3.157 min respectively with linearity in the concentration range of 20-60µg/mL for mupirocin and 10-30µg/mL for metronidazole respectively. The developed reverse phase-high performance liquid chromatographic method was found to be best suitable for pharmacokinetic studies of these mentioned drugs in formulations.

Key words: Mupirocin; Metronidazole; ICH Guidelines

Introduction

Mupirocin[1-4],9-[(E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[[(2S,3S)-3-[(2S,3S)-3-hydroxybutan-2-yl]oxiran-2-yl]methyl]oxan-2-yl]-3-methylbut-2-enoyl]oxynonanoic acid is a natural crotonic acid derivative extracted from Pseudomonas fluorescens is an antibacterial drug which is used to treat small areas of skin infection and also to treat infections caused by bacteria called meticillin-resistant *Staphylococcus aureus* (MRSA). Mupirocin reversibly binds to bacterial isoleucyl-tRNA synthetase, which results in the inhibition of bacterial protein and RNA synthesis. [Figure 1]

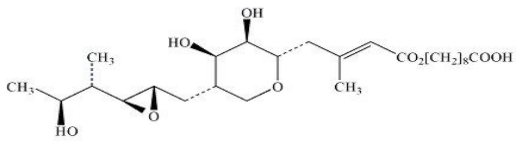

Figure 1: Chemical structure of Mupirocin

Metronidazole[5-10], 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethan-1-ol] is an synthetic antibacterial and antiprotozoal agent used to treat bacterial infections of the vagina, stomach, skin, joints, and respiratory tract[3]. Metronidazole covalently binds to DNA, disrupt its helical structure, inhibiting bacterial nucleic acid synthesis and resulting in bacterial cell death. [Figure 2]

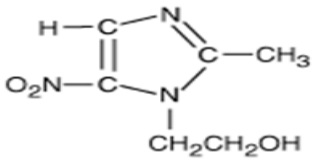

Figure 2: Chemical structure of Metronidazole

Combination of these two drugs is available in the local pharmacy as Mupimet Ointment (label claim 2% of Mupirocin and 1% of Metronidazole) that is used for the treatment of Genital tract infection, Anaerobic infections, Wounds, Skin infection caused by bacteria, Impetigo, Infections of gum and dental cavities and other conditions

Very few HPLC methods were reported for the determination of mupirocin and metronidazole in combination forms[11-12]. With this compliance, it made essential to develop a new reverse phase-high performance liquid chromatographic method for the assay of the above said drugs in combined formulations. In this accord attempts were made by the author to develop simple, precise and accurate reverse phase-high performance liquid chromatographic method for the simultaneous assay of mupirocin and metronidazole and extended it for their determination in formulations.

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Materials and Methods

Instrumentation:

The present chromatographic analysis was carried on Water's 2695 HPLC system provided with Hamilton Syringe, Water's X-bridge C-18 column (4.6 x150mm, 5µ particle size), auto sampler and 2996 Photodiode array detector. The statistical analysis of data was processed with Empower 2 software. Shimazdu electronic weighing balance [Model BL 220 H] was used for weighing the standards and samples. Elico pH meter (Hyderabad, India) LI 120 model was used for pH measurements.

Chemicals and Reagents:

Pharmaceutically grade pure sample of mupirocin and metronidazole were obtained from Spectrum Pharma Research Solutions, Hyderabad as gift samples along with their analytical reports. Commercial formulation of mupirocin and metronidazole in brand name of Mupimet-5.0gms ointment (label claim 2% of Mupirocin and 1% of Metronidazole) was procured from the local pharmacy**.** Milli-Q water, Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Ortho phosphoric acid (GR Grade), Trimethylamine (GR Grade), and Sodium dihydrogen phosphate dihydrate (GR Grade) were obtained from Qualigens Ltd., Mumbai. All dilutions were performed in standard class-A, volumetric glassware.

Mobile Phase Preparation:

The mobile phase was consisted of phosphate buffer (pH 2.5)-acetonitrile (70:30, %v/v). It was filtered through a 0.45 µm membrane filter before use.

Preparation of Phosphate Buffer:

The buffer was prepared was prepared by mixing 0.15% sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.2H_2O$) and 0.4% Triethylamine (TEA) with pH adjusted to 2.5 with 85% phosphoric acid.

Diluent Preparation:

Mobile phase is used as diluent in the present assay.

Preparation of standard solutions:

The standard stock solutions of mupirocin and metronidazole of concentrations 2000µg/mL and 1000µg/mL were prepared individually by weighing accurately 200mg of mupirocin and 100mg of metronidazole working standard and diluted to 100mL with diluent.

Preparation of mixed standard stock and working standard solutions:

10ml of the above standard stock solutions were diluted with mobile phase to obtain final concentrations of 200µg/mL and 100µg/mL of mupirocin and metronidazole respectively. Aliquots of mixed standard stock solution were diluted in range 1.0mL to 2.5mL in a 100mL volumetric flask with mobile phase and volume was made up to mark with mobile phase to obtain concentration ranging from 20-60µg/mL for mupirocin and 10-30µg/mL for metronidazole.

Sample preparation:

For determination of mupirocin and metronidazole in formulation (Mupimet cream), 5.0gm, equivalent to 200mg and 100mg of mupirocin and metronidazole was accurately weighed, transferred to a 100-ml volumetric flask and shaken with 20ml methanol for 25 min with mild warming and later the volume was made up to the mark with the diluent. The content was filtered through whatmann filter paper (No.41). This solution was further diluted with the same diluent to obtain concentration ranging from 20-60µg/mL for mupirocin and 10-30µg/mL for metronidazole respectively. Each of these drug solutions (20µL) was injected six times into the column, the peak area and retention times were recorded.

Results and Discussion

HPLC method development

In the development of the present RP-HPLC method for the selected drugs critical parameters, such as wavelength of detection, selection of column and composition of mobile phase and effect of flow rate on the column were studied in detailed.

Initial trial experiments were performed to select a suitable mobile system for determination of mupirocin and metronidazole respectively. Basing on the property of solubility of both the drugs phosphate buffer (pH adjusted to 2.5 with 85% phosphoric acid) and acetonitrile were selected as components of the mobile system. Different combinations of the above said mobile phases were tried that include phosphate buffer (pH 2.5) and acetonitrile (50:50 %v/v), phosphate buffer (pH 2.5)-acetonitrile (40:60 %v/v phosphate buffer (pH 2.5)-acetonitrile (60:40 %v/v) and phosphate buffer (pH 2.5) acetonitrile (70:30 %v/v), From the above trials it was found that the mobile phase consisting of phosphate buffer (pH 2.5) and acetonitrile in the ratio of 70:30, %v/v resulted in the excellent elution of the mentioned drugs with sharp peaks with low retention and run times.

In choosing the column the above mobile phase was subjected into different columns like C18 column (Inertsil 5µ, 250 mm × 4.6 mm), Water's X-bridge C-18 column (4.6 x150mm, 5µ particle size) and Zorbax SB C8(150X4.6mm,5µ) and

finally, Water's X-bridge C-18 column (4.6 x150mm, 5µ particle size) was found to be the best column for the present assay of the above cited drugs. Simultaneously, the effect of the flow rate (in the range 0.5-1.5 ml/ min) was studied and these studies revealed that the flow rate of 1.0ml/min resulted in maximum resolution of two mentioned drugs. After observing the overlay UV absorption spectrum of both the drugs the isosbestic point of mupirocin and metronidazole was observed at 220nm and this wavelength was chosen as detection wavelength in the present study. The total run time of the analysis was 6 minutes and the retention times of mupirocin and metronidazole were 2.153 and 3.157minutes respectively. The representative chromatogram of standard sample containing mupirocin and metronidazole was illustrated under figure.3 respectively**.**

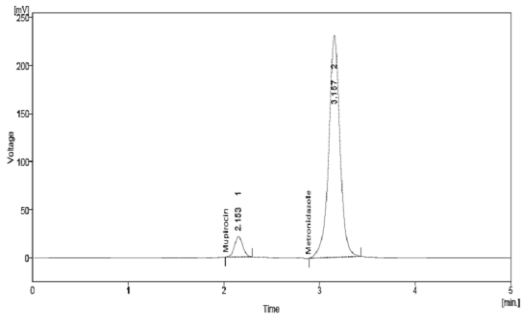

Figure 3: Representative chromatogram of standard sample containing mupirocin and metronidazole

Method Validation:

The above optimized method was fully validated in accordance with the current ICH guidelines[13] in terms of system suitability, specificity, linearity, limits of detection/quantitation precision, and accuracy.

System Suitability:

System suitability parameters like number of theoretical plates, HETP and peak tailing were determined for both the drugs with the proposed method and their values were tabulated in Table 1, respectively. It was found that all the system suitability parameters for developed RP-HPLC method for mupirocin and metronidazole were within the acceptance criteria.

Table 1: Column performance of mupirocin and metronidazole

Parameters	Mupirocin	Metronidazole
No. of theoretical plates	2749	3834
Tailing factor	1.07	0.949
Area	117.546	1875.465
Retention Time	2.153	3.157

Specificity:

The specificity of the proposed method was established by injecting and evaluating blank and placebo solutions using the prescribed optimized chromatographic conditions. It has been observed that there no endogenous peaks for diluent and placebo at the retention times of mupirocin and metronidazole thus, rendering proposed RP-HPLC method more selective and specific.

Linearity and Detector Response:

The linearity was performed by plotting, and calculating linear regression analysis for the standard curves of mupirocin and metronidazole [Figures 4 & 5] respectively. Two standard curves were obtained in the concentration range of 20-60µg/mL for mupirocin and 10-30µg/mL for metronidazole respectively. The slope and intercept value for calibration curve were **y = 1. 9956.x+ 37.6588 (r^2 = 0.9979)** for mupirocin and **y= 60. 813.x+ 629.53 (r^2 = 0.9982))** for metronidazole respectively. It was revealed that an excellent correlation exists between response factor and concentration of cited drugs within the concentration range indicated as above respectively [Table 2].

Table 2: Results of linearity of mupirocin and metronidazole

Mupirocin		Metronidazole	
µg/ml	Peak Area Ratio	µg/ml	Peak Area Ratio
20	79.141	10	1244.648
40	95.165	15	1515.123
60	117.546	20	1875.465
80	138.123	25	2142.362
100	157.443	30	2451.356
Slope,b	1.9956	Slope,b	60.813
Intercept,a	37.6588	Intercept,a	629.53
Correlation, r^2	0.9979	Correlation, r^2	0.9982
LOD	0.046µg/ml	LOD	0.0428µg/ml
LOQ	0.154µg/ml	LOQ	0.142µg/ml

The LOD values for mupirocin and metronidazole were found to be 0.046µg/mL and 0.042µg/mL, respectively and the LOQ values for mupirocin and metronidazole were found to be 0.154µg/mL and 0.142µg/mL respectively suggesting that very low quantities of mupirocin and metronidazole can be estimated accurately using the present developed method [Table 3].

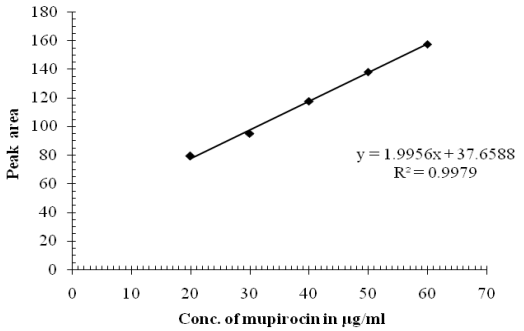

Figure 4: Calibration curve of mupirocin

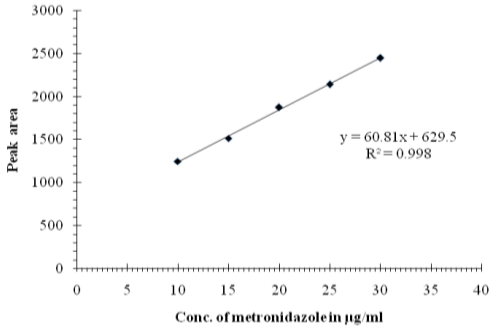

Figure 5: Calibration curve of metronidazole

Precision:

The precision of the developed method was evaluated by carrying out intra-day analysis by injecting six replicate injections of 100% test concentration of the above-mentioned drugs and the results were expressed in terms of standard deviation and %RSD respectively. The results are presented in table 3 revealed that the proposed HPLC method was highly precise.

Table 3: Results of precision studies of mupirocin and metronidazole

	Mupirocin		Metronidazole	
	Rt	Peak Area	Rt	Peak Area
Sample 1	2.177	117.468	3.153	1828.32
Sample 2	2.167	116.785	3.157	1879.004
Sample 3	2.16	118.689	3.187	1874.11
Sample 4	2.143	117.421	3.157	1833.076
Sample 5	2.131	116.354	3.15	1848.682
Sample 6	2.153	116.087	3.147	1865.499
%Mean*	2.15517	117.134	3.1535	1885.174
SD*	0.01659	0.94216	0.018393	9.77
%RSD*	0.76997	0.80435	0.583255	0.518255

*Average of six determinations

Accuracy:

The accuracy of the proposed method was determined at three concentration levels (50,100 and 150%) by recovery experiments which were carried out in triplicate preparations as per the proposed method. Data shown in table 4(99.95-99.99% for mupirocin and 99.97-100.01% for metronidazole) indicated that the developed method exhibited acceptable level of accuracy.

Table 4: Results of accuracy studies of mupirocin and metronidazole

Mupirocin			
S No	**50%Area**	**100%Area**	**150%Area**
Injection-1	78.303	117.444	157.765
Injection-2	79.121	117.789	158.342
Injection-3	78.324	116.567	157.98
Avg *	79	117	158.029
Amt Recovered*	19.98	39.99	59.97
%Recovery*	99.99	99.99	99.95
Metronidazole			
S No	50%Area	100%Area	150%Area
Injection-1	1218.05	1824.03	2462.81
Injection-2	1217.42	1825.12	2433.26
Injection-3	1219.23	1824.98	2446.87
Avg *	1218	1825	2447.64
Amt Recovered	9.97	19.98	30.01
%Recovery*	99.97	99.99	100.01

***Average of three determinations

Robustness Studies:

The robustness studies for mupirocin and metronidazole were established in the mentioned variance conditions (± 2 unit's change in flow rate and detection wavelength). From the results, it was observed none of the above-mentioned modifications caused a significant change in resolution between analytes, tailing factor and theoretical plates. System suitability parameters were also found satisfactory concluding robustness of the developed RP-HPLC method [Table 5].

Table 5: Results of robustness studies of mupirocin and metronidazole

Chromatographic parameters	Changed value	Retention time		Tailing factor	
		Mupirocin	Metronidazole	Mupirocin	Metronidazole
Flow Rate	0.8ml/Min	2.150	3.150	1.077	1.000
	1.2ml/Min	2.137	3.140	1.120	0.923
Wavelength	218nm	2.147	3.140	1.038	1.000
	222nm	2.147	3.143	1.174	1.000

Table 6: Results of ruggedeness studies of mupirocin and metronidazole

	Mupirocin		Metronidazole	
	Rt	Peak Area	Rt	Peak Area
Sample 1	2.153	116.831	3.153	1828.32
Sample 2	2.153	117.398	3.157	1879.004
Sample 3	2.167	118.897	3.187	1874.11
Sample 4	2.153	116.57	3.157	1833.076
Sample 5	2.15	117.783	3.15	1848.682
Sample 6	2.147	117.23	3.147	1865.499
%Mean*	2.153833	117.4515	3.1585	1854.782
SD*	0.006882	0.826238	0.014502	21.37161
%RSD*	0.319539	0.703472	0.459133	1.152244

*Average of six determinations

Ruggedness:

The ruggedness of the proposed RP-HPLC method was evaluated by analyzing samples by two different analysts using different instruments and columns on different days. The % RSD for peak areas of mupirocin and metronidazole was calculated and the experimental results are shown

in table 6 were within the ICH limits, indicating the good ruggedness of the developed RP-HPLC method.

Analysis of marketed formulation:

Analysis of market formulation {Mupimet cream-5.0gm containing mupirocin and metronidazole} was carried using the developed RP-HPLC method. The % drug content of mupirocin and metronidazole in formulation were found to be 99.78 and 99.98%, respectively [Table 7].

Table 7: Analysis of market formulations [Mupimet cream-5.0gms]

Drug	Labal claim (Equivalent weight)	Quantity found*	%RSD	%Assay
Mupirocin (1.0%)	100mg	99.98	0.213	99.98
Metronidazole (2.0%)	200mg	199.99	0.185	99.99

*Average of six determinations

Conclusions

It indicated form the optimized studies that the developed RP-HPLC method eluted mupirocin and metronidazole with the less retention times and with sharper peaks, better repeatability, higher sensitivity and with better efficiency. The validation results cited under results and discussion were within ICH guidelines, concluding that the proposed RP-HPLC method was found to be best suitable for the simultaneous assay of mupirocin and metronidazole in formulations.

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