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Reversed Phase UPLC Method Estimation of Lovastatinin Novel Hydrogel Formulation: Application of method to Pharmacokinetics, Pharmacodynamics and Histopathological samples

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Abstract

Lovastatin is used to treat high blood cholesterol and reduce the risk ofcardiovascular disease. In this study, an ultra-high-performance liquid chromatography (UPLC) method was developed to measure the concentrations of Lovastatin in rat blood, and the method was applied in measuring the pharmacokinetics of the analyte after oral and intravenous administration.The analyte was extracted by solid phase extraction method. A UPLC BEH C18 column (2.1 mm × 100 mm, 1.8 µm particle size) was used for chromatographic separation by gradient elution using acetonitrile-water (0.1% formic acid) as the mobile phase at a flow rate of 0.4 mL/min. Lovastatin was administered to the rats orally at 2 mg/kg and intravenously at 0.05 mg/kg. Blood was collected at various time intervals, and the blood samples were processed after collection and analyzed by UPLC.The intra-day and inter-day accuracy of Lovastatin were 91%-103% and 85%-107%, respectively, and the precision (RSD, %) was less than 15% for both intra-day and inter-day measurements. The matrix effect ranged from 95% to 108%, and the recovery was higher than 70%. Lovastatin has a good linear relationship in the range of 10-500 ng/mL, and the lower limit of quantification was 10 ng/mL. The precision, accuracy, extraction recovery, matrix effect, and stability meet the requirements of the guiding principles.A robust and reliable UPLC method was fully optimized and developed to detect the blood concentration of Lovastatin in rats and the samples were analyzed by Empower software.

Keywords: RP-UPLC, Validation, Hydrogel, Lovastatin, Pharmacokinetics, Pharmacodynamics

Introduction

Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), an enzyme that catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is a required building block for cholesterol biosynthesis and lovastatin interferes with its production by acting as a reversible competitive inhibitor for HMG-CoA, which binds to the HMG-CoA reductase (1). Lovastatin (Fig. 1) is a prodrug, an inactive lactone in its native form, the gamma-lactone closed ring form in which it is administered, is hydrolysed in vivo to the β-hydroxy acid open ring form; which is the active form.Lovastatin and other statins have been studied for their chemo-preventive and chemotherapeutic effects (2).

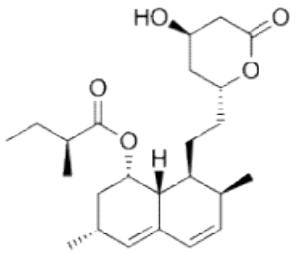

Fig. 1: Chemical structure of Lovastatin

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10.5530/ctbp.2025.4s.13

Several analytical methods have been reported for the analysis of Lovastatin individually by HPLC (3-6) and UPLC-MS (7). A Qbd method also published for Lovastatin nanoemulsion (8). Till date no bioanalytical method was reported for Lovastatin by UPLC. The procurement of clinical data for Lovastatin is also critical as lot of research is still going on for this drug for its efficacy and combined dosage forms.

The objective of the current research is to develop and validate a rapid, reliable, sensitive and simple ultra-performance liquid chromatography method for the quantification of Lovastatin in rat blood. After complete validation, the method was applied to analyze study sample analysis in rats by giving a single oral dose at 3 mg/kg and intravenously at 0.05 mg/kg body weight. In this study we aimed to develop a sensitive method for estimation of Lovastatin in rat blood and also to validate the method as per the ICH and USFDA Guidelines. Simultaneously to study in-vivo pharmacokinetic parameters by UPLC technique.

Materials and Methods

Instrument and chromatographic conditions

UPLC Method

The LC system consisted of a Waters Acquity UPLC with Empower software equipped with a photodiode array detector. A AcquityUPLC BEH RP C18 column (2.1 mm × 100 mm, 1.8 µm particle size) from Waters was used as stationary phase and temperature maintained at 20°C. The mobile phase consisted of Acetonitrile and 0.1% formic acid in gradient mode pumped at a flow rate of 0.4 mL min^{-1}. Analysis was performed for 5 min at the detection wavelength of 238 nm and the injection volume was 2 µL. The autosampler maintained at 4°C.

Chemicals

Lovastatin and internal standard (simvastatin) are purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile and methanol of HPLC grade and all other chemicals were obtained from Merck (Mumbai, India). Formic acid (GR grade) was purchased from Merck Chemicals Ltd., Mumbai. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrix (rat blood) was obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

Preparation of Calibrators and QC Samples

A standard stock solution of Lovastatin was prepared by dissolving standard 50 mg of Lovastatin into 50 mL volumetric flask, to this added 30 mL of methanol and sonicated for 10 minutes at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with methanol to have a solution with a concentration of 1000 µg/mL. Calibration standard and quality control (QC) samples were prepared by adding corresponding working solutions with drug-free rat blood. A volume of 10 mL of appropriate diluted stock solution at different concentrations and 10 mL of IS at a fixed concentration were spiked into 200 µL of rat blood to yield final concentrations of calibration samples 10, 25, 50, 100, 200, 300, 400 and 500 ng/mL. The final concentration of IS was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (10 ng/mL), LQC (50 ng/mL) MQC (200 ng/mL) and HQC (400 ng/mL).

Sample preparation

Analytes were extracted from blood by employing solid phase extraction method and the extract was then cleaned for using the 3 mL Supelclean™ Ultra 2400 SPE cartridge. 2 µL of the supernatant solution was injected for UPLC analysis.

Analytical Validation

All validation experiments were performed according to the Bioanalytical Method Validation Guidance for Industry (9), ICH guidelines (10) and ICH Guidelines (11) on validation of bioanalytical methods.

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10.5530/ctbp.2025.4s.13

Assay Specificity and Selectivity

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed. The specificity of the method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around the retention time.

Linearity

A calibration curve was prepared within the range of 10 to 500 ng/mL Lovastatin in each run. Half of the calibration samples were analyzed at the beginning of therun and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of Lovastatin/IS versus the concentration ofLovastatin. Lovastatin concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within ±20%. Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of quantification (ULOQ, 500 ng/mL), may differ from these specifications. At least 6 concentration levels were represented in eachcurve.

Matrix Effect, Extraction Recovery, and Process Efficiency

The influence of the matrix on the quantification of Lovastatin was monitored using a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (post extraction spiked samples), and (3) the same amount of analyte added to the biological matrixbefore extraction (pre extraction spiked samples).

Total process efficiency was calculated from the ratio of mean peak areas of Lovastatinin extracted validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrixeffect. Extraction recovery was calculated from the ratio of mean peak areas of Lovastatinin extracted validation samples versus blank samples spiked afterextraction. The absolute matrix effect was calculated from the ratio of mean peak areas of Lovastatin in blank samples spiked after extraction versus neat unextracted samples. If the ratio was 85% or 115%, anexogenous matrix effect was inferred.

Matrix Variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank blood samples and also 6 different lots of blank urine samples spiked with IS at the LLOQ level (n = 3 per lot), and blank blood samples with no IS (n = 3/lot) against a calibration curve. The results for the LLOQ samples were considered acceptable if the precision from each matrix lot was ±20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of Lovastatin and IS. Not more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of Lovastatinin the LLOQQCs.

Stability studies

Stability evaluations were performed in both aqueous and matrix based samples. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). Lovastatin stability in blood was evaluated by performing bench top stability, long-term stability, short term stability and freeze-thaw stability. The processed samples were studied for stability

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in auto sampler at 10°C. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples.

LOD and LOQ

Detectable, but not necessarily quantifiable, smallest concentration of an analyte that can be detected is called limit of detection (LOD). LOD is calculated from the formula LOD=3.3 σ/S, where σ is the standard deviation of Y intercept of the regression equation and S is the slope of the regression equation. LOQ is the lowest amount of analyte that can be quantitatively determined with accuracy and precision in the sample. LOQ=10 σ/S, where σ=SD of Y intercept of the regression equation; S =slope of the regression equation

Results and Discussion

Chromatographic and detection parameters

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Symmetry C18, Luna C18 and Zorbax C18. The best results were observed with the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.8 µm particle size) using acetonitrile (A) and 0.1% formic acid (B) (gradient mode) as mobile phase. Gradient flow operated as 95% B upto 1 min, then 1.1–2 min, 50% A, 50% B; 2.1-3 min, 95%A, 5% B; 3.1-4 min, 95%A, 5% B; 4.1–4.5 min, 5% A, 95% B; 4.6–5 min, 5% A, 95% B.Variation of the column temperature between 20 and 30°C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 20°C at a flow rate of 0.4 mL/min. The method allowed the separation of analyte with IS in 5 min (Fig. 2) runtime.

Specificity, Linearity, Accuracy and Precision

The specificity of method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around their retention times (Fig. 3). The eight point calibration curve for the analyte showed a linear correlation between concentration and peak area. Calibration data indicated the linearity (r^2> 0.99) of the detector response for all standard solutions from 10 to 500 ng/mL (Fig. 4). The limits of detection by UPLC was found to be 2 ng/mL and LOQ was found to be 10 ng/mL. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible and showed low standard error. A recovery experiment was performed to confirm the accuracy of the method. Blank blood was spiked with Low QC, Mid QC and High QC levels of the standard stock solution and then extracted and analyzed under

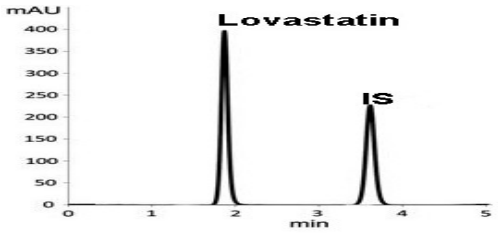

Fig. 2: UPLC separation of Lovastatin and Internal standard

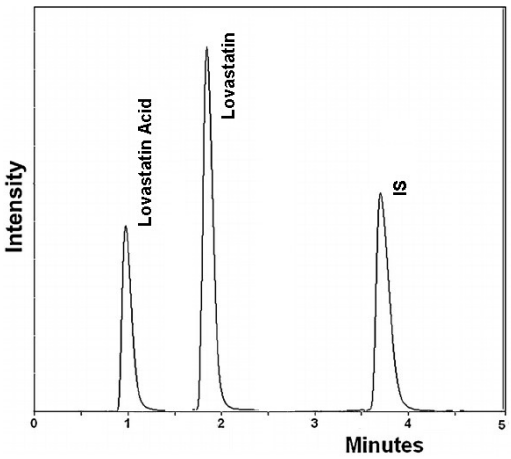

Fig. 3: UPLC separation of Lovastatin acid, Lovastatin and Internal standard

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10.5530/ctbp.2025.4s.13

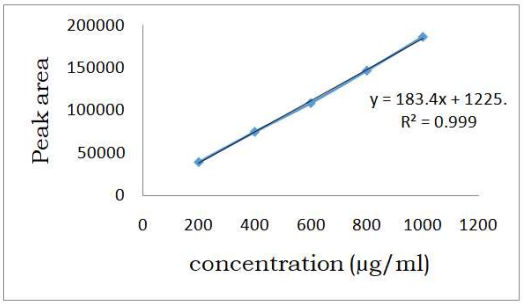

Fig. 4: Calibration curve of Lovastatin in plasma

optimized conditions. The extraction recoveries of all samples from rat blood were in the range of 97.5-108.0% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting. Intra- and inter-day precision of the method was determined by analyzing QC samples on two consecutive days and the obtained intra-day accuracies were in the range of 91.6–103.0% and inter-day accuracies were in the range of 96.4–106.9% (for LLOQ 85.6% was also observed which is acceptable for it). To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration. To demonstrate that the method is suitable for blood sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC. The dilution test was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that samples containing Lovastatin at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 h at room temperature and for 60 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The processed samples were stable up to 36 h in auto sampler at 10°C. The long-term matrix stability was evaluated at −20°C over a period of 60 days. No significant degradation of analytes was observed over the stability duration and conditions. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples. The short-term stability of analyte at room temperature was within 85–115% upto 24 h. Lovastatin was stable upto 10 h on bench top at room temperature and over 3 freeze–thaw cycles. The variability of the matrix effect in rat blood has resulted a very minute changes in the recovery of middle concentration of calibration curve.

Application of the method to pharmacokinetic study in Rats

Rats (200±20 g) used were maintained in a clean room at a temperature between 22±2°C with 12 h light/dark cycles and a relative humidity rate of 50±5%. Rats were housed in cages with a supply of normal laboratory feed with water ad libitum. For all of the studies, the animals (n=6) were deprived of food 12 h before dosing, but had free access to water. In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed UPLC method was successfully applied to a pharmacokinetic study by administration of Lovastatin as single solution to six rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 3 mg/kg body weight.Approximately, 0.2 mL of blood samples from each anesthetized (isoflurane) rat at pre-determined time intervals was collected using a capillary tube

Vol. 19 (Supplementary Issue 4), Oct-Dec 2025, ISSN 0973-8916 (Print)., 2230-7303 (Online)
10.5530/ctbp.2025.4s.13

into pre-labeled eppendorf tubes containing 10% of K_2EDTA anticoagulant (20 µL). The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h (postdose). The total blood volume collected from each rat was approximately 1.7 to 1.9 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight). All the samples were extracted by solid phase extraction method and the obtained supernatant samples were transferred into pre-labeled micro vials.

The blood samples thus obtained were stored at –30°C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and SAS® software version 9.2. All the samples were analyzed by the developed method and the mean concentrations vs time profile of Lovastatinhydrogel is shown in (Figs. 5 and 6), Lovastatin acid is compared with marketed formulation. The pharmacokinetic parameters estimated are shown in (Table 1).

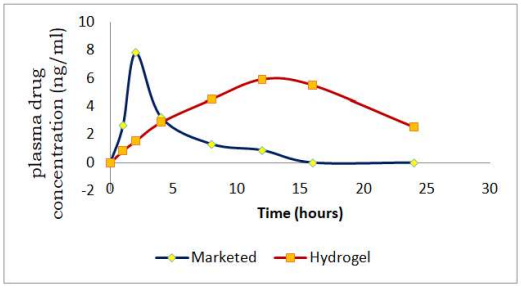

Fig. 5: Plasma concentration-time profile of Lovastatin hydrogels and Marketed formulation

The Pharmacokinetic parameters of Lovastatin Hydrogels were observed from the results as follows: The $C_{max}$was 5.9188 and 5.8822 ng/mL for Lovastatin and its active metabolite with T_{max} of 12 and 8.1 hrs respectively while the marketed formulation's $C_{max}$was 7.81 and 12.2273 ng/mL with T_{max} of 2.0 and 4.1 hrs respectively for Lovastatin and its active metabolite. The relative bioavailability of the Hydrogel formulation was calculated as 61.53%. The Mean Residence Time (MRT) was observed to be 13.6696 hrs which was comparatively higher than the marketed formulation. The active metabolite

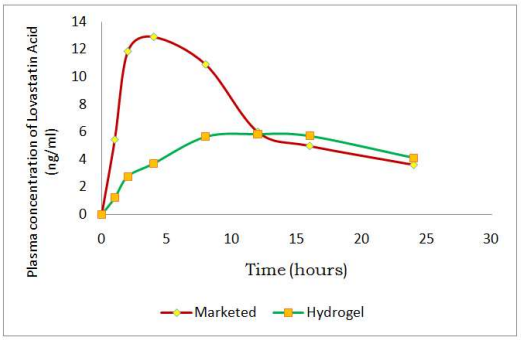

Fig. 6: Plasma concentration-time profile of Lovastatin Acid from hydrogel formulation and Marketed formulation

Table 1: Pharmacokinetic parameters of Lovastatin and Lovastatin acid Hydrogel and Marketed formulations

Parameter	Hydrogel formulation		Marketed formulation	
	Lovastatin	Lovastatin acid	Lovastatin	Lovastatin acid
C_{max}(ng/mL)	5.918±0.11	5.882±0.11	7.8123	12.2273±0.11
T_{max}(H)	12.0±0.11	8.1±0.11	2.0±0.1	4.1±0.11
$AUC_{(0-\infty)}$(ng h/mL)	103.098±0.15	113.14±0.15	32.965±0.1	173.71±0.15
$AUMC_{(0-\infty)}$(ng h/mL)	1409.38±0.15	1458.12±0.15	150.2077±0.15	1656.52±0.15
Fr (%)	----	61.53	----	-----
MRT (H)	13.6696±0.1	12.8877±0.1	4.556882±0.1	3.7794±0.1

All values are expressed as Mean±SD, n=6.
C_{max}: Maximum concentration; T_{max} : Peak Time; $AUC_{(0-\infty)}$: Area under plasma concentration-time curve; $AUMC_{(0-\infty)}$: Area under First moment curve from zero to infinity, Fr (%): Relative Bioavailability;
MRT: Mean residential time.

Reversed Phase UPLC Method Estimation

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10.5530/ctbp.2025.4s.13

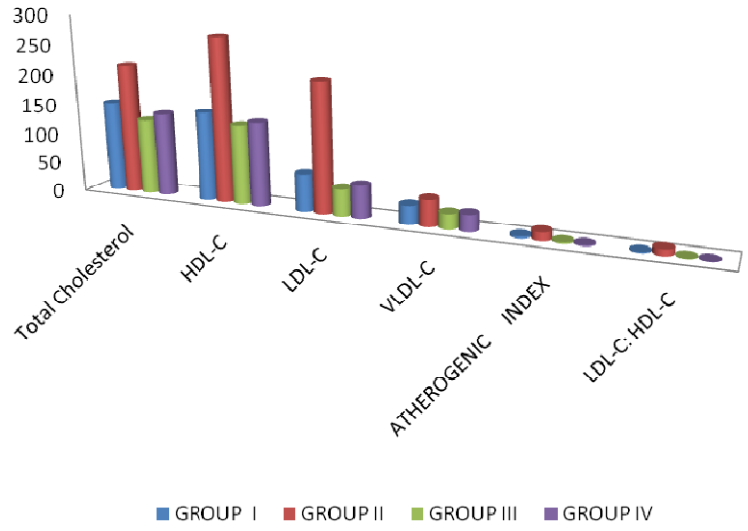

Fig. 7: Comparative Evaluation of Biochemical parameters of Lovastatin Formulations

Lovastatin acid was also estimated to have a higher MRT of 12.88 hrs and the Relative bioavailability was calculated to be 61.53%.

Pharmacodynamic Evaluation of Lovastatin-Eudragit Hydrogels:

The results of the studyshowed a marked increase in the body weights (350 ± 50 mg) of the hyper-lipidemic rats when compared to the normal rats due to cholesterol-rich diet supply. The Total Cholesterol, Triglycerides, and LDL-C levels were also elevated (273.33 ± 9.33, 216.43 ± 0.05, 214.26 ± 9.5 respectively. The HDL-C levels were reduced (20.83 ± 0.71). The rats fed with high cholesterol diet with cholic acid, coconut oil supplemented with the egg served as an experimental model in this study.

The purpose of including Cholesterol and coconut oil was due to accumulation of intracellular cholesterol and its esters in the body as coconut oil contains saturated fats (12).Cholic acid was used to improve the absorption of cholesterol as well as the suppression of Cholesterol-7a-hydroxylase to decrease cholesterol excretion (13).The lipid levels were reduced significantly after the administration of Lovastatin Hydrogels compared to the marketed formulation. The HDL-Cholesterol were increased when the animals were treated with Hydrogel formulation (60.83 ± 2.68) and Marketed formulation (57.74 ± 1.69). The higher levels of HDL-Cholesterol were documented to be physiologically beneficial in reducing the risk of cardiovascular disorders. The levels of TG over 150 leads to the risk of heart disease and stroke. The normal levels of TC. LDL-C and HDL-C (14) are below 200, 130, over 40 respectively. The Atherogenic Index was found to be 2.36 and 2.29 respectively for the hydrogel and marketed formulations respectively. This can be utilized as an important risk factor to prevent atherosclerosis and other cardiovascular disorders.The results revealed that the hydrogel formulation of Lovastatin is better dosage form over the Marketed formulation to control the blood cholesterol levels (Fig. 7).

Histopathological study of Lovastatin-Eudragit Hydrogels

Histological profile of the control animals showed normal hepatocytes as shown in the (Fig. 8). Animals belonging to Hyper-lipidemic Control group (Group II) exhibited intense centrilobular necrosis,

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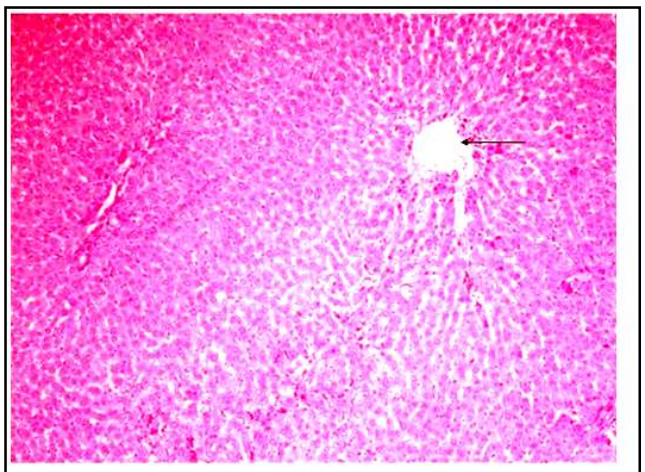
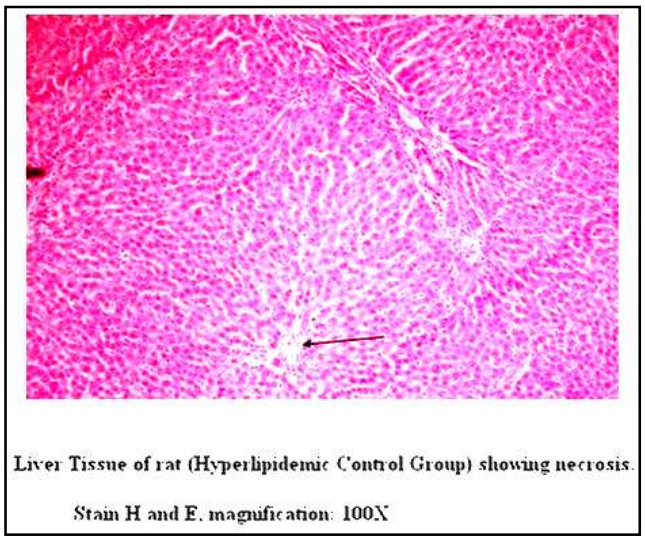
10.5530/ctbp.2025.4s.13



Liver tissue of control animal showing normal histology. Stain H and E, magnification 100X

Fig. 8: Histopathology of Liver tissue of control animal



Liver Tissue of rat (Hyperlipidemic Control Group) showing necrosis.

Stain H and E, magnification: 100X

Fig. 9: Histopathology of Liver tissue of Hyperlipidemic control animal

vacuolization and macro-vesicular fatty change. The sections of liver taken from the animals treated with Lovastatin Hydrogel Formulation showed a significant liver protection when compared to the ones treated with Lovastatin Marketed Formulation which can be evident by the absence of fatty lobules in the former and presence of mild granuloma in the latter (shown in Figs. 9-11). It envisaged that the hepatotoxicity of Lovastatin can be overcome to a certain extent by hydrogel delivery systems. Results are summarized in (Table 2).

Reversed Phase UPLC Method Estimation

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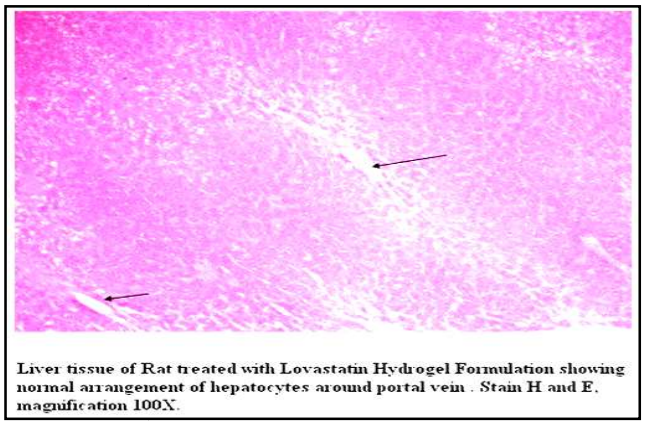

Fig. 10: Histopathology of Liver tissue of animal treated with Lovastatin Hydrogel Formulation

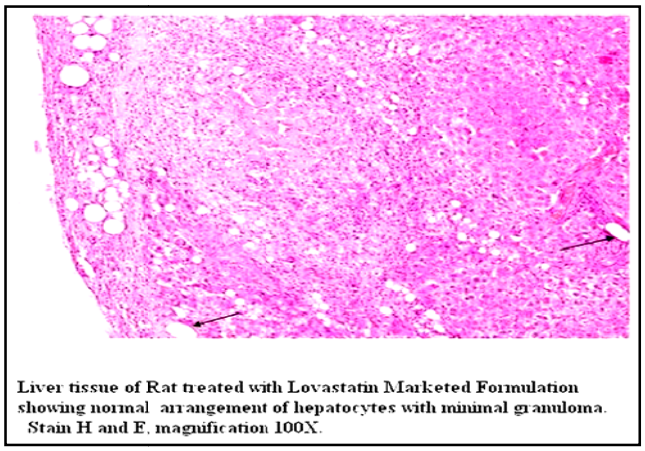

Fig. 11: Histopathology of Liver tissue of animal treated with Lovastatin Marketed Formulation

Table 2: Comparative estimation of Biochemical parameters using Lovastatin Marketed and Hydrogel formulations

Lipid Profile	Group I	Group II	Group III	Group IV
TG	150.42 ± 3.61	216.43 ± 0.05***	126.16± 0.65***	139.24 ± 4.03***
TC	148.57 ± 6.99	273.33 ± 9.33*	132.40± 3.85***	140 ± 4.96***
HDL-C	43.88 ± 4.64	20.83 ± 0.71***	60.83 ± 2.68***	57.74 ±1.69*
LDL-C	61.64 ± 9.92	214.26 ± 9.5*	44.1 ± 3.53***	53.23 ± 1.23***
VLDL-C	30.37 ± 0.72	43.29 ± 0.01***	25.06 ± 0.13***	27.59 ± 0.8***
Atherogenic Index	3.57 ± 0.42	14.15 ± 0.73	2.36 ± 0.14***	2.29 ± 0.32***
LDL-C: HDL-C	1.69 ± 0.42	10.23 ± 0.73	0.82 ± 0.14***	0.71 ± 0.32***

Group I – Normolipidemic control; Group II – Hyperlipidemic control; Group III – Lovastatin Hydrogel formulation; Group IV– Lovastatin Marketed formulation
The values are expressed as Mean ± SEM (n = 6). The statistical test employed by ANOVA followed by Tukey's Test; *p> 0.05; ***p < 0.001; significant as compared to normolipidemic Control and Hyperlipidemic control.

Vol. 19 (Supplementary Issue 4), Oct-Dec 2025, ISSN 0973-8916 (Print)., 2230-7303 (Online)
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Conclusion

A simple RP-UPLC method was developed and validated that can be considered to be economic, precise, specific and robust for the determination of Lovastatin in Hydrogel formulation. The UPLC method utilised lesser organic phase compared to reported methods which believably helpful in reducing organic solvent waste and thereby it will reduce the ecological impact.The UPLC method offers significant advantages over those previously reported, regarding lower sample estimations, simplicity of extraction procedure without any matrix effect. The linear dynamic range established was adequate to measure the concentration of Lovastatin in any preclinical and clinical study involving different biological species. The overall pharmacokinetic parameters were within the range of 80-125% therefore it can be concluded that, the present study provides firm evidence to support in clinical pharmacokinetic studies for further Research of selected drug. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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