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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL RP HPLC METHOD FOR THE ESTIMATION OF METOPROLOL TARTRATE IN RABBIT PLASMA AFTER TRANSDERMAL AND ORAL ADMINISTRATION: APPLICATION IN PHARMACOKINETIC STUDIES

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ABSTRACT

A simple, specific, sensitive and rapid Reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the quantification of Metoprolol Tartrate in small volumes of rabbit plasma. The method was further extended for its pharmacokinetic studies in rabbit plasma samples after transdermal and oral administration. Biological sample preparation involving simple extraction with organic solvent, followed by dilution with mobile phase was adopted to eliminate any chromatographic solvent effects. The method was proven to be linear over a plasma concentration range of 20 ng/ml to100 ng/ml with a mean correlation coefficient of 0.99. The limit of detection and the limit of quantification of the newly developed method were determined to be 5.8ng/mL and 16.1ng/mL, respectively. The method was successfully applied to assess pharmacokinetic parameters of Metoprolol Tartrate in rabbit plasma and found out the comparative bioavailability of MT following oral and transdermal dosage forms. The developed method was established as a rapid analytical tool in a pharmacokinetic study as it required short retention time, high precision, sensitivity and small volumes of plasma for analysis.

Keywords: Metoprolol Tartrate, RP-HPLC, quantification, Rabbit plasma, Pharmacokinetic study, oral, transdermal.

INTRODUCTION

Metoprolol tartrate (MT) is a selective hydrophilic ß-blocking agent for the treatment of mild and moderate hypertension and also for long term management of angina pectoris. MT has a oral bioavailability of only 38 % due to extensive hepatic first-pass metabolism. In the blood circulating system it is in the first step 12% protein bound, then rapidly enters the CNS and has moderate lipid solubility. The metabolism of this drug is hepatically (primarly by CYP2D6). The metabolization occurs also mainly in the liver. Approximately 95% of the drug is excreted renally and less than 5% of the drug is excreted unchanged in urine. Peak plasma concentrations are achieved after 2–3 hours. The half-life of the MT is about 3.2 hours, which makes frequent dosing necessary to maintain the therapeutic blood levels of the drug for long-term treatment.[1-6] Therefore, MT is an ideal drug candidate for transdermal drug delivery. Several methods have been reported for quantification of MT in plasma using high-performance liquid chromatography (HPLC) with UV or fluorescence detection. Many of these methods involve a complex separation step and are non-reproducible. Hence, the purpose of this investigation was to develop a simple, sensitive, selective and reproducible analytical method for the quantitative estimation of MT in a small volume of human plasma. It is also envisaged that this method will be able to provide an efficient solution for pharmacokinetic, bioavailability or bioequivalence studies of MT. This work is performed to ascertain the comparative bioavailability of MT from oral and transdermal dosage forms.[1,7-10]

EXPERIMENTAL

Methodology

In the present work a simple, selective, rapid, precise and economical reverse phase HPLC method have been developed for estimation of Metoprolol tatrate in blood plasma.

Table 1: Solubility of Drug in Different Solvents

SOLVENT	SOLUBILITY
Water	Freely soluble
0.1N HCl	soluble
0.1N NaoH	Insoluble
Methanol	Freely soluble
Acetonitrile	Soluble
Acetate Buffer	Soluble
Phosphate Buffer	Soluble

Solubility[1-2]

Solubility of all three drugs was observed by dissolving them in different solvents. (Table 1)

Selection of Precipitating Agent[12,13]

Selection of precipitation agent is based on the solubility of drug and good protein protein precipitation property. Metoprolol tartrate is soluble in Acetonitrile and having good protein precipitating property so Acetonitrile was selected as the protein precipitation agent.

Selection of Mobile Phase[9-13]

Initially to estimate Metoprolol tartrate, numbers of mobile phase in different ratio were tried. A result was shown in (Table 2).

Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was Acetonitrile: Methanol: 20 mM Ammonium acetate buffer (pH 5.0) in the ratio of 25:55:20 v/v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.

Table 2: Mobile Phase Selection

Mobile Phase	Ratio	Flow rate	Remark
Methanol : water	50 : 50 v/v	1.0 ml/min	Peak Not Found
Acetonitrile : water	50 : 50 v/v	1.0 ml/min	Peak Not Found
Methanol : Acetonitrile	50: 50 v/v	1.0 ml/min	Peak Not Found
20 mM KH_2PO_4 : Acetonitrile (pH Adjust with 4.0 with OPA)	20 : 80 v/v	1.0 ml/min	Poor resolution
20 mM KH_2PO_4 : Acetonitrile (pH Adjust with 3.5 with OPA)	30 : 70 v/v	1.0 ml/min	Poor resolution
20 mM Ammonium acetate buffer (pH 5.0)	25: 55: 20 v/v/v	1.0 ml/min	Satisfactory Result

❖ **Procedure for preparation of mobile phase**

Step-1 preparation of buffer

20 mM Ammonium acetate Buffer in 1000 ml of HPLC grade water, sonicated and pH adjusted to 5 with orthophosphoric acid.

Step-2 preparation of mobile phase

Mixed 55 volume of acetonitrile, 25 volume of methanol and 20 volume of buffer. Filtered through 0.45 µ nylon filter in Millipore unit and degassed by sonication.

Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and no significant effect was observed for retention and resolution of analyte. After various trials Acetonitrile: Methanol: 20 mM Ammonium acetate buffer (pH 5.0) was used as diluents.

Selection of Separation Variable

Table 3: Separation Variable

Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
Acetonitril	25%
Methanol	55%
Phosphate buffer (pH- 5.0)	20%
Diluent	ACN: Methanol: 20mM Ammonium Acetate Buffer pH-5.0 (25:55:20 v/v/v)
Flow rate	1.0 ml/min
Temperature	25 ^{0}C
Sample Size	20 µl
Detection wavelength	274 nm
Retention time	10.792 ± 0.001 min

1. Preparation of stock solution: Accurately weighed 10 mg of MT was transferred into 50 ml volumetric flasks separately and dissolved in 10 ml of plasma, then volume was made up to 50 ml with Acetonitrile and vortex it to get complete precipitation of plasma protein. Stand it aside for few minute, precipitate of protein

settled down then collect the supernatant layer. Centrifuge the collected supernatant layer at 6000 rpm for 7 min at 4°C and then filtered by whatmann filter paper (no.41). Concentration of MT was 200 µg/ml(stock- A).

2. Preparation of Sub Stock Solution: 5 ml of solution was taken from stock-A of METO and transferred into 100 ml volumetric flask separately and diluted up to 100 ml with diluent (Mobile phase) to give concentration of 10 µg/ml (Stock-B).

3. Linearity and Calibration Graph:

To establish the linearity of analytical method, a series of dilution ranging from 20-100 ng/ml was prepared. 0.2ml, 0.4 ml, 0.6ml, 0.8ml and 1.0ml of stock-B was taken separately in 10 ml volumetric flask and volume was made up to 100ml with (Acetonitrile: Methanol: Ammonium acetate buffer (pH 5.0). This gives the solutions of 20ng/ml, 40ng/ml, 60ng/ml, 80ng/ml, 100ng/ml for drug.

All the solution were filtered through 0.2µm membrane filter and injected, chromatograms were recorded at 274nm and it was repeated for six times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

Fabrication of Drug loaded transdermal films[14-17,20,23,24]:

The Drug loaded monolithic matrix type transdermal patches were prepared by film casting technique on mercury substrate using different ratios of ERLPO:Methocel K15M, ERSPO:Methocel K15M, Acrylcoat S100:Methocel K15M and Acrylcoat L100:Methocel K15M (1:4,2:3,3:2,4:1) containing drug Metoprolol Tartrate (15.92 mg/ square centimeter patch). The polymers were weighed in requisite ratios keeping the total polymer weight 500 mg. Hydrophilic materials i.e. Methocel K15M was dissolved in water and hydrophobic materials i.e. Eudragit RLPO, Eudragit RLPO, Acrylcoat S100 or Acrylcoat L100 was dissolved in blend of dichloromethane (DCM) and ethanol (50:50). Then both the solution were mixed and stirred on magnetic stirrer to accomplished homogeneous mixture. The above polymeric dispersion was sonicated for 2 minutes to remove entrapped air bubbles. In this study Lipophilic plasticizers DBP & DBS or hydrophilic plasticizers such as PEG 400 & Propylene Glycol was added for each polymer combination. 2 different permeation enhancers of Terpene class such as limonene and cineole in different percentage alone and in combination (2.5 w/w %, 5.0 w/w %, 7.5 w/w % or 2.5:2.5) was added to each polymer combination. The resulting solution (10 ml) was poured in a petri dish of 9.2 cm diameter containing mercury. The rate of evaporation of the solvent was controlled by placing an inverted funnel over the petri dish. The film formation was noted by observing the mercury surface after complete evaporation of the solvent. Aluminium foil was used as backing film and wax paper as release liner (which could be removed before application of the patch on the skin) were applied to complete the TDDS. The patches were cut with a circular metallic die of 2 cm internal diameter to give an area of 3.14 cm^2 and stored in a desiccator until use.

Different formulations were designed further by adding 2 different permeation enhancers of Terpene class such as limonene and cineole in different percentage alone and in combination.

Percentage of Limonene used: 2.5 w/w %, 5.0 w/w % and 7.5 w/w %

Percentage of Cineole used: 2.5 w/w %, 5.0 w/w % and 7.5 w/w %

Combination of Limonene and cineole used (%): 2.5 w/w %:2.5 w/w %

16 optimized films were obtained (in optimization step-3) which were having good and acceptable permeation enhancing capacity across porcine skin. The above optimized films were obtained after considering permeation enhancing capacity via determination of steady state flux, permeation coefficient and enhancement factor. In all the films it was found that combination of permeation enhancers was more effective in comparison to when they were used alone. Hence only combination of permeation enhancers was used for further development of transdermal films and their evaluation.

Table 4: Formulation of Drug loaded transdermal films EM1-EM8

Formulation code	Drug (mg/ square centimeter patch)	Polymer combination with ratio	Plasticizer type and Percentage	Permeation Enhancer (%w/w of polymer)	
				Limonene	Cineole
EM1	15.92	ERSPO:METHOCEL K15M(1:4)	PEG 400(20%)	2.5	2.5
EM2	15.92	ERSPO: METHOCEL K15M(2:3)	PEG 400 (20%)	2.5	2.5
EM3	15.92	ERSPO: METHOCEL K15M (3:2)	DBS (25%)	2.5	2.5
EM4	15.92	ERSPO: METHOCEL K15M (4:1)	DBS (25%)	2.5	2.5
EM5	15.92	ERLPO: METHOCEL K15M (1:4)	PEG 400 (20%)	2.5	2.5
EM6	15.92	ERLPO: METHOCEL K15M (2:3)	PEG 400 (20%)	2.5	2.5
EM7	15.92	ERLPO: METHOCEL K15M (3:2)	DBS (25%)	2.5	2.5
EM8	15.92	ERLPO: METHOCEL K15M(4:1)	DBS (25%)	2.5	2.5

Table 5: Formulation of Drug loaded transdermal films AM1-AM8

Formulation code	Drug (mg/ square centimeter patch)	Polymer combination with ratio	Plasticizer type and Percentage	Permeation Enhancer (%w/w of polymer)	
				Limonene	Cineole
AM1	15.92	ACRYLCOAT S100: METHOCEL K15M (1:4)	PG (15%)	2.5	2.5
AM2	15.92	ACRYLCOAT S100: METHOCEL K15M (2:3)	PG (15%)	2.5	2.5
AM3	15.92	ACRYLCOAT S100: METHOCEL K15M (3:2)	PG (15%)	2.5	2.5
AM4	15.92	ACRYLCOAT S100: METHOCEL K15M (4:1)	DBT (30%)	2.5	2.5
AM5	15.92	ACRYLCOAT L100: METHOCEL K15M (1:4)	PG (15%)	2.5	2.5
AM6	15.92	ACRYLCOAT L100: METHOCEL K15M (2:3)	PG (15%)	2.5	2.5
AM7	15.92	ACRYLCOAT L100: METHOCEL K15M (3:2)	PG (15%)	2.5	2.5
AM8	15.92	ACRYLCOAT L100: METHOCEL K15M (4:1)	DBT (30%)	2.5	2.5

In vivo pharmacokinetic study in Rabbits[9.10,12-14,18-24]

Study Procedure

The In vivo pharmacokinetic study will be performed on twelve healthy male albino rabbits weighing between 2.5 to 3.0 kg. The dose of the drug was calculated according to the body surface area of the animal. The rabbits were fasted overnight but water was allowed ad libitum. The rabbits were divided into three groups of four rabbits each. The rabbits were kept in cages with husk bedding. The hair of a dorsal skin surface of around 50.0 cm^2 shaved and care taken to avoid skin damage during shaving. On the next morning **Group A** rabbits orally administered Metoprolol Tartrate (1.7mg/kg) 2 times with 0.5–1.0ml saline by feeding tube at 12 hour interval, **Group B** rabbits were applied the 1st optimized medicated transdermal patch AM2 to the shaved skin surface of rabbit. **Group C** rabbits were applied the 2nd optimized medicated transdermal path EM6 to the shaved skin surface of rabbit. The patches were placed over the skin with the help of surgical adhesive tape. The optimized patches were loaded with same amount of drug as oral.

Sampling

The blood samples (1.0ml) withdrawn from the marginal ear vein of the animals. The blood samples were collected at 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, 12, 16, 20, and 24 hr and transferred into heparinized test tubes to prevent coagulation of blood. The devices were removed after 24 hr of sampling. The blood samples (1.0ml) will be extracted and centrifuged. The organic layer will be separated and evaporated under a gentle stream of nitrogen at 45°C. The residue will be constituted in mobile phase and aliquot injected into the HPLC to determine the drug concentration.

Ethical approval for the handling of experimental animals was obtained from the Institutional Animal Ethical Committee.

***In-Vivo* Data Analysis**

The plasma concentration of Metoprolol Tartrate at different time intervals was subjected to pharmacokinetic analysis to calculate various parameters: maximum plasma concentration (Cmax), time to reach maximum concentration (Tmax), and area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$). The values of Cmax and Tmax were read directly from the arithmetic plot of time vs plasma concentration of Metoprolol Tartrate. The AUC was calculated by using the trapezoidal rule. The elimination rate constant (Ke) was calculated by regression analysis from the slope of the line, and the half-life (t1/2) was obtained by 0.693/Ke.

Result and Discussion

Mobile phase containing plasma was run through the column to obtain peaks for plasma at Rt

2.568 minutes.

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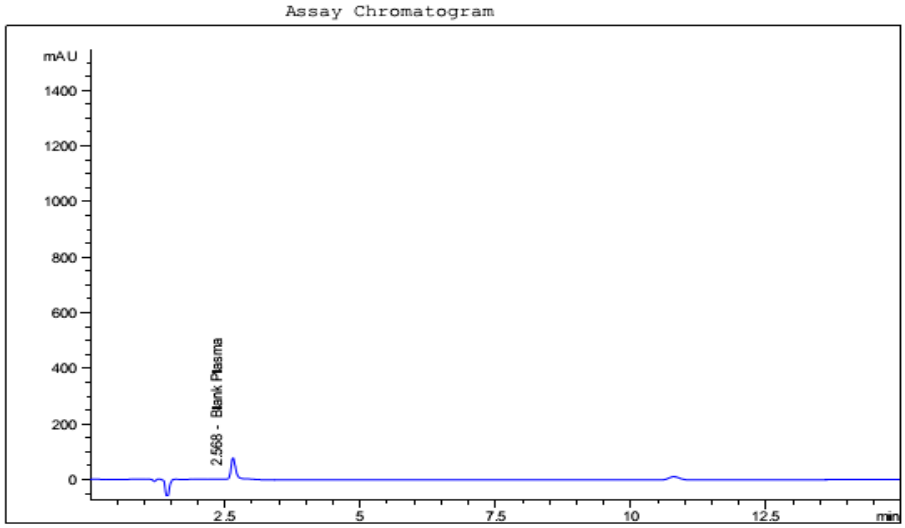

Figure 1: Chromatogram of blank plasma

Standard Curve Graph of Metoprolol in Plasma

A sample chromatogram of Metoprolol in plasma is shown in fig and Retention time for Metoprolol in plasma was found to be 10.792 ±0.001 minutes.

Standard graph of Metoprolol with plasma was also plotted which shows a linearity range of 20 ng/ml to100 ng/ml and regression of 0.99. The data of standard curve for Metoprolol in blood plasma is given in table and figure.

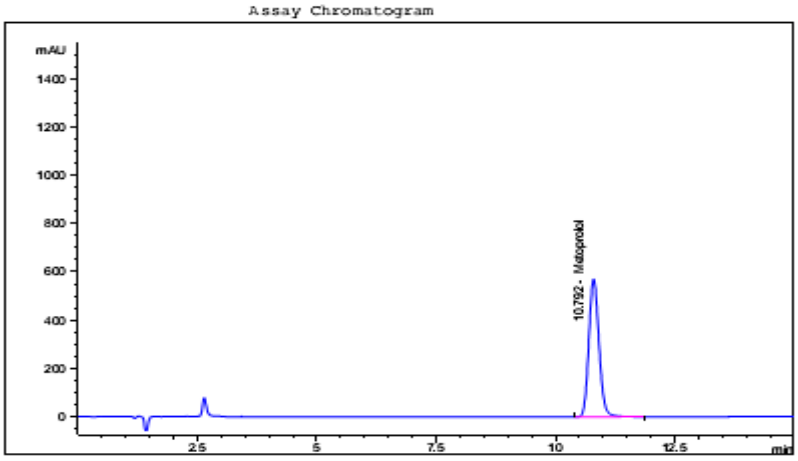

Figure 2: Chromatogram of pure Metoprolol in blood plasma

Table 6: Standard curve of Metoprolol in Plasma

Standard Concentration ng/ml	Area under Curve (AUC) at RT (10.792 ± 0.001 min)						Mean±SD*
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
20	855.265	854.225	868.256	856.236	860.569	848.236	857.1311±6.744395
40	1605.26	1599.27	1603.147	1612.548	1602.258	1608.589	1605.179±4.764753
60	2415.24	2412.22	2425.16	2450.256	2415.569	2411.254	2421.617±7.660787
80	3150.27	3147.27	3145.27	3140.548	3125.654	3150.256	3143.211±9.332913
100	4018.26	4012.25	4020.15	4015.587	4023.547	4018.874	4018.111±3.873858
Correl Coeff (r^2)	0.999	0.999	0.999	0.999	0.999	0.999	0.999±0.00
Slope (m)	39.35	39.32	39.23	39.23	39.24	39.41	39.2966±0.075829
Intercept (c)	47.55	45.83	58.62	61.02	50.71	42.55	51.04667±7.32898

**Standard deviation*

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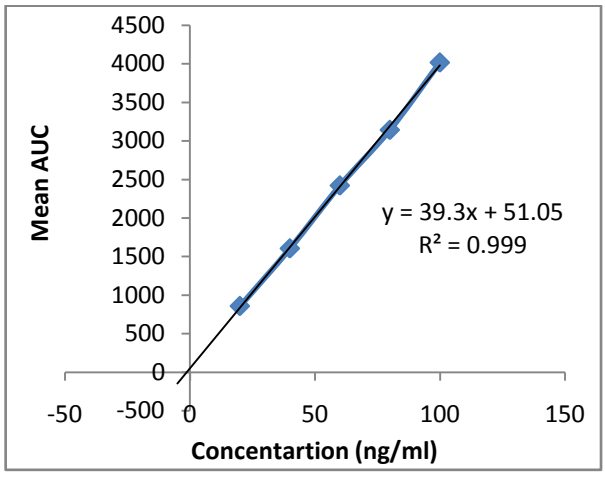

Figure 3: Calibration curve of Metoprolol tartrate

Table 7: HPLC data for pure Metoprolol in rabbit plasma

Parameter	Rabbit Plasma
Retention time (min)	10.792±0.001
Linearity range (ng/ml)	20-100
R^2value	0.999
Equation for linearity	y = 39.3x + 51.05
RSD%	0.096-0.7868

System Suitability Parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min. After complete saturation of column, six replicates of working standard of Metoprolol tartrate 100 ng/ml was injected separately. Peak report and column performance report were recorded for all chromatogram.

Table 8: System Suitability Parameters of Metoprolol

System suitability Parameter →	RT	AUC	No. of theoretical plates	Tailing factor	HETP
Rep-1	10.792	4018.26	2954	1.78	0.08463
Rep-2	10.793	4012.25	2953	1.77	0.08466
Rep-3	10.794	4020.15	2963	1.77	0.08437
Rep-4	10.794	4015.587	2955	1.74	0.08460
Rep-5	10.792	4023.547	2952	1.78	0.08469
Rep-6	10.793	4018.874	2951	1.77	0.08472
Mean	10.793	4018.111	2954.67	1.77	0.084612
S.D.*	0.001	3.873858	4.320	0.015	0.000126
RSD%**	0.009265	0.09641	0.146	0.832	0.148637

*** % Relative Standard deviation *Standard deviation*

Validation of Developed Method

A. Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test, which are directly proportional to area of analyte in the sample. The calibration plot was contracted after analysis of five different (from 20 to 100 ng/ ml) concentrations and areas for each concentration was recorded five times, and mean area was calculated. The regression equation and correlation coefficient of curve are given and the standard calibration curve of the drug is shown in figure. From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration (Table 9).

Table 9: Response Ratio Data for Linearity of Metoprolol

Replicates	Concentration (ng/ml)	Mean AUC	Response Ratio
Rep-1	**20**	857.131	42.85
Rep-2	**40**	1605.179	40.12
Rep-3	**60**	2421.617	40.36
Rep-4	**80**	3143.211	39.29
Rep-5	**100**	4018.111	40.18
Mean			40.56
SD			1.344
%RSD			3.313

*** % Relative Standard deviation *Standard deviation*

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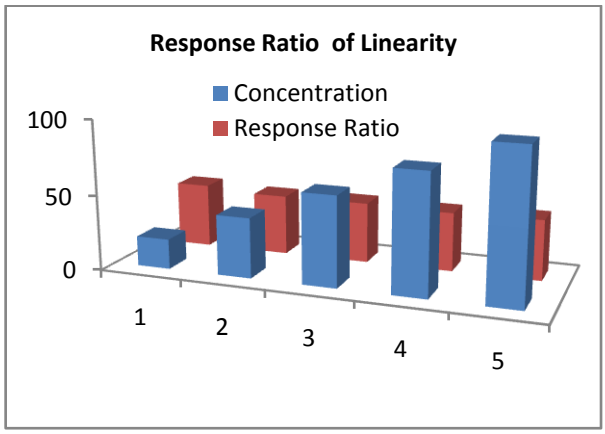

Figure 4: 3D Response Ratio Curve of Metoprolol tartrate

B. Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

C. Accuracy

Recovery studies were performed to validate the accuracy of developed method. To pre-analysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed.

Table 10: Recovery Study of Metoprolol (80% Level)

Conc. of sample (ng/ml)	Amt. Added (ng/ml)	Conc. Found. (ng/ml)			% conc. Found			Mean % conc.
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	
20	**16**	16.1	15.9	16.0	100.625	99.375	100.000	100.000
40	**32**	31.9	31.8	32.1	99.688	99.375	100.313	99.792
60	**48**	47.8	48.1	47.9	99.583	100.208	99.792	99.861
				SD			**MEAN**	99.884
								0.106
							% RSD	0.106

*** % Relative Standard deviation *Standard deviation*

Table 11: Recovery Study of Metoprolol (100% Level)

Conc. of sample (ng/ml)	Amt. Added (ng/ml)	Conc. Found. (ng/ml)			% conc. Found			Mean %conc
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	
20	**20**	19.8	18.8	19.2	99.000	94.000	96.000	96.333
40	**40**	39.6	38.9	39.3	99.000	97.250	48.250	98.167
60	**60**	59.4	58.9	59.7	99.000	98.167	99.500	98.889
				SD			**MEAN**	**97.769**
								1.317
							% RSD	**1.347**

*** % Relative Standard deviation *Standard deviation*

Table 12: Recovery Study of Metoprolol (120% Level)

Conc. of sample (ng/ml)	Amt. Added (ng/ml)	Conc. Found. (ng/ml)			% conc. Found			Mean %conc
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	
20	**24**	24.1	23.8	23.5	100.417	99.167	97.917	99.167
40	**48**	47.8	47.9	47.5	99.583	99.792	98.958	99.444
60	**72**	71.5	71.8	71.5	99.306	99.722	99.306	99.444
							MEAN	**99.352**
							SD	**0.160**
							% RSD	**0.161**

*** % Relative Standard deviation *Standard deviation*

D. Precision

The precision are established in three differences:

1. Repeatability
2. Intermediate precision
   a) Day to Day
   b) Analyst to Analyst

3. Reproducibility

1. Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 20, 40, 60, 80 and 100 ng/ml for MT indicates the precision under the same operating condition over short interval time. Results of repeatability are reported in table- respectively.

Table 13: Repeatability of Metoprolol

CONC. / REP.	CONCENTRATION FOUND (ng/ml)					MEAN
	20	**40**	**60**	**80**	**100**	
Replicate-1	20.9	39.1	60.2	80.2	98.9	
Replicate-2	21.0	39.8	59.8	79.1	100.2	
Replicate-3	20.0	38.9	59.7	81.2	100.3	
Replicate-4	20.2	36.7	58.7	78.9	97.3	
Replicate-5	20.3	37.6	59.8	78.2	97.8	
MEAN	20.48	38.42	59.64	79.52	98.9	
% MEAN	102.4	96.05	99.40	99.40	98.90	**99.23**
SD	0.443	1.24	0.055	0.118	0.136	**0.095**
% RSD	0.043	0.129	0.056	0.118	0.137	**0.097**

*** % Relative Standard deviation *Standard deviation*

2. Intermidiate Precision

a) Day To Day Precision

Intermediate precision was also performed within laboratory variation on different days in five replicate at five concentrations. Results of day to day intermediate precision for METO reported in table 14respectively.

Table 14: Day-To-Day Variation of Metoprolol

CONC. / REP.	CONCENTRATION FOUND (ng/ml)					MEAN
	20	**40**	**60**	**80**	**100**	
Replicate-1	20.7	40.3	57.8	76.7	96.8	
Replicate-2	19.2	40.3	59.9	78.8	99.3	
Replicate-3	19.3	38.8	58.9	75.6	99.4	
Replicate-4	19.8	35.5	55.9	78.3	99.7	
Replicate-5	17.8	39.8	58.3	79.8	93.9	
MEAN	193.6	38.94	58.16	77.84	97.82	
% MEAN	96.8	97.35	96.93333	97.3	97.82	**97.241**
SD	0.105	0.202	0.149	0.168	0.248	**0.174**
% RSD	0.1090	0.2073	0.1533	0.1727	0.2537	**0.179**

*** % Relative Standard deviation *Standard deviation*

b) Analyst- To- Analyst Precision

Analyst to analyst variation was performed by different analyst in five replicate at five concentrations.

Table 15: Analyst- To-Analyst Variation of Metoprolol

CONC. / REP.	CONCENTRATION FOUND (ng/ml)					MEAN
	20	**40**	**60**	**80**	**100**	
Replicate-1	18.1	38.2	59.41	80.3	99.8	
Replicate-2	18.2	38.9	59.32	77.9	99.9	
Replicate-3	18.3	38.1	59.01	78.9	98.3	
Replicate-4	16.1	37.3	60.31	77.2	95.8	
Replicate-5	22.3	40.3	60.20	78.8	98.8	
MEAN	18.6	38.56	59.65	78.62	98.52	
% MEAN	93	96.4	99.41667	98.275	98.52	97.122
SD	0.226	0.113	0.057	0.117	0.166	0.136
% RSD	0.2431	0.1168	0.0577	0.1190	0.1688	0.141

*** % Relative Standard deviation *Standard deviation*

3. Reproducibility

The reproducibility was performed by chemical to chemical (use of rankem chemicals in place of merck chemicals) variation in five replicate at five concentrations.

Table 16: Reproducibility of Metoprolol

CONC. / REP.	CONCENTRATION FOUND (ng/ml)					MEAN
	20	40	60	80	100	
Replicate-1	19.1	39.2	59.4	78.9	99.6	
Replicate-2	18.7	39.4	57.9	79.3	98.9	
Replicate-3	19.4	39.3	58.7	78.3	97.8	
Replicate-4	18.5	36.7	59.1	78.9	96.9	
Replicate-5	19.7	38.6	58.9	79.9	97.9	
MEAN	19.08	38.64	58.80	79.06	98.22	
% MEAN	95.400	96.600	98.000	98.825	98.220	**97.409**
SD	0.049	0.113	0.057	0.059	0.105	**0.076**
% RSD	0.052	0.117	0.058	0.060	0.107	**0.078**

*** % Relative Standard deviation *Standard deviation*

Robustness

As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, ACN: Methanol: Ammonium Acetate Buffer pH- 5 (25:55:20 % V/V/V), to (25:54:21 % V/V/V).Results of robustness are reported in table-

Table 17: Robustness of Metoprolol

CONC. / REP.	CONCENTRATION FOUND (ng/ml)					MEAN
	20	40	60	80	100	
Replicate-1	18.9	38.9	59.8	87.7	99.2	
Replicate-2	18.4	39.9	57.4	88.9	99.2	
Replicate-3	19.3	37.3	58.3	87.9	99.6	
Replicate-4	18.3	36.7	58.9	89.5	99.5	
Replicate-5	19.9	37.3	53.4	88.9	99.3	
MEAN	19.14	38.02	57.56	78.64	99.36	
% MEAN	95.700	95.050	95.933	98.300	99.360	**96.869**
SD	0.048	0.133	0.248	0.080	0.018	**0.105**
% RSD	0.050	0.140	0.259	0.081	0.018	**0.110**

*** % Relative Standard deviation *Standard deviation*

a. Detection Limit and Quantitation Limit

The LOD and LOQ of developed method was calculated based on the standard deviation of response and slope of the linearity curve. (Table 18)

Table 18: LOD and LOQ of Metoprolol

Name	LOD (ng/ml) ±SD*	LOQ (ng/ml) ±SD*
Metoprolol Tartrate	5.8±0.005	16.1±0.003

***Standard deviation**

Plasma Estimation of Metoprolol in Rabbits

The plasma concentrations of MT vs. time are shown in Fig. and the pharmacokinetic parameters are presented in Table . The C_{max} and t_{max} after oral administration of MT were 94.24±0.19 ng/ml and 2.000±0.00 h, respectively. In case of transdermal patches, the C_{max} (91.160±0.16 to 93.160±0.13 ng/ml) and t_{max} (8 h) values were significantly different compared to oral route. Measurable concentrations of the drug were obtained within an hour of application of the patch and relatively steady plasma concentration of drug was observed for over 24 h. The biological half-life ($t_{1/2}$) of MT was prolonged to about 6 h (oral: 2.419±1.02 h) in Rabbits.

Table 19: Plasma Estimation of Metoprolol in Rabbits

Time (hrs)	Drug concentration in blood plasma (ng/ml)		
	Pure Drug administered orally	Optimized Transdermal film AM2*	Optimized Transdermal film EM6*
0	0	0	0
0.5	46.21±1.23	17.73±2.31	19.15±2.26
1	68.44±1.55	36.11±2.11	38.59±2.42
2	94.24±1.20	45.62±2.51	48.17±2.12
4	51.51±1.77	61.77±1.52	64.53±2.31
6	32.11±2.31	83.82±1.44	85.33±1.71
8	19.43±1.11	91.16±1.91	93.15±1.46
12	5.12±1.81	90.23±1.43	91.32±1.21
14	93.14±1.41	87.41±1.22	90.55±1.20
18	41.25±1.09	82.52±1.81	88.13±2.51
20	22.31±1.33	63.79±1.55	71.44±2.09
24	8.77±2.45	41.76±1.91	44.11±2.31

**Significant compared to MT-Oral ($p<0.05$); each point represents Mean±SE;n=3*

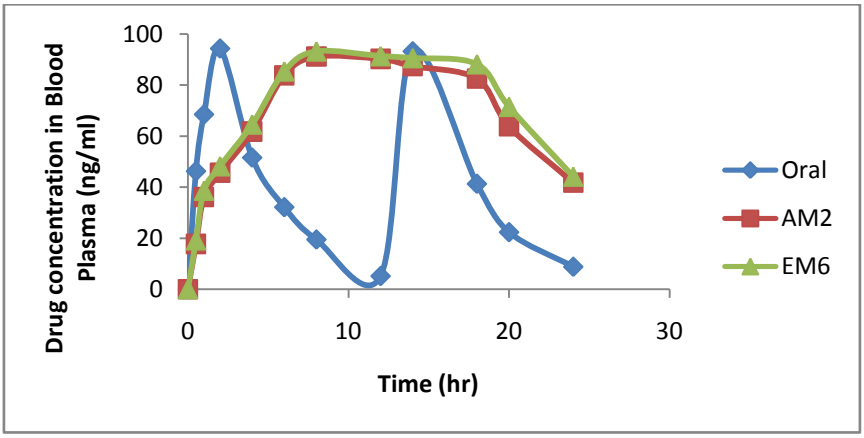

Figure 5: Plasma concentration–time profile of MT after oral and transdermal patch treatment in Rabbits

Table 20: Pharmacokinetic parameters after oral and transdermal treatment of MT

S.No.	Pharmacokinetic parameter	Pure metoprolol administered orally	Optimized formulation AM2	Optimized formulation EM6
1	$t_{1/2}$ (hr)	2.419±1.02	6.165±0.15*	5.970±0.13*
2	Ke(h^{-1})	0.2864±0.003	0.112±0.002*	0.116±0.002*
3	T_{max} (hr)	2.000±0.00	8.000±0.00*	8.000±0.00*
4	C_{max} (ng/ml)	94.24±0.19	91.160±0.16*	93.160±0.13*
5	AUC $_{0-t}$ (ng/ml*h)	451.565±4.22	1726.408±4.61*	1805.513±4.35*
6	$AUC_{0-\infty}$ (ng/ml*h)	469.439±4.19	2097.836±4.43*	2185.458±4.11*
7	$AUMC_{0-\infty}$ (ng/ml*h^2)	2009.104±6.03	33282.570±6.21*	34600.480±6.12*
8	MRT (hr)	4.279±1.23	15.865±0.32*	15.832±0.41*

All values are expressed as Mean±SE, n=3

C_{max}=Maximum concentration; t_{max}=Time of maximum concentration; K_e=Elimination rate constant; AUC=Area under plasma concentration–time curve; AUMC= Area under plasma First Moment concentration–time curve; $t_{1/2}$=Elimination half-life; MRT=Mean residential time,

*Significant compared to oral MT ($p<0.05$)

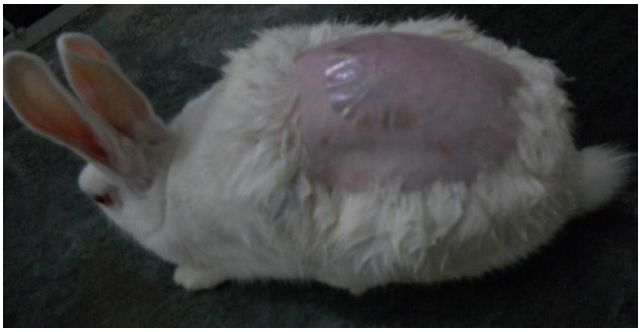

Figure 6: Application of Optimized Transdermal Patches

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CONCLUSION:

A simple, rapid, reproducible, and sensitive HPLC method has been developed for analysis of MT in human plasma. The pharmacokinetic parameters obtained with transdermal patches were significantly ($p<0.05$) different from those obtained with oral administration. The *In vivo* pharmacokinetic results from the oral administration of drug metoprolol tartrate solution indicate that the drug is rapidly absorbed from the rabbit GI tract, whereas drugs through transdermal route are slowly but continuously absorbed. Though the rise in drug concentration was slower than oral administration, the drug concentration in plasma remained high for longer period with transdermal patches. The calculated pharmacokinetic parameters indicate that the biological half life ($t_{1/2}$) of drug is prolonged in rabbits by transdermal application in comparison to oral dose. Hence, the drug administered through transdermal patch will remain for longer period of time in the body and thus exert a sustained the action. Moreover, the improved performance of the designed optimized transdermal films of drug is also reflected by area under the curve (AUC) measurement as no trough and peaks in drug plasma level was recorded. The high AUC values observed with the patches also indicate increased bioavailability of the drug, this may be due to bypass of the hepatic first pass effects and avoidance from gastric degradation. The T_{max} value was considerably high. Maximum plasma concentration (C_{max}) of the optimized transdermal films was found to be less in comparison to oral dose. The significantly less elimination rate constant (Ke) and high mean residence time (MRT) values of drug by transdermal application in comparison to oral dose, further supports the sustained action of the drug from transdermal patches.

On the whole, transdermal patches of MT showed better in vivo effectiveness in rabbits compared to oral administration. This could be due to slow and continuous supply of drug at a desirable rate to systemic circulation, which could better control the hypertension in hypertensive subjects.

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