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Comparative *in vivo* evaluation of simvastatin after oral and transdermal administration in rabbits

B. Manjula*, V. Rama Mohan Gupta and K. B. Chandra Sekhar

[1]Research Scholor, JNTU Ananthapuram, Ananthapuram
[2]Department of Pharmaceutics, G. Pulla Reddy College of Pharmacy, Hyderabad
[3]Vice Chancellor, Krishna University, Machilipatnam, Andhra Pradesh
*Corresponding author: betholimanjula@gmail.com

Abstract

Membrane-moderated transdermal systems of Simvastatin liposomes were prepared by incorporating the drug reservoir within a shallow compartment moulded from a drug-impermeable backing membrane and 2% w/v cellulose acetate rate-controlling membrane. The pharmacodynamic and pharmacokinetic performance of Simvastatin following transdermal administration was compared with that of oral administration. This study was carried out in a randomized cross-over design in male New Zealand albino rabbits. The estimation of Simvastatinin plasma was carried out by LC-MS/MS method. The parameters such as maximum plasma concentration (C max), time for peak plasma concentration (t max), mean residence time (MRT) and area under curve (AUC 0 - ∞) were significantly (P< 0.001) differed following transdermal administration compared to oral administration. The relative bioavailability of Simvastatin was increased about nine fold after transdermal administration as compared to oral delivery. This may be due to the avoidance of first pass effect of Simvastatin. The concentration of Simvastatin in plasma was found to be stabilized and maintained in a narrow range over the study period up to 24 hrs for transdermal formulation where as the concentration was decreased rapidly up on oral administration. It was concluded that the relative rate of extensive first pass metabolism was significantly reduced in transdermal administration, resulted in increased relative bioavailability and reduced frequency of administration.

Keywords: Simvastatin, Liposomes, Transdermal Systems, LC-MS/MS, *In vivo* Studies.

Introduction

The transdermal route of drug delivery has gained great interest of pharmaceutical research, as it circumvents number of problems associated with oral route of drug administration. The barrier nature of skin inhibits the penetration of most drugs. The use of lipid vesicles as delivery system for skin treatment has gained attention in recent years(1). Liposomes are microscopic or submicroscopic particles and are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids.Liposomes are microscopic vesicles that contain amphipathic phospholipids arranged in one or more concentric bilayers enclosing an equal number of aqueous compartments. The thermo-dynamically stable, lamellar structures form spontaneously when a lipid is brought into contact with an aqueous phase(2).

The aim of the present study was to develop and evaluate the potential use of liposome vesicles in the transdermal drug delivery for delivery of Simvastatin .Simvastatin is an effective drug in the treatment of hyperlipidemic patients, simvastatin is a methylated derivative of lovastatin that acts by competitively inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-COA) reductase, the enzyme that catalyzes the rate limiting step in cholesterol biosynthesis. Administration of conventional tablets of simvastatin has been reported to exhibit

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fluctuations in plasma drug levels, results either in manifestation of side effects or reduction in drug concentration at the receptor sites also is a cholesterol-lowering agent and is structurally similar to the HMG, a substituent of the endogenous substrate of HMG-CoA reductase. Simvastatin lowers hepatic cholesterol synthesis by competitively inhibiting HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in the cholesterol biosynthesis pathway via the mevalonic acid pathway(3). Due to short biological half life (5.3 hours) and low bioavailability (5%) due to extensive first pass metabolism makes it suitable candidate for transdermal drug delivery system. An in vivo evaluation study was conducted to ascertain pharmacokinetic parameters in rabbits after oral and transdermal administration of of simvastatin in rabbits.

Materials and Methods

The *in vivo* study of the optimized formulations were performed as per the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of social Justice and Empowerment, Government of India. Prior approval by Institutional animals ethics committee was obtained for conduction of experiments (Ref: IAEC/IX/10/ACOP/ CPCSEA, Dated 21-12-19).

Preparation of Liposomes by Thin Film Hydration Technique: Liposomes were prepared by rotary evaporation-sonication method. [4]The lipid mixture (500mg) consist of phospholipid (Soya Lecithin), edge activator (Tween 80) and drug (10 mg/ml) in different ratios was dissolved in an organic solvent mixture consist of chloroform and methanol (2:1, v/v) then placed in a clean, dry round bottom flask. The organic solvent was carefully evaporated by rotary evaporation (Buchi rotavapor R-3000, Switzerland) under reduced pressure above the lipid transition temperature(at 600c for 1 hr) to form a lipid film on the wall of the flask. The final traces of the solvents were removed by subjecting the flask to vacuum over night. The dried thin lipid film deposited on the wall of the flask was hydrated with a phosphate buffer solution (pH 6.4) by rotation for 1hr at room temperature at 60 rpm. The resulting vesicles were swollen for 2 hrs at room temperature to getlarge multilamellar vesicles. To prepare small Liposome vesicles, the resulting vesicles were sonicated at 100 kHz, 80 Amp for 30 minutes at pulse on 30sec and pulse off 50 sec using a probe sonicator (OrchidScientifics, Nasik).The obtained suspension was passed through a series of 0.45µ and0.22µ polycarbonate filters and then stored at 4°C(4).

***Preparation of Rate Controlling Membrane*:** Solvent evaporation technique was employed in the present work for the preparation of Cellulose acetate films. The polymer solutions were prepared by dissolving the polymer (2% w/w Cellulose acetate) in 50 ml of Ethyl acetate Methanol (8:2). Dibutyl phthalate at a concentration of 40% w/w of the polymer was used as a plasticizer. 20 ml of the polymer solution was poured in a Petri plate (9.4 cm diameter) placed on a horizontal flat surface. The rate of evaporation was controlled by inverting a funnel over the Petriplate. After 24 hours the dried films were taken out and stored in a desiccators(5).

***Preparation of Liposomes Loaded Gels*:** Accurately weighed quantity of 500 mg of Hydroxy propyl methyl cellulose was dispersed in 5 ml of distilled water and was allowed for swelling over night. The swollen carbopol was stirred for 60 minutes at 800 rpm. The previously prepared required Simvastatin equivalent Liposomes, methylparaben and propylparaben were incorporated into the polymer dispersion with stirring at 500 rpm by a magnetic stirrer for 1 hour. The pHof above mixture was adjusted to 7.4 with tri ethanolamine (0.5%). The gel was transferred in to a measuring cylinder and the volume was made up to 10ml with distilled water(6).

***Design of Membrane Moderated Transdermal Therapeutic System*:** A circular silicon rubber ring with an internal diameter of 2.5 cm and a thickness of 3mm was fixed on to a backing membrane (an imperforated

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adhesive strip was supplied by Johnson and Johnson Limited ,Mumbai). This serves as a compartment for drug reservoir. Gel equivalent to 40 mg of Simvastatin was taken into the compartment as a drug reservoir. Cellulose acetate membrane of known thickness was fixed onthe ring with glue to form a membrane moderated therapeutic systems. A double sided adhesive strip was fixed on the rim of the ring above Cellulose acetate membrane(7).

In Vivo Evaluation

***Subject Selection*:** Twelve New Zealand healthy rabbits with a mean age of 10±2 weeks and with a mean body weight of 3±0.2 kg were used in this study. Each group consisted of six rabbits (n=6) each and were subjected for overnight fasting, it was taken care that there was no stress on the animals. Rabbits were randomly divided into two groups for different sampling time and each group was housed in one cage. Food and water were available ad libitum at all times during the experiment. The study was conducted in a crossover design with 2 weeks washout periods in between the two experiments. The animal dose of Simvastatin was calculated relevant to human dose by using the following formula(8).

Human dose of Simvastatin = 40 mg.

Animal dose = $\frac{Human\ Dose \times Animal\ Weight}{Human\ Weight}$ =

40x3/70= 1.71 mg /kg

***Blood Sampling*:** About 1 ml of blood samples were collected from the tracheal lobular vein of the rabbit using and the blood was stored in screw top heparinized plastic tubes, the sampling time for blood was done at 0 min (predose), 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 hr. The plasma was immediately separated by aspiration after centrifugation at 4000 rpm for 5 minutes and frozen at -20°C until analysed by LC-MS/MS method(9).

***Determination of Pharmacokinetic Parameters*:** Various pharmacokinetic parameters such as peak plasma concentration (C_{max}), time at which peak occurred (T_{max}), area under the curve (AUC), elimination rate constant (K_{el}), biological half-life ($t_{½}$) and mean residence time (MRT) were calculated using the noncompartmental pharmacokinetics data analysis software PK Solutions 2.0™(Summit Research Services, Montrose, CO, USA) (10).

***Statistical Analysis of the Pharmacokinetic Parameters*:** The pharmacokinetic parameters of the tested formulations were statistically analyzed using paired sample's t-test for normal distributed results of C_{max}, K_a, K_e, MRT and $AUC_{0-\alpha}$ values. All tests were performed at 0.001 level of significance(10).

Estimation of Simvastatin in Rabbit Plasma:

***LC-MS/MS Method*:** A summary of the chromatographic and mass spectrometric conditions is as follows:

UPLC: WATERS

Mass spectrometer: API 2000

Ion source: Heated nebulizer

Polarity: Negative

Detection ions

Simvastatin: 240.2 * amu (parent), 196.0* amu (product), d3-Simvastatin: 244.2* amu (parent), 200.2 amu (product)

Column: Phenomenax,synergi 4 µ Polar- RP80 A, 4.6x75 mm

Column oven temperature: 35°C

Peltier temperature: 10°C

Mobile phase: Ammonium formate: Acetonitrile (30:70)

Flow rate: 1 ml/min.

Volume of injection: 10 µL

Retention time: Simvastatin - 0.5 to 1.20 minutes: ISTD - 0.5 to 1.20 minutes

Run time: 2.00 minutes

Preparation of Working Standard Solutions:

***1. Preparation of Simvastatin Standard Stock Solution*:** Simvastatin working standard equivalent to 5 mg

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Simvastatin was accurately weighed and transferred into a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Simvastatin. The solutions were labeled and stored in a cold store at 2-8°C.

***2. Preparation of Internal Standard Stock Solution*:** 0.005 g of d3-Simvastatinwas weighed accurately and transferred in to a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Simvastatin–d4. The solutions were labeled and stored in a cold store at 2-8°C. Stock solution was diluted with 60% Acetonitrile in water solution to get a concentration of 75.00 µg/ml.

Calibration Curve Standards:

***1. Preparation of Stock Dilutions of Standard Simvastatin Solution*:** Stock solution of Simvastatin was diluted with 60% Acetonitrile in water solution to get a concentrations ranging from 1 to 140 µg/ml (Figure 1).

***2. Spiking of Plasma for Calibration Curve Standards*:** Concentrations of Simvastatin ranging from 50 to 7000 ng/ml were prepared with plasma and labeled them as CC1 to CC8 in (Table 1). The calibration

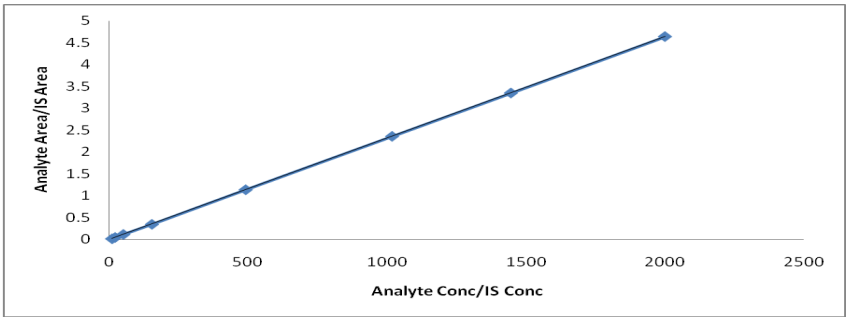

Fig 1. Calibration Curve for Estimation of Simvastatin in Plasma

Table 1: Analyte Concentrations of Stock Dilutions of Standard Simvastatin Solution with Plasma

S No	Sample Name	Analyte Concentration (ng/ml)	Analyte peak area	IS Peak Area	Area Ratio	Calculated Concentration (ng/ml)	Accuracy (%)
1	Plasma Blank	0	0	0	0	N/A	N/A
2	Blank+ISTD	0	254	513643	0	N/A	N/A
3	CC1	50.05	3879	528938	0.01	50.407	100.71
4	CC2	100.15	6997	506198	0.01	97.939	97.79
5	CC3	250.300	17284	484351	0.04	258.119	103.12
6	CC4	500.650	34646	504084	0.07	500.215	99.91
7	CC5	1001.250	77371	577631	0.13	978.007	97.68
8	CC6	2002.500	134349	520675	0.26	1887.085	94.24
9	CC7	5006.250	345626	529110	0.65	4782.401	95.53
10	CC8	7008.750	578109	544120	1.06	7780.670	111.01

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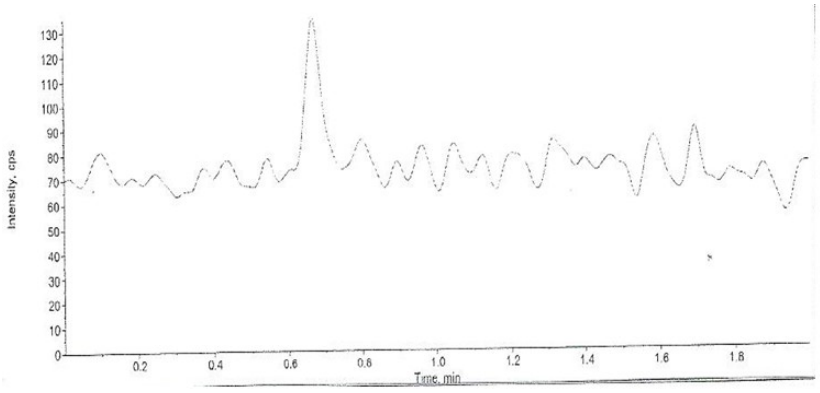
curve standards were prepared freshly for each validation run.

***Extraction Procedure*:**

***Step 1*:** Blank, calibration curve standards and the subject samples were withdrawn from the deep freezer and allowed them to thaw. The thawed samples were vortexed to ensure complete mixing of contents. To 0.25 ml of plasma sample in a ria vial, 25 ul of d3-Simvastatin standard (75 µg/ml) was added. To plasma blank, 25 ul of 60% Acetonitrile in water solution was added and vortexed the samples to ensure complete mixing of contents.

***Step 2*:** Add 2.5 ml of tertitary butyl methyl ether, place on a shaker for 15 minutes and centrifuge for 10 minutes at 4000 rpm at 20°C. Transfer supernatant (organic layer) into the another rial vial. Evaporate this layer under a stream of nitrogen at 45°C. The residue was reconstituted with 0.5 ml of mobile phase and vortexed. The samples were transferred in to auto-injector vials and loaded the vials in to auto sampler. 10 ul of sample was injected in to LC-MS/MS system.

***Data Processing*:** The chromatograms were obtained by using the computer-based Analyst 1.4.2 version software supplied by the Applied Biosystems, Canada. The concentrations of the unknown samples were calculated from the equation using regression analysis of spiked plasma calibration standard with $1/x^2$ as weighting factor. y = mx + c Where, y = Ratio of Simvastatin peak area and ISTD peak area (analyte area / ISTD area); x = Concentration of Simvastatin; m = Slope of the calibration curve; c = y-axis intercept value. Linear regression analysis equation of stock dilutions of standard Simvastatin solution with plasma is, y = 0.000136x + 0.000454.



Sample Name	: Plasma blank Simvastatin	Mass(es)	: 240.2/196.0amu
Concentration	: 0.000 ng/ml	Calculated conc	: N/A ng/ml
Proc. Algorithm	: Analyst classic	Bunching factor	: 2
Noise Threshold	: 10.00	Area Threshold	: 100.00
Num Smooths	: 10	Sep .Width	: 0.20
Sep. Height	: 0.01	Exp. Peak Ratio	: 5.00
Exp. Adj Ratio	: 4.00	Exp. Val Ratio	: 3.00
RT Window	: 30.0	Expected RT	: 2.00
Use Relative RT	: False	Int. Type	: No Peak
Retention Time	: 0.00	Area	: 0
Height	: 0	Area Ratio	: 0.000
Start Time	: 0.00	End Time	: 0.00

Fig. 2. Chromatograms of Blank Plasma

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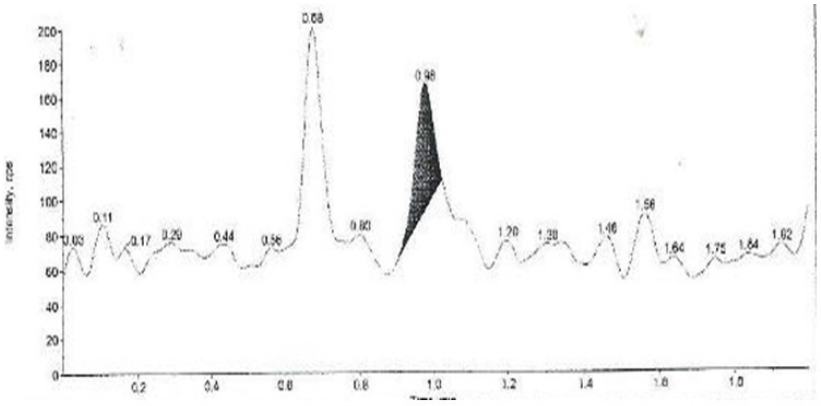
Results and Discussion

The *in vivo* experiments were conducted as per the protocol and procedure described earlier. The ability of core in cup tablet as a drug delivery system to release drugs in a predetermined time release manner was investigated in rabbits after oral administrations was investigated. Bioanalytical methods employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play a significant role in evaluation and interpretation of pharmacokinetic data. For the successful conduct of pharmacokinetic study, the development of selective and sensitive bioanalytical methods plays an important role for the quantitative evaluation of drugs and their metabolites (analytes). The LC-MS/MS methods were highly sensitive and suitable for the detection of drug in plasma even in low concentrations. Various pharmacokinetic parameters such as peak plasma concentration (C_{max}), time at which peak occurred (T_{max}), area under the curve (AUC), elimination rate constant (K_{el}), biological half-life ($t_{½}$) and mean residence time (MRT) were calculated using the noncompartmental pharmacokinetics data analysis software PK Solutions 2.0™(Summit Research Services, Montrose, CO, USA).

The pharmacokinetic parameters of the tested formulations were statistically analyzed using paired sample's t-test for normal distributed results of C_{max}, K_a, K_e, MRT and $AUC_{0-\alpha}$ values. All tests were performed at 0.001 level of significance.

Calibration curves were constructed from blank sample (plasma sample processed without IS), blank+IS samples and eight point calibration standards for Simvastatin in plasma. Plasma concentrations of Simvastatin at different times were calculated. Pharmacokinetic parameters such as absorption rate constant, elimination rate constant, half life, AUC, and MRT were calculated from the plot of time versus plasma concentration and subjected to statistical analysis and the results were shown in (Table 2).



Sample Name	:	Plasma blank+ISTD	Mass(es)	:	240.2/196.0amu
Concentration	:	0.000 ng/ml	Calculated conc	:	N/A ng/ml
Proc.Algorithm	:	Analyst classic	Bunching factor	:	2
Noise Threshold	:	10.00	Area Threshold	:	100.00
Num Smooths	:	10	Sep .Width	:	0.20
Sep. Height	:	0.01	Exp. Peak Ratio	:	5.00
Exp. Adj Ratio	:	4.00	Exp. Val Ratio	:	3.00
RT Window	:	30.0	Expected RT	:	2.00
Use Relative RT	:	False	Int. Type	:	No Peak
Retention Time	:	0.00	Area	:	0
Height	:	0	Area Ratio	:	0.00
Start Time	:	0.00	End Time	:	0.00

Fig. 3. Chromatogram of Stock Solution of Standard Simvastatin Solution with Plasma

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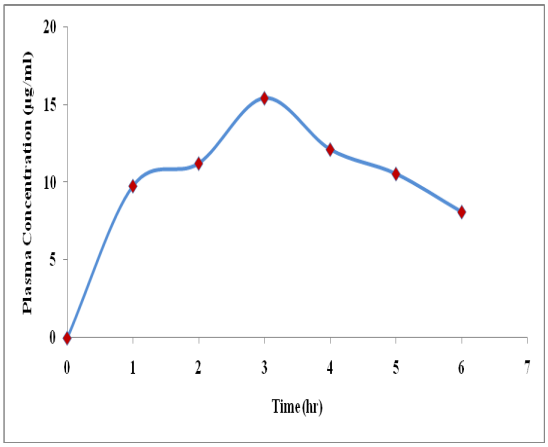

Fig. 4. Plasma Concentration-Time Curve of Simvastatin Following Oral Administration of Oral Suspension

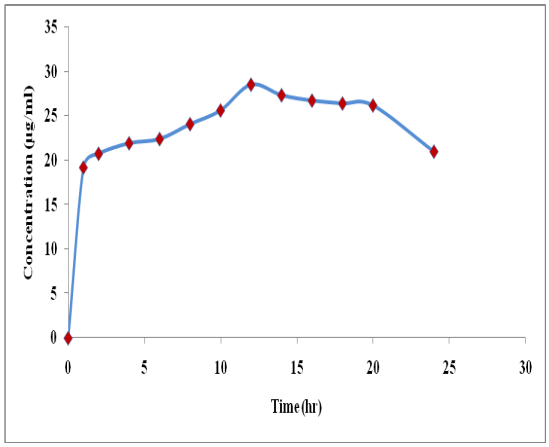

Fig. 5. Plasma Concentration-Time Curve of Simvastatin Following Topical Administration of Optimized Transdermal Formulation

Table 2: Statistical Treatment of Pharmacokinetic Parameters (Mean ± S.D.) of Following Oral Administration of Oral Suspension and Optimized Transdermal Formulation of Simvastatin

Pharmacokinetic Parameter	Oral Suspension	Optimized Transdermal Formulation	Calculated Value of 't'
C_{max} (ng/ml)	23.4 ± 0.31	28.6 ± 0.41	16.70***
Tmax (h)	3± 0.13	12± 0.08	21.36***
MRT (h)	5.1 ± 0.05	24.3± 0.15	25.50***
$t_{1/2}$ (h)	2.62± 0.03	12.52 ± 0.013	7. 75***
K_{el} (h^{-1})	0.26 ± 0.04	0.055 ± 0.007	3.87***
K_a (h^{-1})	1.14 ± 0.01	0.33 ± 0.02	12.67***
$AUC_{0-□}$ (ng h/ml)	105.9± 1.56	958.0.±3.07	156.60***
Null hypothesis (H_o): There is no significant difference between the pharmacokinetic parameters of oral administration of oral suspension and optimized transdermal formulations of Simvastatin. Table value of 't' with 10 DF at the 0.001 level is 4.587.			
Result: H_o is not accepted as the calculated 't' value more than the table Value of't' with 10 DF at 0.001 levels of significance. It was therefore concluded that there was significant difference between the pharmacokinetic parameters of oral administration of oral suspension and optimized transdermal formulations of Simvastatin.			
Values are presented in Mean ± SD			

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Plasma Concentration of Simvastatin following oral and transdermal administration in rabbits at different times were calculated. The results from the oral administration of Simvastatin indicated the maximum plasma concentration (C_{max}) 23.4 ± 0.31 ng/ml at 3 hrs (t_{max}) while transdermal administration exhibited the maximum plasma concentration (C_{max}) of 28.6 ± 0.41 ng/ml at 12 hrs (t_{max}). The oral administration of Simvastatin resulted in a low and quite variable AUC of 105.9± 1.56 ng.hr/ml, where as the transdermal resulted in AUC of 958.0 ± 3.07 ng.hr/ml. The mean residence time of transdermal administration (24.3± 0.15 hrs) was found to be more than oral administration (5.1 ± 0.05 hrs). The results indicated that the parameters significantly differed following transdermal administration, compared to oral administration. The concentration of selected drugs in plasma was found to be stabilized and maintained in a narrow range over the study period up to 24 hrs for transdermal formulation where as the concentration was decreased rapidly up on oral administration. The maximum plasma concentration (C_{max}) was attained at 3 hrs after oral administration and it was observed after 12 h upon application of transdermal formulation of same dose.

The *in vivo* pharmacokinetic studies revealed that the transdermal formulations of Simvastatin exhibited controlled release and absorption kinetics over longer periods of time which in turn maintained the desired plasma concentrations over longer periods of time.

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