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Jitesha Patel
Faculty of Pharmacy, G.H. Patel Building, Donor's Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Parth Patel
Faculty of Pharmacy, G.H. Patel Building, Donor's Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Rajashree Mashru
Faculty of Pharmacy, G.H. Patel Building, Donor's Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Corresponding Author:
Rajashree Mashru
Faculty of Pharmacy, G.H. Patel Building, Donor's Plaza, The M.S. University of Baroda, Vadodara, Gujarat, India

Novel UV-spectrophotometric & RP-HPLC method development and validation of simultaneous estimation of ranolazine and metformin HCL: A statistical analysis

Jitesha Patel, Parth Patel and Rajashree Mashru

Abstract
Reversed phase High-performance liquid chromatographic (RP-HPLC) and UV spectrophotometric methods were developed and validated for quantitative determination of Ranolazine and Metformin HCl. Different analytical validation parameter such as linearity, precision, accuracy, limit of detection, limit of quantification were determined according to ICH Q2[R1] guidelines. Separation was achieved by using Analytical Technologies Limited C18 (250 mm x 4.6 mm, 5µm) as stationary phase. The retention time for Ranolazine and Metformin HCl was 6.0±0.10 min and 3.4±0.15 min respectively. The UV Spectrophotometric method is based on first order derivative spectroscopy. Metformin HCl determined at 251nm (Zero crossing point of Ranolazine) and Ranolazine determined at 237nm (Zero crossing point of Metformin HCl) using methanol as a diluent. This method was validated as per ICH guideline. The Linearity of the calibration curve for each analyte in the desired concentration range was good (r^2>0.989) by both the RP-HPLC and UV methods. The method showed good reproducibility and recovery with % RSD less than 2%.The proposed methods are highly sensitive, precise and accurate and hence successfully applied for the estimating assay of synthetic mixture.

Keywords: ranolazine, metformin HCL, RP-HPLC, first derivative ZCP, anova

Introduction
Combination of Ranolazine (RANO) and Metformin HCl is used for the treatment of patient suffering from chronic angina and co-morbid type 2 diabetes mellitus. Metformin HCl (MET), is chemically known as 1-carbamimidamido-N, N-dimethylmethanimidamide1 is an effective biguanide antidiabetic agent. It is used for the treatment of noninsulin-dependent diabetes mellitus as a first-line drug. It act by using improving Glycaemic control by lowering glucose absorption, decreasing hepatic glucose production, and growing the insulin-mediated uptake of glucose. MET is the most effective antidiabetic drug that has been conclusively proven to prevent the cardiovascular complications of diabetes. It is also used in the treatment of polycystic ovary syndrome and additionally has been investigated for different diseases where insulin resistance may be a crucial factor [1].
RANO is N-(2, 6-dimethylphenyl)-2-{4-[2-hydroxy-3-(2-methoxyphenoxy) propyl] piperazin-1- yl} acetamide, is class of antianginal drug Ranolazine (RANO) is used for the treatment of cardiac ischemia it affects sodium dependent calcium channels during myocardial ischemia. Ranolazine is a piperazine derivative, is an antianginal agent. Ranolazine inhibit the cardiac late sodium current, which may affect the electrolyte balance in the myocardium, relieving angina symptoms [2].

Statistical analysis
Statistics may be defined as the collection, Presentation, analysis and interpretation of numerical data. Analysis of Variance is a technique of separating the total variability in a set of data into components parts, represented by a statistical model. If more than two assay methods are to be compared, the correct statistical procedure to compare the means is the one way analysis of variance (ANOVA). P value in ANOVA is the probability of that random sampling would lead to a difference between sample means as large or longer than you observed. P value threshold is fixed to the value same as alpha probability level. i.e. 0.05 Results of % Assay obtained by two developed methods were Subjected to ANOVA.

Chemical structure of ranolazine and metformin HCl

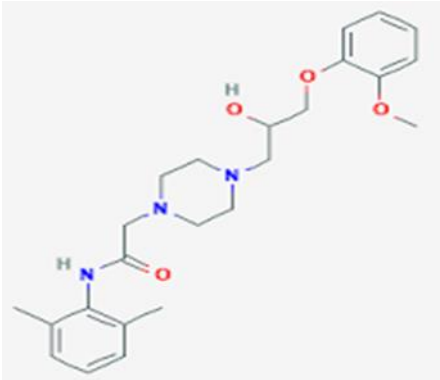

Fig 1: Chemical structure of Ranolazine [3]

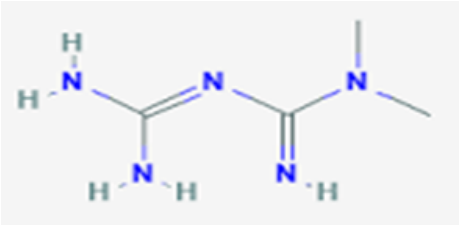

Fig 2: Chemical structure of metformin HCl [4]

Ranolazine and metformin HCl Combination study running in phase-3 clinical trial. Co-administration of Ranolazine and metformin HCl was well tolerated in these T2DM subject, with no serious side effect [5]. MET is official in IP, [6], USP [7], BP [8], JP [9] and RANO is not in official in any pharmacopeia. Various analytical methods are reported such as HPLC [10-18], UV Spectrophotometry [19-20] for the estimation of RANO and MET individually and its combination with other drug. However, still no any HPLC method has been developed for the simultaneous estimation of these two drugs. So the present work discusses the UV- spectrophotometric method development and RP-HPLC method development and validation for the simultaneous estimation of RANO and MET. The proposed method was validated as per ICH Q2 [R1] guideline [21].

Experimental part

RP-HPLC method

Instrumentation

Chromatography was performed on shimadzu chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD20AV absorbance detector. Data acquisition and integration was performed using Spinchrome software. Analytical Technologies Limited C18 column (4.6mm x 250mm, 5µm) used as stationary phase.

Chromatographic condition

The optimized mobile phase consisting of Acetonitrile and methanol and Ammonium Formate pH 6 in the ratio of 25:40:35(v/v).The mobile phase was filtered through nylon 0.2µm membrane filter and mobile phase degassed by ultra-sonication for 15min. Injection volume was 20µL with run time of 10minutes. Flow rate was set to 1mL/min and detection of both drugs was carried out at 228nm.

Preparation of buffer

0.05M Ammonium Formate pH 6 was used for method development. Buffer was prepared by dissolving 0.315g of ammonium formate in 100ml double distilled water. The pH was adjusted by glacial acetic acid using pH meter. The prepared buffer was passed through 0.22µm membrane filter and the degassed by ultra-sonication for 10min.

Preparation of mobile phase

Mobile phase was prepared by mixing 0.05M ammonium formate buffer (pH 6) and acetonitrile and methanol in ratio of (35:25:40v/v).Before use mobile phase was filter through 0.22µm membrane filter and the degassed by ultra-sonication for 15min.

Preparation of standard stock solutions and test solutions:

Weighed 10mg of standard Ranolazine and Metformin, transferred to 10mL volumetric flask separately, dissolved in methanol and then volume were made up to the mark with methanol, to obtain solution containing 1000µg/mL. Aliquots of the stock solution were appropriately diluted with methanol to obtain working standards of 500µg/mL solution of Ranolazine and metformin. The linearity of the method was investigated by using concentration in range 50-150 µg/ml by diluting appropriate volume of the stock solution with methanol.

Preparation of calibration curve

The calibration curve was prepared by injecting concentration of 50-150 µg/ml for both MET and RANO binary mixture solution manually in triplicate to the HPLC system at detection wavelength of 228 nm. The calibration curve was constructed by plotting concentration of Ranolazine and Metformin HCl versus peak area, and the regression equations were calculated.

UV Spectrophotometric method

Instrumentation

The UV method was performed on SHIMADZU double beam spectrophotometer with using 10mm quartz cuvettes. Data acquisition was done by using UV-probe software. The absorption spectra of standard and test solution was carried out in range of 200-400nm.

Determination of wavelength of maximum absorbance of Ranolazine and metformin

Solutions of 5 µg/ml of RANO and MET were separately prepared and scanned in the UV range of 200nm to 400nm and were stored in the memory of the instrument and transformed to first derivative with $\Delta\lambda$ = 10nm and scaling factor 1.After observing overlay first order derivative spectra, zero crossing point of drug were selected. At 237nm, Metformin HCl shows zero crossing point and hence Ranolazine can be determined. At 251nm, Ranolazine shows zero crossing point and hence Metformin HCl can be determined. Selection of the wavelength spectra is demonstrated in figure 3.

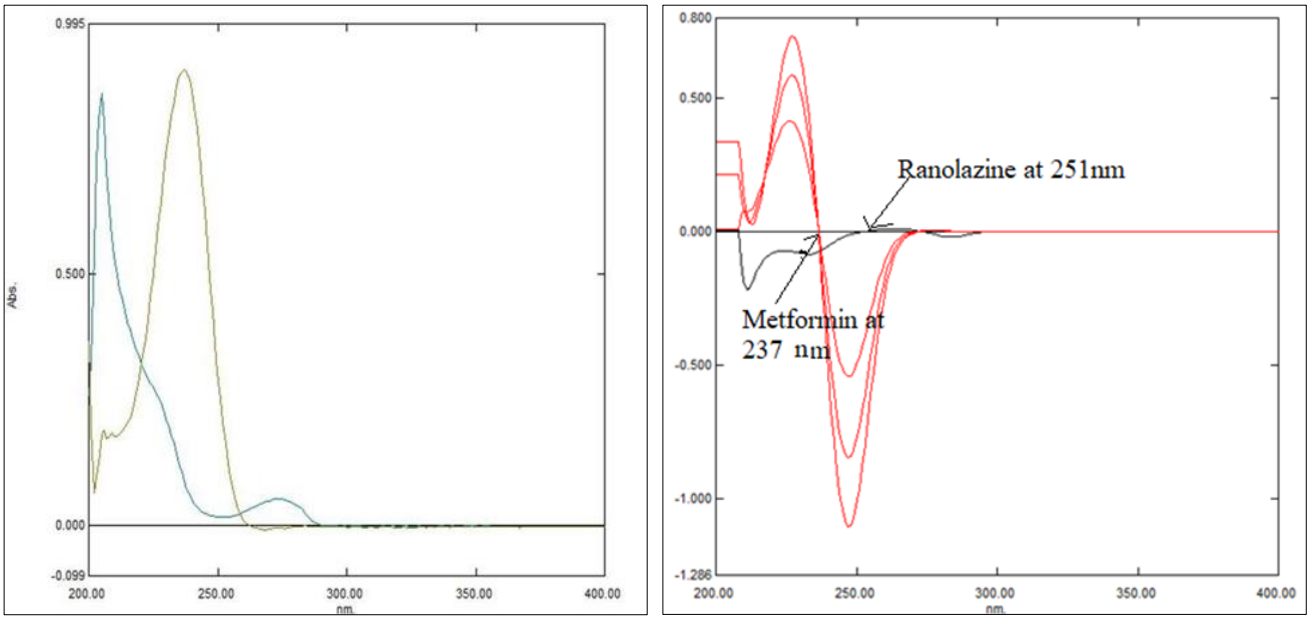

Fig 3: Wavelength selection spectrum of ranolazine and metformin HCL

Preparation of standard stock solution and test solution
Both UV Spectrophotometric method 10mg each of Ranolazine and Metformin were weighed accurately and transferred into a 10 ml volumetric flask, dissolved in methanol and then volume were made up to mark with methanol to produce a stock solution containing 1000 µg/ml of Ranolazine and Metformin respectively. Aliquots of the stock solution were appropriately diluted with methanol to obtain working standards of 500µg/mL solution of Ranolazine and metformin. The linearity of the method was investigated by using concentration in range 5-25 µg/ml by diluting appropriate volume of the stock solution with methanol.

Preparation of calibration curve
The calibration curve was prepared by scanning test sample ranging from 5-25 µg/ml at 237 nm and 251 nm. The calibration curve was constructed by plotting concentration of Ranolazine and Metformin HCl versus peak area, and the regression equations were calculated.

First order zero crossing point method
The absorption spectra of the solutions of Metformin HCl and Ranolazine were recorded in the range of 200 nm to 400 nm and were stored in the memory of the instrument and transformed to first derivative with $\Delta\lambda$ = 10nm and scaling factor 1. Calibration curves were constructed with six different concentrations in the range between 5-25 µg/ml for both metformin HCl and Ranolazine.
Statistical Analysis

Method validation
The HPLC method and UV method was validated in terms of linearity, sensitivity, precision and accuracy, robustness in accordance with ICH Q2 (R1) guideline and system suitability test as per USP [22].

Linearity
The linearity of an analytical procedure is its ability (within given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The calibration curve was constructed by plotting peak area versus concentration and the linearity was evaluated by least square regression analysis. Calibration curve were prepared with appropriate volume of working standard solutions (500 µg/ml) for both UV and RP-HPLC with the range of 5-25 µg/ml and 50-150 µg/ml respectively.

Precision
Precision of the developed method was studied by performing intra-day and inter- day precision. The intraday precision was determined by performing three measurements of different concentration on the same day at different time interval. The inter-day precision of method was checked by repeating the study on three consecutive days. The % RSD (Relative Standard Deviation) was calculated.

Accuracy
Accuracy of the method was estimated by using standard addition method at three different levels by recovery experiments. The recovery is calculated from the test results as the percentage of analyte recovered by the assay. The
Known amounts of standard solutions of Ranolazine and metformin were added to a pre evaluate test solutions of Ranolazine and mesalamine. Each solution was injected in triplicate, and the recovery was calculated.

Limit of detection
It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. The limit of detection (LOD) of the drugs was derived by calculating the signal to noise ratio (S/N, i.e., 3.3. Limit of detection can be calculated using the following equation as per ICH guidelines.

$LOD = 3.3 \times s/S$

Where, s = the standard deviation of response and S = Slope of calibration curve.

Limit of quantification
It is the lowest concentration of an analyte in a sample that can be determined with the acceptable precision and accuracy under stated experimental conditions. The limit of quantification (LOQ) of the drugs was derived by calculating

the signal to noise ratio (S/N, i.e., 10 for LOQ) using the following equation as per International Conference on Harmonization (ICH) guidelines.

LOQ = 10 ×s/S

Where, s = the standard deviation of response and S = Slope of the calibration curve.

Robustness

The robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variation in method parameter and provide an indication of its reliability during normal usage.

- PH
- Flow rate
- Concentration of methanol

Ruggedness

The ruggedness is an analytical method of the degree of reproducibility of samples results obtained by analysis of the same samples under a different conditions for example in different PH, different temperature and different mobile composition.

Assay of combination dosage form

Combination dosage form was prepared by equivalent to take 15 mg for both RANO and MET with common tablet excipients in appropriate amount. This mixture was diluted with methanol to make concentration 15µg/mL for both drugs.

Result and discussion

RP-HPLC and UV-method validation

Linearity

RP-HPLC and UV-Spectrophotometric methods were developed for MET and RANO which can be conveniently Employed for routine analysis in pharmaceutical dosage forms. The chromatographic conditions were optimized in Order to provide a good performance of the assay. The Retention time for RANO and MET was found to be 6.0 min and 3.4 min respectively. The chromatograms have been shown in Fig.4.A seven point calibration curve was constructed with working standard and found linear for each of the analytes over their calibration ranges. The slopes were calculated using the plot of drug concentration versus peak area of the chromatogram. Figure 5 shows overlay spectra of both drugs of the UV-Spectrophotometric method. The regression coefficient of the correlation equation was greater than 0.989 and the method was validated by using MET and RANO drugs with less than 2% RSD.

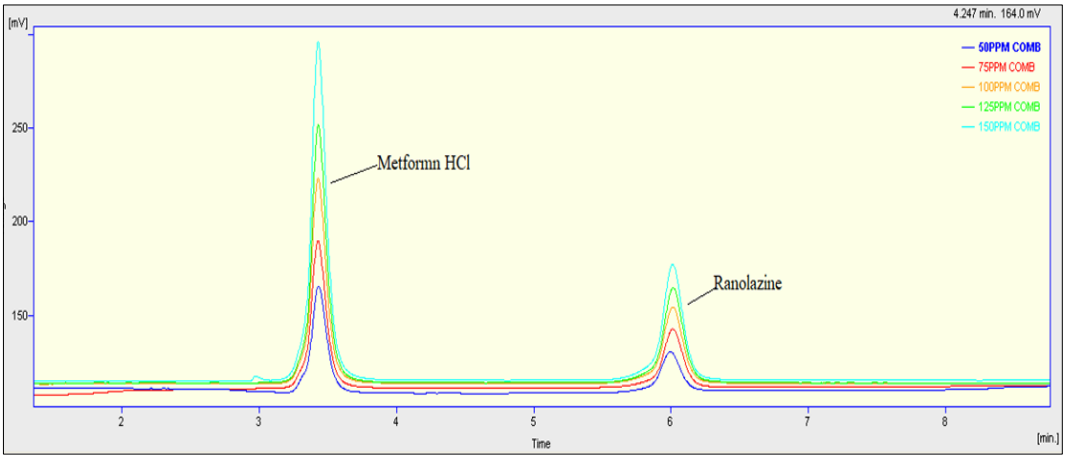

Fig 4: Linearity Spectra of MET and RANO by RP-HPLC Method

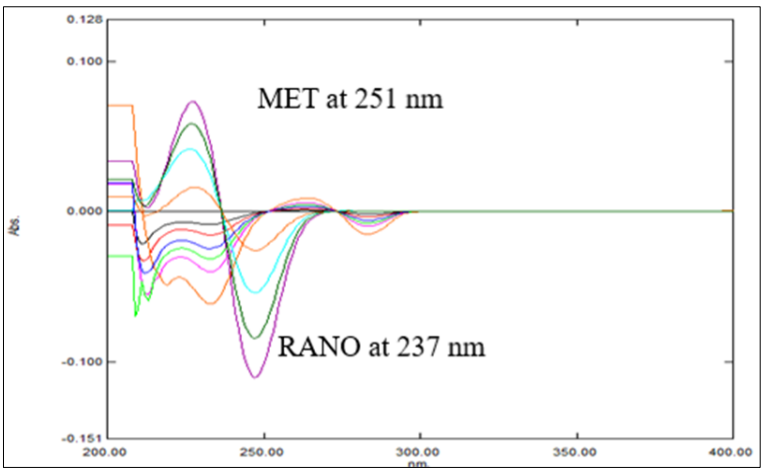

Fig 5: Linearity Spectra of MET and RANO by UV-Method

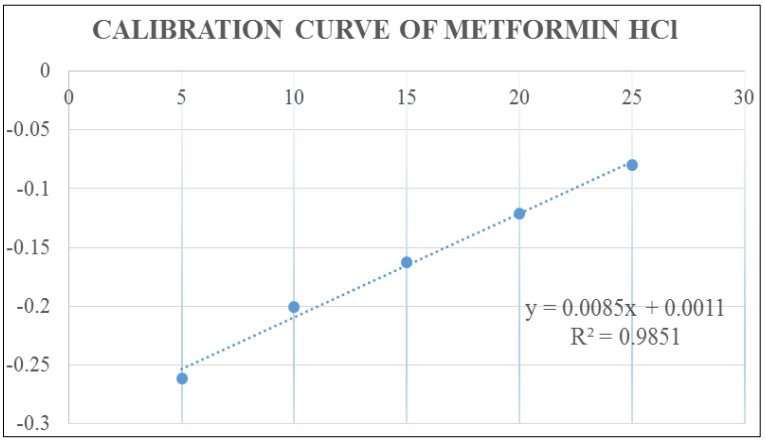

Fig 6: Calibration Graph of First Derivative of MET

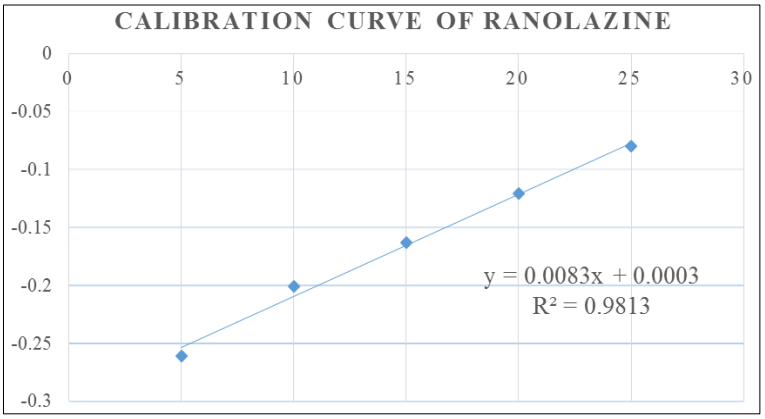

Fig 7: Calibration Graph of First Derivative of RANO

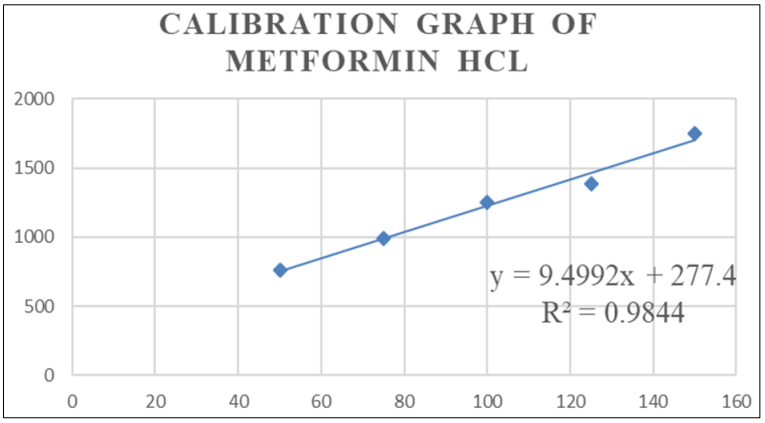

Fig 8: Calibration Graph of MET

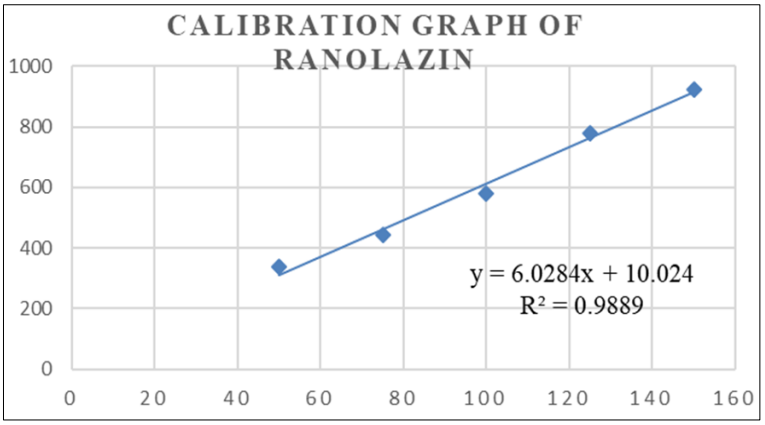

Fig 9: Calibration Graph of RANO

LOD and LOQ

LOD and LOQ of RANO and MET were determined by formula. LOD and LOQ for UV method were found to be 0.09 µg/mL and 0.28 µg/mL for Ranolazine and 0.26 µg/mL and 0.80 µg/mL for metformin HCl, respectively. LOD and LOQ for RP-HPLC method were found to be 0.19 µg/mL and 0.57 µg/mL for Metformin HCl and 0.8 µg/mL and 0.28 µg/mL for Ranolazine, respectively.

Accuracy

Accuracy for the RP-HPLC method and UV-method was estimated using the standard addition method at three different level by recovery experiment. For UV-method accuracy was obtained between 98.41% to 100.02% and 98.25% to 99.42% for RANO and MET respectively. For RP-HPLC method accuracy was obtained between 99.28% to 101.29% and 99.63% to 101.76% for MET and RANO respectively. The result of the recovery study are shown in Table 1. Thus method is accurate as per recovery study.

Table 1: Result of recovery study by RP-HPLC and UV method

Method	Drug	Spiked level	Amt. Present (µg/mL)	Amt. added (µg/mL)	Amt. recovered (µg/mL)	%Recovery ±SD (n=3)
RP-HPLC	RANO	50%	100	50	150.29	99.63±0.36
Method		100%	100	100	198.11	101.76±0.32
		150%	100	150	249.83	99.58±0.48
	MET	50%	100	50	149.51	101.28±0.41
		100%	100	100	199.86	99.71±0.38
		150%	100	150	249.05	99.28±0.64
UV	RANO	80%	100	80	179.61	98.41±0.000164
Method		100%	100	100	199.94	100.02±0.00021
		120%	100	120	218.88	100.16±0.00065
	MET	80%	100	80	180.65	98.62±0.00028
		100%	100	100	198.92	98.25±0.00016
		120%	100	120	220.71	99.42±0.00076

Precision

Intraday and Interday precision was measured in terms of % RSD. The experiment was repeated 3 times a day for intraday and for 3 different days for inter-day precision. Absorbance was determined and result found satisfactory as RSD<2 for both UV-method and RP-HPLC method. The result of the precision are presented in table 2, 3 and the method was found to be precise.

Table 2: Data of Intraday and Interday precision for RANO and MET by UV-method

Precision		Intraday precision(n=3)		Interday precision (n=3)	
Drug	Level (%)	Absorbance (Mean±SD)	%RSD	Absorbance (Mean±SD)	%RSD
	80	0.0211±0.0004	1.44	0.0210±0.0003	1.43
RANO	100	0.0264±0.0006	1.68	0.0261±0.0005	1.63
	120	0.0321±0.0003	1.18	0.0320±0.0003	1.20
MET	80	0.0472±0.0001	1.34	0.0481±0.0002	1.47
	100	0.0532±0.0002	1.42	0.0627±0.0004	1.52
	120	0.0782±0.0003	0.69	0.0795±0.0003	0.74

Table 3: Data of Intraday and Interday precision for RANO and MET by RP-HPLC method

Precision		Intraday precision(n=3)		Interday precision (n=3)	
Drug	Level (%)	Area (Mean±SD)	%RSD	Area (Mean±SD)	%RSD
	50	748.13±4.72	0.63	760.09±7.94	1.04
RANO	75	989.05±8.55	0.83	975.67±17.91	0.54
	100	1236.33±5.42	1.44	1248.63±9.84	0.79
MET	50	353.42±6.97	1.87	356.79±3.93	1.10
	75	448.60±6.40	1.43	460.74±4.49	0.97
	100	581.43±8.22	1.41	587.84±5.22	0.89

Robustness

Making a deliberate change in wavelength for UV-Method and change in mobile phase composition and PH of mobile phase for RP-HPLC and the %RSD found to be less than 2, specify that the both method are robust. The Result are demonstrated in Table 4 and 5

Table 4: Robustness study for RANO and MET by UV-Method

Conc (µg/mL)	Absorbance at different wavelength (RANO)			Absorbance at different wavelength (MET)		
	236 nm	237 nm	238 nm	250 nm	251 nm	252 nm
	0.028	0.033	0.025	0.062	0.064	0.057
15 µg/mL	0.032	0.031	0.028	0.058	0.053	0.062
	0.030	0.034	0.038	0.052	0.061	0.056
Mean ± SD	0.035 ±0.003	0.032 ±0.002	0.065 ±0.005	0.057 ±0.001	0.059 ±0.002	0.058 ±0.004
RSD	1.85	1.78	1.92	1.64	1.76	1.88

Table 5: Robustness study for RANO and MET by RP-HPLC Method

Factors		Peak Area	
	Level of change	**MET**	**RANO**
PH	0.45	1256.61	586.85
	0.5	1275.22	580.18
	0.55	1280.50	584.17
	Mean±SD	1270±4.44	583.73±7.94
	%RSD	0.58	0.86
Mobile Phase	Methanol: ACN: Amm. Formate (42:23: 35)	1244.89	584.85
	Methanol: ACN: Amm. Formate (40:29: 33)	1239.88	568.43
	Methanol: ACN: Amm. Formate (41:24: 35)	1247.46	575.66
	Mean±SD	1244.07±9.68	576.31±9.68
	%RSD	0.77	0.93

Assay of Synthetic mixture

Result of % Drug content of RANO and MET in combine dosage form was found 100.58%±0.047 and 100.40%±0.062 by UV-method and 100.87%±0.382 and 100.76%±0.427 by RP-HPLC method, respectively.

Table 6: Assay of combine dosage form by RP-HPLC and UV-Method

Method	Drug	%Assay (Avg ± SD) (n=3)	%RSD
UV-Method	RANO	100.58±0.047	0.483
	MET	100.40±0.062	0.751
RP-HPLC Method	RANO	100.87±0.382	0.348
	MET	100.76±0.427	0.642

Statistical analysis of the developed methods

The analysis done three times by each method (Count-3). Data analysis was done using Microsoft Excel for Mac Version16.34. It was used for applying ANOVA test. Results of ANOVA for RANO and MET are shown in table 7.

Table 7: ANOVA for comparision of different methods for RANO & MET

Anova: Single Factor						
Summary						
Groups	Count	Sum	Average	Variance		
Column 1	2	201.45	100.725	0.04205		
Column 2	2	201.16	100.58	0.0648		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.021025	1	0.021025	0.39354235	0.59451464	18.5128205
Within Groups	0.10685	2	0.053425			
Total	0.127875	3				

Conclusion

Simple, rapid, accurate and precise RP-HPLC as well as UV-spectrophotometric methods have been developed and validated for the routine analysis of RANO and MET in API and synthetic mixture forms. This method was validated as per ICH guideline. The value of standard deviation and coefficient of variation calculated were satisfactory low, indicating the suitability of the proposed methods for routine estimation of RANO and MET. Statistical analysis of all the two methods were done. It can be seen that P-value for both RANO & MET was greater than α=0.05 and observed F value was lower than Fcritical values, hence there was no significant difference between two methods for RANO & MET.

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