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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR METOPROLOL SUCCINATE AND TELMISARTAN USING UV SPECTRO-PHOTOMETRY AND RP-HPLC

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ABSTRACT

An accurate, precise and reproducible bioanalytical UV-spectrophotometric and liquid chromatographic assay method were developed and validated for the determination of metoprolol succinate (MET) and telmisartan (TEL) in blood plasma using protein precipitation technique. Spectrophotometric estimation was done by simultaneous equation method followed by first order derivative method using 50% methanol as solvent. In this method λ_{max} for MET and TEL were selected at 240.5 nm and 237.5 nm respectively. RP-HPLC analysis was carried out using Prontosil C-18 column (4.6 x 250 mm, 5 µ particle size) and mobile phase composed of methanol:acetonitrile:phosphate buffer (pH-5) in ratio of 35:35:30 v/v/v, at a flow rate of 1.0 mL min^{-1} and chromatogram was recorded at 225 nm. Linearity of drugs for blood plasma was evaluated over the concentration range of 5-25 µg mL^{-1} and 8-40 µg mL^{-1} for MET and TEL in both UV spectrophotometric and RP-HPLC method (the value of r^2= 0.999 by both the methods for MET and TEL). The developed methods were validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values. Therefore, both the methods can be used for routine monitoring of MET and TEL in industry in the assay of bulk drug, in plasma and tablets.

Key words: *Metoprolol succinate; Telmisartan; UV-Spectroscopy; RP-HPLC; Simultaneous equation method; First derivative method.*

INTRODUCTION

Metoprolol succinate (MET)[1], (±)1-(Isopropylamino)-3-[*p*-(2-methoxyethyl)phenoxy]-2 propanol succinate (2:1) (salt), is orally administered β-adrenergic receptor blocking agent widely used in the treatment of hypertension. This preferential effect is not absolute, however and at higher doses, metoprolol also inhibits β-2 adrenoreceptors, chiefly located in the bronchial and vascular musculature. It may be used alone or in combination with other antihypertensive agents.[2]

Telmisartan[3], 4′-[[4-Methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl]biphenyl-2-carboxylic acid, is an orally active nonpeptide angiotensin II antagonist that acts on the AT1 receptor subtype. Angiotensin II is the principal precursor of the renin-angiotensin system, with effects that include vasoconstriction, stimulation of synthesis and release of aldosterone, cardiac stimulation and renal reabsorption of sodium. Telmisartan works by blocking the vasoconstrictor and aldosterone secretory effects of angiotensin II [4].

Tablet dosage forms containing MET and TEL in ratio of 25mg:40 mg tablets of various brands are available in market. Several analytical methods that have been reported for the estimation of metoprolol succinate in biological fluids and/or pharmaceutical formulations include spectrophotometric[5,6], high-performance liquid chromatography (HPLC)[7,8] and stability indicating HPTLC[9], spectrofluorometry[10] while TEL determinations have been reported by UV-Vis spectrophotometry[11], HPLC[12-14] and HPTLC[15], stability indicating UPLC[16] and forced degradation behaviour by RP-HPLC[17]. However there is no method available for the simultaneous determination of these two drugs. Therefore, an attempt was made to develop a new, rapid and sensitive method for the simultaneous determination of MET and TEL in plasma by precipitation method. To assess the reproducibility and wide applicability of the developed method, it was validated as per ICH guidlines[18,19].

EXPERIMENTAL

Instrument

In UV-spectrophotometric method, Shimadzu model-1700 series was used, which is a double beam double detector spectrophotometer, having spectral bandwidth 3 nm and of wavelength accuracy ±1 nm, with 1cm quartz cells.

Liquid chromatographic system from Young Lin 9100 comprising of manual injector, YL 9111 quaternary pump for constant flow and constant pressure delivery and photodiode array detector (YL 9160 detector) connected to software YL clarity for controlling the instrumentation as well as processing the generated data. Cooling centrifuge used was RemiC-23 BL. Min Sell.

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Reagents and chemicals

Pure sample of MET and TEL was obtained as gift sample from Unichem Laboratories Ltd. (Unichem Bhavan, Prabhat Estate, Off S.V.Road, Jogeshwari (West), Mumbai) Tablet, TELMA-X of Glenmark, Nasik containing MET and TEL in ratio of 25 mg:40 mg respectively was purchased from local market. Acetonitrile (HPLC Grade), methanol (HPLC Grade), potassium dihydrogen phosphate (AR grade) supplied by Rankem. Milli-Q was used to prepare water used in UV spectroscopy and RP-HPLC method. Methanol used in UV spectrophotometry and a mixture of methanol:acetonitrile:phosphate buffer (pH5) in ratio of 35:35:30 v/v/v in RP-HPLC as diluents.

Linearity range and calibration graph

Extraction of drug sample

Accurately weighed 10 mg of MET and TEL were transferred into 50 mL volumetric flasks separately and dissolved in 10 mL of plasma, then volume was made up to 50 mL with methanol and mixture were shaken vigorously, this gave complete precipitation of plasma protein. The mixture was kept for few minutes until precipitate of protein settled down then supernatant layer was collected. 10 mL methanol was added in order to precipitate protein completely. Collected supernatant layer was centrifuged at 6000 rpm for 7 min at 4°C and then filtered by whatman filter paper (no.41). Concentration of MET and TEL in methanol was 200 µg mL^{-1}. (Stock- A)

Preparation of Standard Stock Solutions of MET and TEL

Standard stock solutions were prepared by dissolving separately 100 mg of each drug in 100 mL of diluents used in UV spectrophotometry method (i.e. methanol) and RP-HPLC method (i.e. A mixture of methanol: acetonitrile:phosphate buffer (pH 5) in ratio of 35:35:30 v/v/v) and the flask was sonicated for about 10 min to solubilize the drugs (Stock-A).

Preparation of Sub Stock Solution

5 mL of solution was taken from stock-A of **MET and TEL** and transferred into 50 mL volumetric flask separately and diluted up to 50 mL with diluent to give concentration of 100 µg mL^{-1} (i.e. Stock-B).

Preparation of working standard solution for calibration curve

0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL and 2.5 mL of stock-B of MET was taken separately in 10 mL volumetric flask and volume was made up to 10mL with diluent. This gives the solutions of 5 µg mL^{-1}, 10 µg mL^{-1}, 15 µg mL^{-1}, 20 µg mL^{-1} and 25 µg mL^{-1} for MET.

Likewise 0.8 mL, 1.6 mL, 2.4 mL, 3.2 mL, 4.0 mL of stock-B of TEL was taken separately in 10 mL volumetric flask and volume was made up to 10 mL with diluent. This gives the solutions of 8µg mL^{-1}, 16µg mL^{-1}, 24µg mL^{-1}, 32µg mL^{-1} and 25µg mL^{-1} for TEL.

For spectrophotometric method, UV spectrum was recorded in the range of 200-400nm (Fig.1) in the concentration range of 5-25 µg mL^{-1} for MET and 8-40 µg m^{L-1} for TEL. Calibration curve was plotted between concentration versus absorbance at λmax for MET and TEL i.e. 240.5 nm and 237.5 nm for MET and TEL respectively (Fig.2, and Fig. 3). The result of their optical characteristics and linearity data of both drugs are reported in the Table 1.

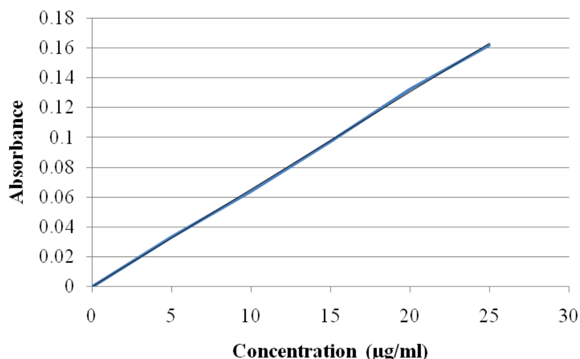

Fig. 2: *Calibration curve of MET in UV spectrophotometric method*

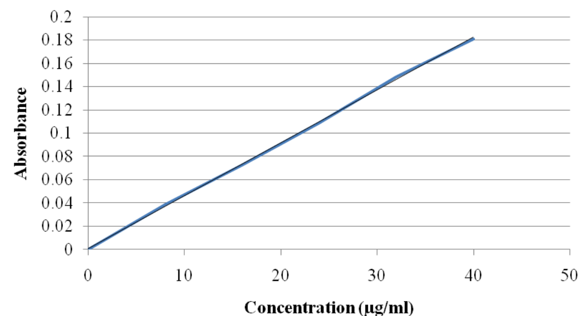

Fig. 3: *Calibration curve of TEL in UV spectrophotometric method*

Table 1: *Optical characteristics and linearity data of MET and TEL*

Sr.No.	Parameters	UV Spectrophotometric Method		RP-HPLC Method	
		MET	TEL	MET	TEL
1	Working λ	240.5	237.5	225	225
2	Beer's law limit (µg/ml)	5-25	8-16	4-20	8-40
3	Correlation Coefficient (r^2)*	0.9997	0.9995	0.9998	0.9998
4	Slope (m)*	0.0065	0.0045	26.73	102.11
5	Intercept (c)*	0.04	0.0008	-2.14	26.05

*Average of five determination

For HPLC method, the chromatogram was recorded at 225 nm (Fig. 4A and 4B) for both the drugs in the concentration range 5-25 µg mL^{-1} for MET and 8-40 µg mL^{-1} for TEL. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph (Fig. 5, and Fig. 6). The result of their optical characteristics and linearity data of both drugs is reported in Table 1.

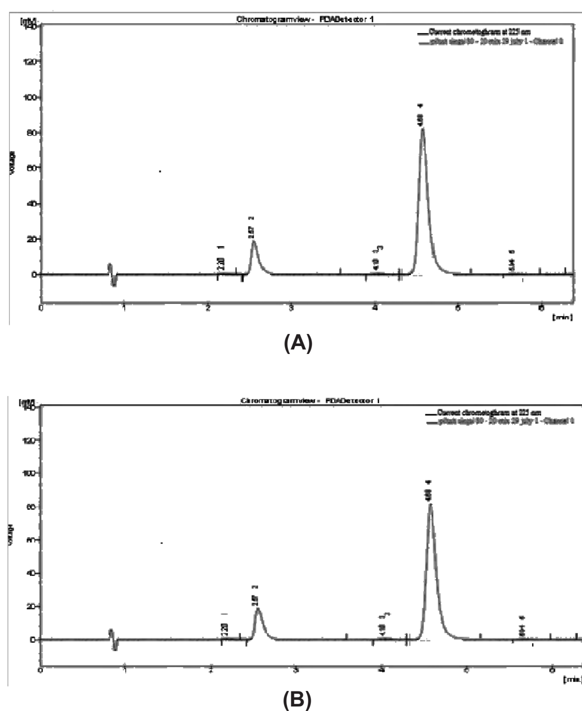

Fig. 4: *(A) Chromatogram of MET &TEL with plasma extraction (B) Chromatogram of MET &TEL AT 225 nm*

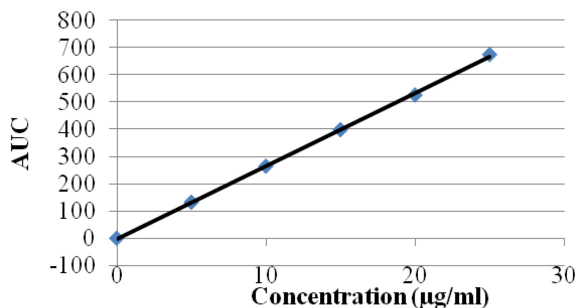

Fig. 5: *Calibration curve of MET in RP-HPLC method*

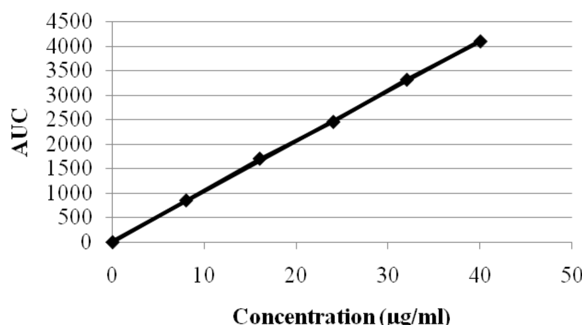

Fig. 6: *Calibration Curve of TEL in RP-HPLC Method*

UV Spectrophotometric Method

Study of overlay spectra of drugs and selection of method

The overlay spectra exhibit that the absorbance maxima of MET (i.e 223nm and 278 nm) is overshadow by the TEL (i.e λ_{max} is 293 nm) Fig. 1(A). To identify the absorbance maxima of each drug, the UV spectrum of each drug was derivatized in first order then simultaneous equation method was applied for quantitative determination of MET and TEL Fig. 1(B, C). Due to difference in absorbance maxima after derivatization and having no interference with each other, both drugs were simultaneously estimated by simultaneous equation method[20].

Vierordt's simultaneous equation method

The wavelength 237.5nm (λ_{max} of MET) and 240.5nm (λ_{max} of TEL) was selected for quantitative estimation of drug using simultaneous equation method after first order derivatization. Overlay spectra is shown in Fig. 1(D).

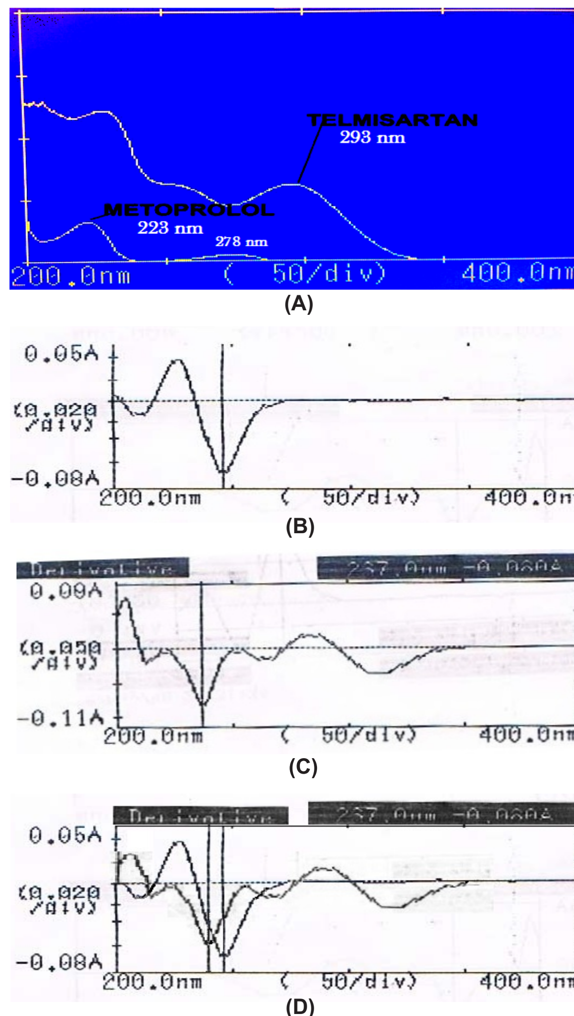

Fig. 1: *(A) UV spectra of MET and TEL*
First order derivative UV spectra of (B) MET (C) TEL
(D) First order derivative overlay UV spectra of MET and TEL

1) The absorptivity of metoprolol at λ_1 (237.5nm) and λ_2 (240.5 nm) are ax_1 and ax_2 respectively (Table 2).
2) The absorptivity of telmisartan at λ_1 (237.5 nm) and λ_2(240.5 nm) are ay_1 and ay_2 respectively.
3) The absorbance of the diluted sample at λ1 and λ2 are A_1 and A_2 respectively.

Let Cx and Cy be the concentrations of metoprolol and telmisartan respectively in the dilute sample. Two equations are constructed based upon the fact i.e. at λ_1 and λ_2 the absorbance of the mixture is the sum of individual absorbance of X and Y[24].

The quantification analysis of MET and TEL in a binary mixture was performed by using **Eqn-1** and **Eqn-2**.

$C_X = A_2ay_1 - A_1ay_2 / ax_2ay_1 - ax_1ay_2$ ……**Eqn-1**

$C_Y = A_1ax_2 - A_2ax_1 / ax_2ay_1 - ax_1ay_2$ ……**Eqn-2**

Table 2 : *Absorptivities of MET (x) and TEL (y) at λ_1 and λ_2*

	Simultaneous Equation Method			
Drug	**237.5 nm (λ_1)**		**240.5 nm (λ_2)**	
MET	ax_1	0.0041	ax_2	0.0066
TEL	ay_1	0.0045	ay_2	0.0022

RP-HPLC Method

System Suitability

The system suitability parameter was carried out to verify that the analytical system was working properly and could give accurate and precise result. The six replicates of reference standard were injected separately and chromatogram was recorded. The result of system suitability parameter is reported in Table 3.

Table 3 : *Results of system suitability parameters*

Sr. No.	PARAMETERS	MET (RT=2.74min)	TEL (RT=6.7min)
1	No. of Theoretical Plates	2954.67±4.32	5919.83±3.43
2	HETP	0.08±0.00	0.04±0.00
3	Tailing Factor	1.77±0.015	1.39±0.03
4	Resolution	2.64	2.01
5	Capacity Factor	1.609	1.272

N=6

Analysis of Marketed Formulation

Twenty tablets containing MET and TEL (TELMA-X) were weighed and ground to a fine powder; an equivalent amount to 25 mg of MET was taken in 100 ml volumetric flask. The TEL present in this amount of tablet powder was 40 mg. This was dissolve in 25 ml of diluents by sonication for about 10 minutes. The volume was made up to the mark by diluents as per the UV spectrophotometry method and RP-HPLC method. The solutions were filtered (whatman filter paper no.41). The filtrate was used to prepare samples of different concentration. The statistical evaluation of tablet analysis by both methods is reported in Table 4.

Table 4 : *Results and statistical parameters for tablet analysis (TELMA-X)*

S. No	Drug	Label Claim	Amount Found	MEAN*	S.D.*	%COV*	Std. Error*
UV Spectrophotometry Method	MET	50	49.39	98.788	1.336	1.353	0.232
	TEL	40	39.52	98.805	0.850	0.860	0.342
RP-HPLC	MET	50	49.25	98.511	1.135	1.153	0.245
	TEL	40	39.516	98.791	0.921	0.932	0.354

**Average of five determination*

Validation Parameters

Bioanalytical method validation includes all the procedures to demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix such as blood, plasma, serum and urine are reliable and reproducible for the intended use. The process by which a specific bioanalytical method is developed, validated and used in routine sample analysis can be divided into:

1. Reference standard preparation for calibration curve;
2. Bioanalytical method, development and establishment of assay procedure;
3. Application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run/batch.

In Quantitative Bioanalytical procedures, following validation parameters should be evaluated: selectivity, the calibration model (linearity), stability, accuracy (bias), precision (repeatability, intermediate precision) and the lower limit of quantification (LLOQ). Additional parameters which may be relevant include limit of detection (LOD), recovery, reproducibility, and ruggedness (robustness). For qualitative procedures, a general validation guideline is currently not available, but there seems to be agreement that at least selectivity and the LOD should be evaluated and that additional parameters like precision, recovery and ruggedness (robustness) might also be important[21,22].

Linearity

Linearity was studied by analyzing five standard solutions (n=5) in the range of 5-25 µg mL^{-1} and 8-40 µg mL^{-1} for MET and TEL respectively in both UV spectrophotometric and HPLC method. Calibration curves with concentration verses absorbance or peak area was plotted for each method and the obtained data were subjected to regression analysis using the least squares method. Linearity of MET and TEL was established by response ratios of drug. Response ratio of both drugs was calculated by dividing the absorbance or peak area with respective concentration (Table 5).

Table 5 : *Response ratios of MET and TEL*

S.No	Concentration (µg/ml)		UV Spectrophotometric				RP-HPLC			
	MET	TEL	ABS	RR	ABS	RR	AUC	RR	AUC	RR
1.	5	8	0.033	0.0066	0.036	0.0045	132	26.4	852	106.5
2.	10	16	0.066	0.0066	0.072	0.0045	264	26.4	1704	106.5
3.	15	24	0.099	0.0066	0.108	0.0045	396	26.4	2556	106.5
4.	20	32	0.132	0.0066	0.144	0.0045	528	26.4	3408	106.5
5.	25	40	0.165	0.0066	0.18	0.0045	660	26.4	4260	106.5

Accuracy

The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80 %, 100 % and 120 %. The recovery studies were carried out by adding known amount of standard solution of MET and TEL to preanalysed tablet solutions. The resulting solutions were then re-analysed by proposed methods.

In UV Spectrophotometric method, the value of mean recoveries was found to be in the range of 98.06 % to 98.25 % for MET and 98.11 % to 98.13 % for TEL. The value of SD and %RSD less than 2 indicate the accuracy of the method.

In RP-HPLC method, the value of mean recoveries was found in the range of 98.24 % to 99.35 % for MET and 98.21 % to 99.84 % for TEL. Total amount of drug found and percentage recovery was calculated. Results of recovery studies are reported in Table 6.

Table 6 : *Results of recovery studies on marketed formulations*

Recovery Level %	% Recovery (Mean±SD)*			
	UV Spectrophotometric method		RP-HPLC method	
	MET	TEL	MET	TEL
80	98.19± 0.52	98.13±0.30	98.240±1.156	98.21±0.409
100	98.25± 0.34	98.11±0.28	99.118±0.526	99.840±0.044
120	98.06±0.40	98.11±0.28	99.351±0.305	99.267±0.36

**Average of five determination*

Precision

Precision of the methods was studied at three levels i.e. repeatability, intermediate precision (day to day and analyst to analyst) and reproducibility (Table 7).

Table 7 : *Results of validation (MEAN±SD)*

Parameter	UV Spectrophotometric method				RP-HPLC method			
Precision (Mean±SD)*	MET	% RSD	TEL	% RSD	MET	% RSD	TEL	% RSD
Repeatability	98.30±0.07	0.074	98.66±0.18	0.188	99.42±0.16	1.22	99.16±0.40	1.88
Day to Day	98.35±0.09	0.092	98.18±0.10	0.111	98.89 ±0.17	1.40	98.23±0.35	1.92
Analyst to Analyst	98.29±0.08	0.083	98.27±0.13	0.134	98.06±0.14	1.66	99.38±0.12	0.57
Reproducibility	98.21±0.05	0.055	98.21±0.11	0.112	98.96±0.07	0.64	99.31±0.14	0.83
Robustness*	98.16±0.10	0.108	98.26±0.14	0.148	98.62±0.10	0.95	99.03±0.21	1.20

**Average of 5 replicate and 5 concentration.*

Robustness

For the robustness of the analytical method we changed the ratio of mobile phase. As a replacement for the methanol:acetonitrile:phosphate buffer (pH 5) in a ratio of 35:35:30 v/v/v, methanol: acetonitrile:phosphate buffer (pH 5) in a ratio of 32:38:30 v/v/v were used as solvent (Results are shown in Table 7).

LOD and LOQ

Detection limit and quantitation limit of described method were observed as 0.102µg mL^{-1} and 0.312µg mL^{-1} for metoprolol and 0.023µg mL^{-1} and 0.723µg mL^{-1} for telmisartan respectively in UV spectrophotometric method and RP-HPLC method, based on the SD of response and slope, which meet the requirement of new method.

Stability

Bench top stability

The bench top stability was determined at lower (5 µg mL^{-1} for MET and 8 µg mL^{-1} for TEL) and higher (25 µg mL^{-1} for MET and 40 µg mL^{-1} for TEL) quality control sample by evaluating 5 replicate samples at each level. The samples were processed after keeping them on the bench top (room temperature) for about 6 hours and then analysed against freshly spiked calibration curve standards. Results of bench top stability are reported in Table 8.

Table 8 : *Bench top stability*

Parameter	Bench Top stability			
	LQC		HQC	
	MET	TEL	MET	TEL
Mean	98.09%	98.74%	98.97%	98.04%
SD	0.04	0.035	0.029	0.030
%RSD	0.041	0.036	0.030	0.031

Freeze thaw stability

The freeze thaw stability in matrix was assessed by assaying 6 replicates of samples at low (5 µg mL^{-1} for MET and 8 µg mL^{-1} for TEL) and high (25 µg mL^{-1} for MET and 40 µg mL^{-1} for TEL) concentrations previously frozen and thawed over three cycle against freshly prepared spiked calibration standards. The samples were frozen at -27°C for at least 40 h followed by unassisted thawing at room temperature. The samples were again frozen for 24 h under the same condition. This freeze-thaw cycle was repeated two times more and then the sample was processed after the third cycle and analysed. Results of freeze thaw stability are reported in Table 9.

Table 9 : *Freez thaw stability*

Parameter	Freez thaw stability			
	LQC		HQC	
	Metoprolol	Telmisarten	Metoprolol	Telmisarten
Mean	98.00%	98.75%	97.93%	99.24%
SD	0.04	0.035	0.029	0.030
%RSD	0.041	0.036	0.030	0.031

RESULTS AND DISCUSSIONS

The aim of the present work was to develop simple and reproducible bioanalytical UV-spectrophotometric and RP-HPLC method for the simultaneous determination of MET and TEL in plasma as well as solid pharmaceutical dosage forms. As the MET and TEL were sparingly soluble in water therefore a mixture of methanol in water (50:50 v/v) might be used in spectrophotometry as solvent and precipitating agent for the preparation of all standard and sample solutions as easily available and cost effective. The mixture of methanol:acetonitrile: phosphate buffer (pH 5) in ratio of 35:35:30 v/v/v was used in RP-HPLC analysis, which improved resolution and peak shape.

UV-Spectrophotometric Method

Based on the solubility and stability and spectral characteristics of the drugs, 50% methanol was used for spectrophotometry method as solvent. MET and TEL showed maximum absorbances at 240.5 nm and 237.5 nm respectively after first order derivatization. MET and TEL follows the Beer's law in the concentration range of 5-25 µg/ml and 8-40 µg/ml respectively (r^2= 0.9998 and 0.9998). Simultaneous equation method followed by first order derivatization was used for the quantitative estimation of both drugs. The optimized methods showed good reproducibility and recovery in the range of 98.06% to 98.25 % for MET and 98.11% to 98.13% for TEL. The mean percent label claims of tablet dosage by spectrophotometric method were found to be 98.788±1.336 and 98.805±0.850 for MET and TEL respectively. The standard deviation, coefficient of variance and standard error were found satisfactorily low for MET and TEL. The Result of precision at different levels were found to be within acceptable limits (i.e. RSD<2).

RP-HPLC Method

In RP-HPLC method, several attempts were made in order to get a satisfactory resolution of MET and TEL in different mobile phases with various ratios of a mixture of organic phases and buffer by using a C_{18} column. Initially the mobile phase used was a mixture of water and methanol followed by water and acetonitrile in different ratios. Further methanol:acetonitrile:phosphate buffer (pH 5) in a ratio of 35:35:30 (v/v/v), mobile phase was

used by isocratic elution at a flow rate of 1.0 mL/min which shows satisfactory and good resolution. The resolution was found reproducible and satisfactory. MET and TEL follows the linearity in the concentration range of 5-25 µg mL^{-1} and 8-40 µg mL^{-1} respectively (r^2= 0.9998 and 0.9998) and chromatogram was recorded at 225 nm. The values of mean recoveries were found between 98.24% and 99.35% for MET & 98.21% and 99.84% for TEL. The mean percent label claims of tablet dosage by RP-HPLC method were found to be 98.511±1.135 and 98.791±0.921 for MET and TEL respectively. The bench top stability and freeze thaw stability were determined at lower (5 µg mL^{-1} for MET and 8 µg mL^{-1} for TEL) and higher (25 µg mL^{-1} for MET and 40 µg mL^{-1} for TEL) quality control sample and result was found to be satisfactory. The standard deviation, coefficient of variance and standard error for MET and TEL were satisfactorily low. The Result of precision at different levels were found within acceptable limits (RSD<2).

CONCLUSION

In conclusion, an RP-HPLC and simple reproducible UV-spectrophotometric methods were developed and validated for the simultaneous determination of MET and TEL in plasma as well as solid dosage forms. The advantage of UV method over HPLC method is that the proposed UV method does not require the elaborate treatment and procedures usually associated with chromatography method. It is less time consuming and economical as a precipitating agent was economic. Statistical comparison of the quantitative determination of MET and TEL shows that RP-HPLC method was more accurate and precise than UV method. The results indicate HPLC and UV spectrophotometry methods are adequate methods to quantify MET and TEL in pure form, in plasma and its dosage form. There was no interference in the analysis by the plasma and excipients of the tablets. Since these methods are simple, specific, rapid, precise and accurate, they may be successfully and conveniently adopted for routine quality control analysis of MET and TEL in plasma and pharmaceutical dosage form.

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