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Original Article

Development and Validation of RP-HPLC Method for Determination of Eprosartan Mesylate in Rat Plasma: Application to Preclinical Pharmacokinetic Study

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ABSTRACT

A selective and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the determination of Eprosartan mesylate in rat plasma. A single step protein precipitation was carried out with acetonitrile to deproteinized plasma sample. A chromatographic separation was achieved on a Phenomenox, Gemini C_{18} (250x4.6 mm, 5 µm) column using acetonitrile and water (45:55) as a mobile phase with a pH adjusted to 3.4 with orthophsophoric acid (85%) at a flow rate 1 mL/min. The intensity of peak was monitored at 235 nm. The proposed chromatographic condition shows good symmetry and resolution of peaks. The retention time of Eprosartan mesylate and Olemesartan medoxamil (Internal standard) were appropriately 2.2 ± 0.5 and 3.1 ± 0.5 min, respectively. The validation studies performed as per ICH guidelines indicated high degree of accuracy, precision, with good degree of sensitivity and robustness of the proposed method. Furthermore, no interference was observed with plasma suggesting its utility for the pharmacokinetic analysis and bioavailability study of eprosartan mesylate in rat plasma.

Key words: Eprosartan mesylate, Reverse phase high performance liquid chromatography, Pharmacokinetic analysis, Protein Precipitation, Validation, ICH guidelines.

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INTRODUCTION

Eprosartan mesylate (EPM) is (E) -α-[{2-butyl-1-[(4-carboxyphenyl) methyl]-1H imidazole-5yl} methylene]-2-thiophenepropanoic acid monomethane sulfonate [1,2], and its structural formula is shown in (Figure 1). Eprosartan is an angiotensin II receptor antagonist used in the treatment of hypertension.[3] It is a highly selective angotensin II type 1 (AT_1) receptor antagonist with higher affinity for AT_1 than AT_2 receptor and no selectivity for adrenergic, serotonergic or other receptor types.[4,5] On the other hand, Olmesartan medoxamil (OLM) which is used as an internal standard (IS) is (5-methyl-2oxo-1, 3-dioxol-4yl) methyl ester of 4-(1-hydroxy-1-methylethyl)-2-propyl-1{[2(1H-tetrazol 5-yl) biphenyl -4yl] methyl}-1H-imidazole-5-carboxylate.[6,7] Several analytical methods are reported for estimation of Eprosartan mesylate in bulk and tablet formulations by UV spectrophotometry[8], RP-HPLC[9], EPM with Hydrochlorthiazide by RP-HPLC.[10] Bioanalytical methods reported include determination of EPM in human plasma by HPLC[11-13], HPTLC[14] and LC-MS.[15] However, to the best of our knowledge no LC method has been reported for the determination of EPM in rat plasma. The purpose of the present study was to develop a simple and accurate RP-HPLC method for determination EPM in rat plasma to support its pharmacokinetic study after oral administration.

MATERIALS AND METHODS

Materials and reagents

Eprosartan mesylate was a kind gift of Mylan Pharmaceutical Inc. (Nashik, India), Olmesartan medoxamil was provided by Ajanta Pharmaceutical Ltd. (Mumbai, India). HPLC grade Acetonitrile and Orthophsophoric acid was procured from Merck, (Mumbai, India). Triple distilled de-ionized water was used throughout the study. All solutions were filtered through a Millipore vacuum filter system (0.42µm) and degassed by sonicator.

Instrumentation

High performance liquid chromatography (HPLC) system consisted of LC solution data handling system (Shimadzu LC-20AD, Kyoto, Japan) equipped with SPD-20A Shimadzu UV detector (sensitivity of 0.005 absorbance unit full scale) and Rheodyne injector with 20 µL sample loop. A 25 µL Hamilton syringe was used for injecting the samples. Data acquisition was performed by using LC 2010 solution software. Ultrasonic bath sonicator was used for degassing of the mobile phase. Weighing of the materials were carried on a Shimadzu balance with high accuracy.

Chromatographic conditions

Chromatographic separation was carried out with Phenomenox, Gemini C_{18} (250x4.6 mm i.d., 5 µm) column at ambient temperature (28°C) with Security Guard (4.0x3.0 mm i.d., 5 µm) column (Phenomenox, Torrance, CA, USA). The mobile phase for isocratic elution consisted of water and acetonitrile (55:45 v/v), pH of which is maintained at 3.4 using orthophosphoric acid (85%). The mobile phase was prepared freshly and filtered through a Millipore vacuum filter system equipped with 0.42 µm filter for each run and degassed by sonicator. The isocratic elution was performed by pumping the mobile phase at a constant flow rate of 1.0 mL/min. The run time for elution was adjusted at 8 min. A sample of 20 µL was injected onto the analytical column and detected by measuring UV absorbance at 235 nm.

Eprosartan mesylate standard, QC and IS preparation

Primary stock solutions of eprosartan mesylate (1 mg/mL) and olmesartan medoxomil (0.5 mg/mL) were prepared in the mobile phase. Appropriate dilutions were made into 10 mL volumetric flasks with the mobile phase to prepare the working solutions for calibration curve and quality control (QC) samples. Calibration standards of eprosartan mesylate in rat plasma (100, 300, 600, 900, 1200, 1500, 1800 ng/mL) were prepared by

spiking the working standard into a pool of drug free samples along with the corresponding internal standard (IS). The QC standards were prepared by spiking the drug free plasma with eprosartan mesylate to obtain final concentrations of 100 (LQC), 900 (MQC) and 1800 (HQC) ng/mL respectively. All the sample solutions were stored at -20°C until the analysis.

Animal testing and collection of blood

All experiments and protocols described in the study were approved by the Institutional Animal Ethical Committee (Reg no. KNCOP/R&D/AN-PROT/14-15/04), Kamla Nehru College of Pharmacy, Butibori, Nagpur, India and were in accordance with guidelines of the Committee for purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. The healthy male Wistar rat weighing 220-250 g were selected for the study. The animals were fasted overnight prior to the experiment but had free access to water. The blood samples (approximately 500 µL) were collected from retro orbital plexus of the rat.

Plasma sample preparation

A single step protein precipitation method was employed for extraction of EPM from rat plasma. In brief, 100 µL of drug spiked plasma sample was pipetted into heparinized centrifuge tube. The plasma was then deproteinized, to free the bound EPM, using acetonitrile with a ratio of 1:3 (v/v) acetonitrile: plasma. The plasma: acetonitrile mixtures were vortex mixed for 3 min and then centrifuged at 5,000 rpm for 5 min. Clear supernatant samples were collected and injected into the HPLC system.

Method validation

Method validation was carried out as per the guidelines given by International Conference on Harmonization[16] and United States Pharmacopeia.[17] Various validation parameters were studied and validated as per fallowing procedure.

Selectivity and specificity

Selectivity of the developed method can be defined as quantification of intended analyte is not affected by the presence of proteins and other impurities in the bio-matrix. Six blank plasma samples were investigated for interference to judge selectivity and specificity of the method.

Linearity

The linearity of the method was determined at seven point calibration curve. Spiked concentrations were plotted against peak area ratios of EPM to IS and best fit line was calculated. Calibration standards of eprosartan mesylate in rat plasma (100, 300, 600, 900, 1200, 1500, 1800 ng/mL) were constructed and linearity parameters were evaluated.

Accuracy

The accuracy of the proposed method was determined by analyzing three quality control samples of EPM in plasma i.e. 100 (LQC), 900 (MQC) and 1800 (HQC) ng/mL respectively.

Precision

The precision of the method was evaluated by inter day and intraday variation studies. Three QC standard samples in triplicate were prepared twice in a day and studied for intraday variation (n=6). The same protocol was fallowed for three different days to study inter day variation and percentage relative standards (%RSD) were calculated.

Limit of quantification

Limit of quantification (LOQ) is defined as the lowest concentration of EPM in plasma samples that can be quantified with less than 20% RSD. To estimate LOQ, six independent plasma samples containing 300 ng/mL EPM were prepared and analyzed using developed method. Mean concentration and %RSD for these six samples were determined.

Recovery

The mean recovery was determined by comparing the peak area obtained from extracted plasma samples with the unextracted samples that represent 100% recovery. For recovery study, plasma extracted samples were prepared by spiking EPM at three different concentration levels LQC, MQC and HQC (100, 900, 1800 ng/mL, respectively) in triplicate. The precision of EPM recovery at each level (n=3) was determined.

Stability

The stability of EPM in rat plasma was assessed by analyzing QC samples (LQC, MQC and HQC) at three concentrations levels. Stability was tested by subjecting the QC samples to three freeze thaw cycles and room temperature (4 h). Stability was also checked by extracting appropriate QC which had been maintained at specific temperature (4°C) for the specific time and analyzing the extracts for EPM.

Pharmacokinetic study

Wistar rats weighing (220-250 g) were housed with free access to food and water. The rats were fasted overnight before the dosing with free access to water. EPM (10 mg/kg body weight of rat) was suspended in 0.5% sodium carboxyl methyl cellulose and administered to rats by oral gavage. After oral administration, blood samples of 0.5 ml were collected from retro-orbital plexus at 0.5, 1, 2, 4, 6, 8, 12, and 24 h time point. Plasma was separated from each blood sample by centrifugation at 10,000 rpm for 10 min and stored at -20°C until analysis. Aliquots of 100 µL serum samples were processed and analyzed for EPM concentrations.

The pharmacokinetic parameters were calculated from measured EPM plasma concentrations versus time profile after oral administration with non-compartment model with linear trapezoidal method using PK solver 2.0 software (An add- in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel).

RESULTS AND DISCUSSION

Method development

Mobile phase consisting of acetonitrile: water (45:55% v/v) with pH 3.4 adjusted with orthophsophoric acid (85% v/v), at a flow rate of 1mL/min was selected as optimal condition for developed method. With optimized chromatographic conditions, the retention time of EPM was found to be 2.2 ± 0.20 min with an asymmetric factor 1.1 ± 0.15 (Figure 2). Alteration in a pH showed peak broadening and change in retention time. Decrease in acetonitrile concentration from 45% resulted in diminished height of peak. The employed method gave good symmetry and resolution of EPM.

Selectivity and specificity

Single one step protein precipitation technique was found to be effective for determination of EPM in rat plasma. No significant endogenous interference was observed in processed test samples as well as blank plasma sample at a retention time of the EPM (Figure 3). The typical chromatogram of EPM with IS in rat plasma are shown in (Figure 4). Thus, the proposed method was found to be simple, specific and selective for determination of EPM in rat plasma.

Linearity

The chromatograms representing respective areas of EPM with concentration range 100-1800 ng/mL in 7 points calibration curve with fixed concentration of OLM (IS) are shown in (Figure 5). The plasma calibration curve was found to be linear over the concentration range, Y=111.3x +18974.The coefficient of regression (r^2) = 0.997, indicating the good correlation between the peak area for each concentration of EPM.

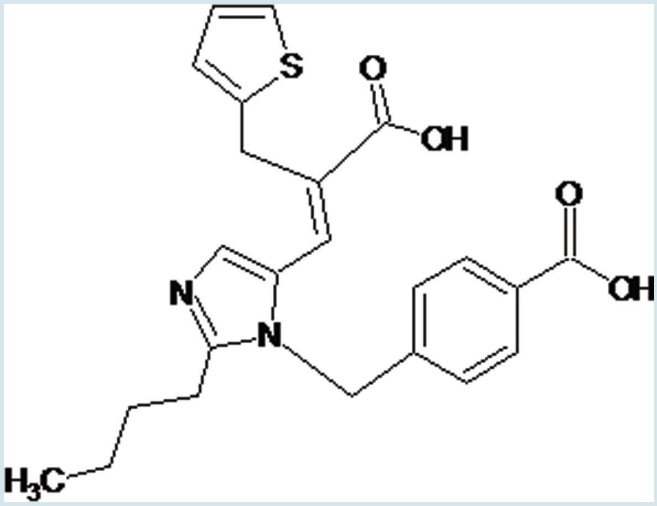

Figure 1: Structural formula of eprosartan mesylate

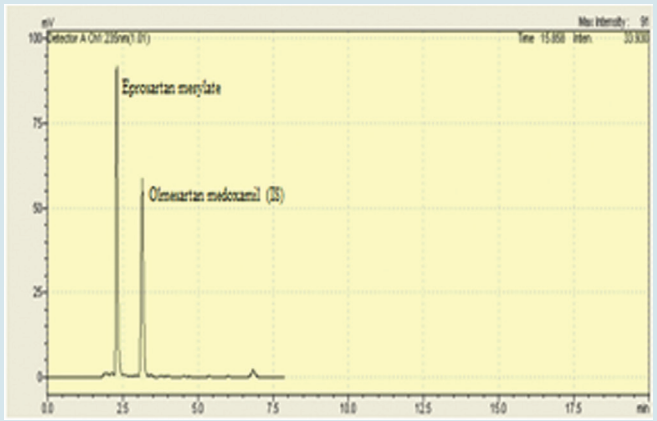

Figure 4: A typical chromatogram of EPM with OLM (IS) showing well resolved peaks in rat plasma

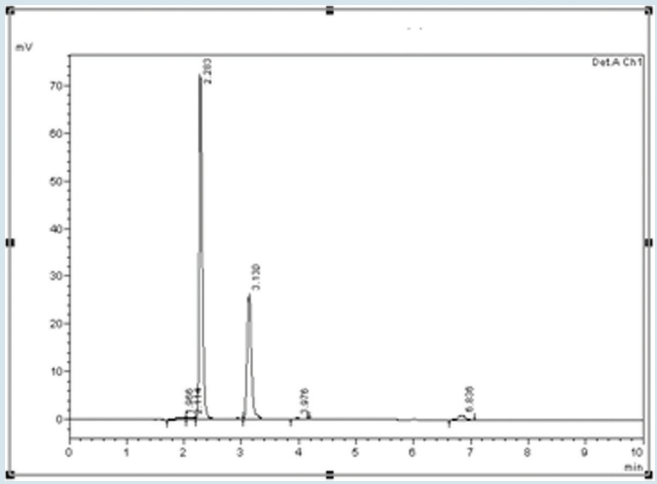

Figure 2: A representative chromatogram of EPM with OLM (IS) having RT 2.2 and 3.1 respectively

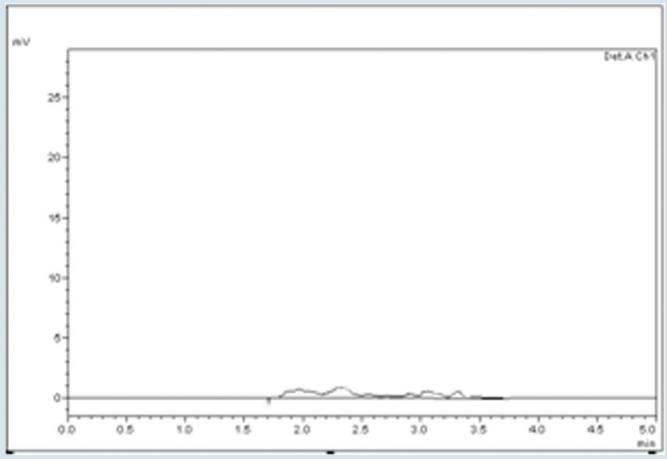

Figure 3: Chromatogram scan of rat plasma sample without any spiked analyte

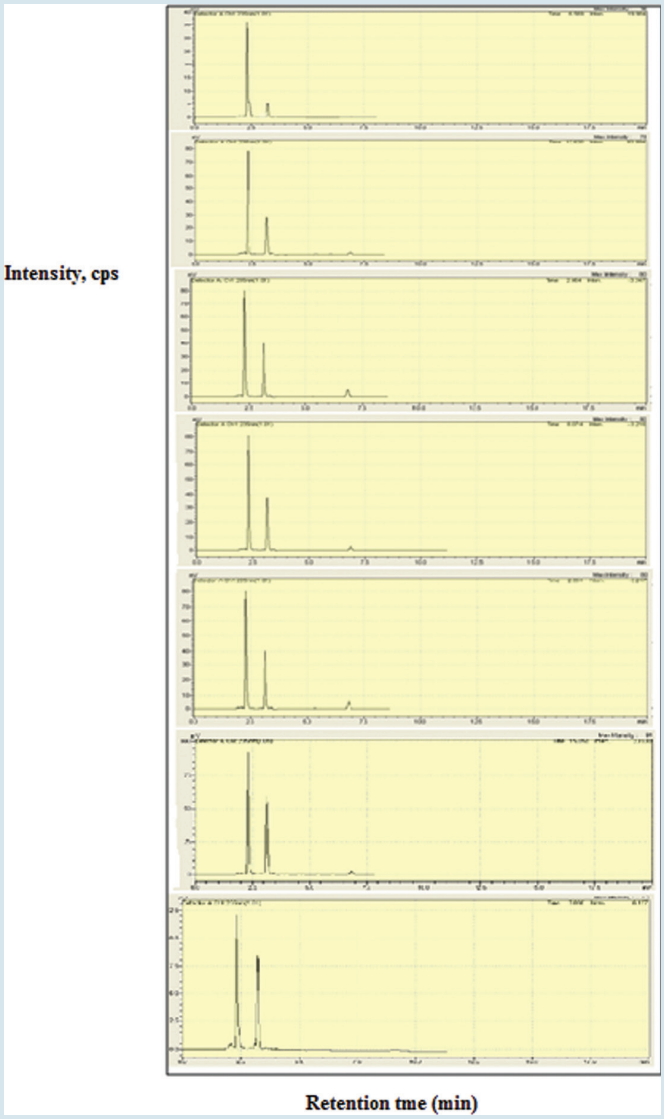

Figure 5: Chromatograms of spiked plasma with standard EPM of 100-1800 ng/mL with corresponding to OLM (IS) for standardization

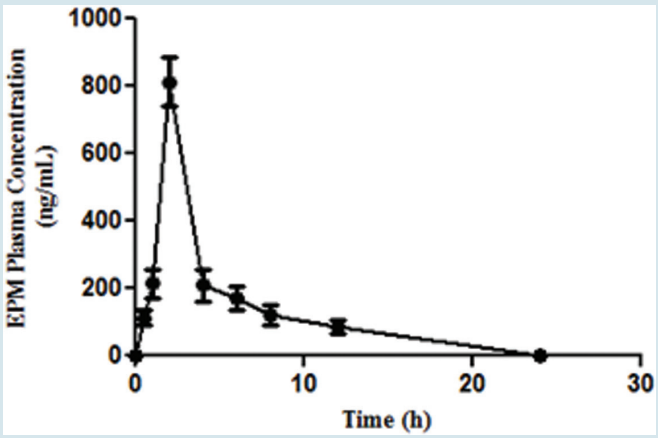

Figure 6: The mean plasma concentration-time profile of EPM in rats after oral administration (Mean ±SD, n=6).

Table 1: Accuracy data of the proposed method in rat plasma

Sr. No.	QC samples of EPM	Recovered amount [a] (SD ±) (ng/mL)	RSD (%)	Accuracy (%)
1	LQC	92.54±3.2	3.4	92.54
2	MQC	845.23±2.6	2.7	93.91
3	HQC	1739.64±4.1	4.2	96.61

*EPM- Eprosartan mesylate; LQC- Low quality control; MQC- Medium quality control; HQC- High quality control; SD- Standard deviation; RSD- Relative standard deviation; [a] Each value is a mean of six independent determinations (n=6).

Table 2: Precision study of developed method for EPM in rat plasma (Inter and Intra day)

Sr. No.	Quality control samples of EPM	Inter-day Precision		Intra-day Precision	
		Recovered amount [a] (±SD) (ng/mL)	% RSD	Recovered amount [a] (±SD) (ng/mL)	% RSD
1	LQC	91..56±3.2	3.4	93.78±2.4	2.5
2	MQC	832.87±4.1	4.4	846.37±2.9	3.0
3	HQC	1729.26±4.7	4.8	1735.45±3.4	3.5

*EPM- Eprosartan mesylate; LQC- Low quality control; MQC- Medium quality control; HQC- High quality control; SD- Standard deviation; RSD- Relative standard deviation; [a] Each value is a mean of six independent determinations (n=6).

Accuracy

The accuracy of analytical procedure measures the closeness of measured values to the true value. Quality control samples (100 (LQC), 900 (MQC) and 1800 (HQC) ng/ml respectively) showed an accuracy ranging from 92.54 to 96.61% with maximum % RSD of 4.2 across all QC samples, indicating the accuracy of the proposed method for EPM determination in rat plasma Table 1.

Precision

In precision study, the %RSD ranged from 2.5 to 4.8 across all QC samples Table 2. In the inter-day variation study the %RSD were found to be not more than 4.8 and for intra-day variation %RSD were less than 3.5. The low %RSD values indicated the repeatability and intermediate precision of the proposed method.

Limit of quantification

The %RSD and mean percentage accuracy of six independent samples of 100 ng/mL was found to be 3.4 and 92.54%. Hence, the concentration, 100 ng/ml was considered as lowest limit of quantification (LLOQ) for the proposed method.

Table 3: Absolute recovery of EPM from plasma samples fallowing protein precipitation method

Sr. No.	QC samples of EPM	Actual amount (100%) Un-Extracted (ng/mL)	Recovered amount [a] (SD ±) Extracted (ng/mL)	Mean % recovery	RSD (%)
1	LQC	100	91.53±3.1	91.53	3.3
2	MQC	900	853.45±3.9	94.82	4.1
3	HQC	1800	1738.34±4.5	96.57	4.6

*EPM- Eprosartan mesylate; LQC- Low quality control; MQC- Medium quality control; HQC- High quality control; SD- Standard deviation; RSD- Relative standard deviation; [a] Each value is a mean of three independent determinations (n=3).

Table 4: The plasma pharmacokinetic parameters of EPM in rats after oral administrations (Mean ±SD, n=6)

Sr. No.	Pharmacokinetic Parameters	Value
1	AUC_{0-t} (ng/mL* h)	2725.251±536.261
2	$AUC_{0-\infty}$ (ng/mL* h)	3462.933±614.435
3	C_{max} (ng/mL)	812±42.5
4	T_{max} (h)	2.0±0.0
5	$t_{1/2}$ (h)	6.05±1.2

AUC_{0-t} – area under the concentration time profile curve until last observation, $AUC_{0-\infty}$ -- area under the concentration time profile curve extrapolated to infinity, C_{max} – peak of maximum concentration, T_{max} – time of peak concentration, $t_{1/2}$ – elimination half life.

Recovery

The extraction recovery of EPM from spiked rat plasma samples, when compared with unextracted samples of same concentration was found to be 91.53 to 96.57 % with % RSD less than 4.6 at each concentration levels Table 3. The high mean percentage recovery and low %RSD values indicated the good extraction efficiency of the selected solvent for precipitation and also robustness of the proposed method.

Stability

The results of stability study showed that, there was no deterioration for EPM in rat plasma at QC levels for the three freeze-thaw cycles, indicating that drug concentrations can be accurately determined in samples that had thawed up to three times before the analysis or that have been thawed and kept at ambient temperature for up to 4h. The stability of EPM in the extract was also assessed after 24 h storage at 4°C to ensure sample reinjection and holding off. The results showed that EPM in rat plasma was stable fallowing storage at 4°C at 24 h.

Pharmacokinetic application

The proposed method was successfully applied in pharmacokinetic study after a single oral dose of 10 mg/kg of EPM to Wistar rats. The mean plasma concentration versus time profile of EPM obtained after an oral administration is illustrated (Figure 6). The pharmacokinetic parameters obtained from non-compartment analysis using linear trapezoidal method are summarized in Table 4.

CONCLUSION

A simple, selective and accurate RP-HPLC method was developed and validated for estimation of EPM in Wistar rat plasma. The proposed method was found to be rapid, specific, reproducible and cost effective. A single step protein precipitation with acetonitrile was found to be

efficient with no endogenous interference with plasma. Furthermore, the drug was found to be stable under various processing and storage conditions. The method involves simple procedures for sample preparations and relatively short run time. The developed method can find its importance in bioequivalence, bioavailability and drug interaction studies of Eprosatan mesylate.

CONFLICT OF INTEREST

The Authors declares there is no conflict of interest.

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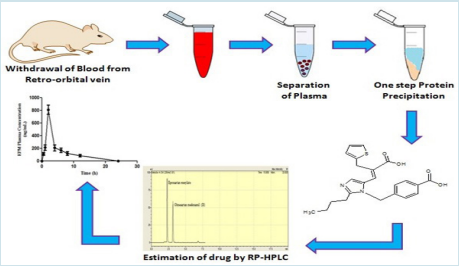
PICTORIAL ABSTRACT



SUMMARY

- A simple, selective and accurate RP-HPLC method was developed and validated for estimation of Eprosartan mesylate in Wistar rat plasma.
- A single step protein precipitation with acetonitrile was found to be efficient with no endogenous interference with plasma.
- The drug was found to be stable under various processing and storage conditions.
- The developed method can find its importance in bioequivalence, bioavailability and drug interaction studies of Eprosatan mesylate.

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