

Original Article

**SIMULTANEOUS DETERMINATION OF ATORVASTATIN CALCIUM AND OLMESARTAN MEDOXOMIL IN RAT PLASMA BY LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY AND ITS APPLICATION TO PHARMACOKINETICS IN RATS**

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Received: 31 Aug 2014 Revised and Accepted: 01 Oct 2014

ABSTRACT

**Objective:** A novel LC-ESI-MS/MS method was developed and validated for simultaneous determination of atorvastatin calcium (ATR) and olmesartan medoxomil (OLM) in rat plasma and evaluated pharmacokinetic study in rats.

**Methods:** The analytes were separated on aquasil C<sub>18</sub> reversed phase column and the detection of target compounds was done in Multiple Reaction Monitoring (MRM) mode using an ion trap mass spectrometer. The method was validated according to the ICH guidelines.

**Results:** The limit of detections (LODs) and the limit of quantifications (LOQs) were 0.15ng/mL and 1.0ng/mL for ATR and 0.21ng/mL and 1.0ng/mL for OLM respectively. The determined intra- and inter-day precisions were less than 10% and the accuracy was within 90 - 110 %. The stability and extraction recoveries of the analytes were in the acceptable range.

**Conclusion:** This method was successfully applied to the pharmacokinetic study of ATR and OLM in rats.

**Keywords:** Atorvastatin calcium, Olmesartan medoxomil, Electron spray Ionization, Multiple Reaction Monitoring, Pharmacokinetic study.

INTRODUCTION

Control of hypertension and hyperlipidaemia are important for the prevention of cardio vascular risk factors. The combination of atorvastatin calcium (ATR) along with olmesartan medoxomil (OLM) is used in the treatment of coexisting hypertension and hyperlipidemia in adult patients. Atorvastatin calcium (ATR) is chemically described as calcium salt of ( $\beta$  R, 8 R)-2-(4 - fluoro-phenyl) -  $\alpha$ ,  $\delta$  di hydroxyl 5(1 methyl ethyl) 1, 3, phenyl, 4 (phenyl amino) carbonyl) -1 H pyrroleheptanoic acid tri hydrate used as antihyperlipidaemic [1]. ATR is liver selective competitive inhibitor of 3-hydroxy-3-methylglutaryl Co enzyme A (HMG Co A) reductase, the rate limiting enzyme that converts 3-hydroxy-3-methylglutaryl Co enzyme A to mevalonate, a precursor of cholesterol biosynthesis. It also lowers elevated total and LDL cholesterol, apolipoprotein-B, and triglyceride levels in patients with primary hypercholesterolemia and mixed dislipidemia [2,3]. ATR is rapidly absorbed after oral administration due to presystemic clearance in gastro intestinal mucosa and metabolism in liver. Its absolute bioavailability is approximately 12% and low plasma concentration is achieved following administration of the drug [4].

Olmesartan is administered as an ester prodrug, olmesartan medoxomil (CS-866) which is hydrolyzed to active olmesartan during absorption from gastrointestinal tract that acts as an angiotensin II receptor blocker effective in lowering blood pressure in hypertensive patients [5-7]. Chemically it is known as 2,3-dihydroxy-2-butenyl-4-[1-hydroxy-1-methylethyl]-2-propyl-1-[p(o-1H-tetrazol-5-ylphenyl)benzyl]imidazole-5-carboxylate, cyclic 2,3-carbonate [8]. The peak plasma concentration ( $C_{max}$ ) of olmesartan is reached after one to two hours after oral administration. The bioavailability of olmesartan is not affected by food. Olmesartan is eliminated in a biphasic manner, with a terminal elimination half-life of approximately 13 hours. It is highly bound to plasma proteins (99%) and does not penetrate red blood cells [9].

Several analytical methods including LC-UV and LC-MS have been reported for bioavailability of ATR [10-20] and OLM [21-26] either individually or in combination with other drugs. However, so far, no single and precise analytical method was reported for the simultaneous estimation of ATR and OLM in rat plasma. Hence it is important to determine the bioavailability of ATR and OLM when

these two drugs were administered in combination. Therefore, the aim of this study was to develop a new, simple, rapid, precise and economic method for the simultaneous determination of these two drugs in rat plasma using liquid chromatography electrospray ionization tandem mass spectrometry. The developed bio analytical method has been validated according to the ICH guidelines [27] and applied successfully for pharmacokinetic study in rats.

MATERIALS AND METHODS

Apparatus and Chemicals

The reference standards of Olmesartan medoxomil (OLM) was gift sample from Ranbaxy Laboratories Ltd. (Gurgaon, India). Atorvastatin calcium (ATV) and Losartan (LOS) were obtained from Aurobindo Pharma Ltd. (Hyderabad, India). Their structures and purities were confirmed by HPLC, nuclear magnetic resonance and high resolution mass spectrometric methods. All the chemicals were showed purity more than 99 %. High purity deionized water was obtained using a Direct Q ultra-pure water system from Millipore (Milford, MA, USA). HPLC grade acetonitrile and methanol were purchased from E-Merck (Mumbai, India). Analytical grade formic acid and glacial acetic acid were purchased from SD-Fine Chemicals Ltd. (Mumbai, India). 0.45 $\mu$ m Syringe filters with nylon membrane was obtained from Pall scientific, (Bangalore, KA, USA). Spinix Vortex shaker was obtained from Jaibro Scientific Works (New Delhi, India). Biofuge refrigerated centrifuge was purchased from Her-aeus (Germany). The animals were procured from National Institute of Nutrition, Tarnaka, Hyderabad.

Standard solutions and fortification

Standard solutions of ATV, OLM and LOS were prepared at 1mg/mL concentration by dissolving 10 mg of each drug in 10 mL of acetonitrile and water (50:50, v/v). They were further diluted with acetonitrile to obtain working standard solutions for spiking purposes. Calibration standard and quality control samples in plasma were prepared by fortifying the corresponding working standard solutions with drug-free rat plasma. A volume of 10  $\mu$ L of each working standard solution was spiked to 100  $\mu$ L of blank plasma samples to produce final concentrations equivalent to 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000ng/mL. A volume of 10  $\mu$ L

internal standard losartan was added to each concentration to produce a final concentration of 20ng/mL. The QC samples were prepared in the same way as calibration samples to yield low, medium and high concentration levels of 1, 5, 50 and 500 ng/mL for ATV and OLM respectively. Linearity of the two drugs was evaluated using least square linear regression analysis and regression equations were used for determination of concentrations via back calculations.

#### Extraction procedure

Analytes were extracted from plasma by employing protein precipitation method. A volume of 200  $\mu$ L of chilled acetonitrile (-20  $^{\circ}$ C) was added as a protein precipitating agent, vortexed for 1 min and then centrifuged at 10,000 rpm for 10 min on Biofuge refrigerated centrifuge at 4 $^{\circ}$ C. The supernatant layer was separated and filtered through 0.45  $\mu$ m syringe filters and 20  $\mu$ L of the sample solution was injected for LC-MS/MS analysis.

#### LC-ESI-MS/MS analysis

A Thermo Finnegan LCQ Advantage Max ion trap mass spectrometer coupled to a Thermo Finnegan HPLC system containing surveyor LC quaternary pump plus, surveyor auto-sampler plus (Thermo Scientific, San Jose, CA, USA). The Xcalibur software (version 2.1) was used for data acquisition and analysis.

The separation of the analytes was carried out on an Aquasil-C<sub>18</sub> (50 mm length 2.1 mm internal diameter and 5  $\mu$ m particle size) column protected with Phenomenex security guard column C<sub>18</sub> (4 mm $\times$ 2 mm ID) (Phenomenex, India) and was operated at ambient temperature. The mobile phase composed with acetonitrile (pump-A) and water containing 0.04 % glacial acetic acid (pump-B) used in (80:20, v/v) isocratic mode at an optimum flow rate of 0.25 mL/min for 3 min.

Mass Spectral determination was done in electrospray ionization positive ion mode on an ion trap mass spectrometer. The full scan MS and MS/MS spectra of each analyte were obtained by direct infusion of the respected sample solution at a concentration of 10 $\mu$ g/mL solution prepared in the mobile phase.

The flow rates of sheath gas and auxiliary gas were optimized and set to 30 psi and 5 psi respectively. The needle spray voltage was set to 4.5 kV. Helium was used as damping and collision gas tuned for each analyte to obtain good signal intensity in MS<sup>2</sup> experiment.

The structures and ESI-MS/MS spectra of analytes were presented in Fig. 1. The quantitative analysis of drugs was done in multiple reactions monitoring (MRM) mode. The precursor ions, product ions, and LC-ESI-MS/MS parameters were depicted in Table 1.

Table 1: LC-ESI-MRM parameters of all the analytes

Analyte	Retention time	ESI mode	MRM transition	Collision Energy
losartan	1.20 $\pm$ 0.3	positive	423 $\rightarrow$ 405	30
atorvastatin	1.68 $\pm$ 0.3	positive	559 $\rightarrow$ 440	44
olmesartan	1.83 $\pm$ 0.3	positive	559 $\rightarrow$ 541	30

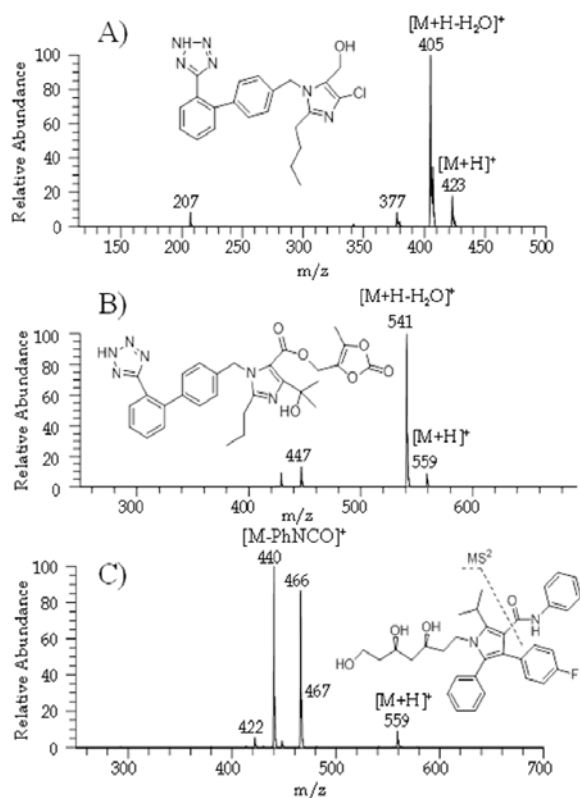


Fig. 1: LC-ESI-MS/MS spectra of A) losartan, B) olmesartan and C) atorvastatin and their corresponding structures

#### Method validation

The bio analytical method was validated according to the FDA guidelines (US Food and Drug Administration, May 2001). The method was validated in terms of selectivity, specificity, linearity,

limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, recovery, and matrix effect.

The selectivity and specificity were assessed by comparing the chromatograms of six different sources of blank rat plasma with those of the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and LC-MS/MS conditions to ensure no interference of ATV, OLM and the ISs from blank plasma. LODs of the drugs were determined based on signal intensity three times more than baseline noise (S/N=3) and LOQs of the drugs were determined based on intensity of signal which was ten times more than the noise (S/N=10).

The linearity of the assay was evaluated by constructing calibration curves with different concentrations ranging from 1 to 1000ng/mL for atorvastatin and olmesartan. The calibration curves were constructed by plotting the each respective peak area ratios of ATV and OLM to the LOS (IS) against the concentrations of ATV and OLM respectively, using the weighting factor of 1/x<sup>2</sup>.

Quality Control (QC) samples were prepared in blank plasma at the concentrations of 1 (LLOQ), 5, 50 (MLOQs) and 500ng/mL (HLOQ) for ATV and OLM in six replicates (n=6) for assessing the accuracy, intra- and inter-batch precisions (reproducibility) of the method. All QC samples were prepared freshly on three consecutive days and analyzed in each analytical batch along with the unknown samples.

Extraction recoveries for drugs were determined by post extraction and pre extraction spiking at four concentration levels 1, 5, 50 and 500ng/mL. In post extraction, 100  $\mu$ L blank plasma samples were extracted with 200  $\mu$ L of acetonitrile, drugs mixture solutions were spiked into the blank extracts along with the IS at 10 $\mu$ g/mL. In pre extraction, similar concentration levels were spiked in to 100  $\mu$ L blank plasma samples prior to extraction. The samples were prepared and analyzed in six replicates. The extraction recovery values and validation results of two drugs were showed in Table 2.

The matrix effect (ME) of the method was measured by extracting 100  $\mu$ L of blank plasma samples. The residues after evaporation were reconstituted with 200  $\mu$ L of standard solution containing the drugs at four concentration levels 1, 5, 50 and 500ng/mL along with the internal standard 20ng/mL. The percentage ME was calculated by comparing the relative area ratios (analyte/IS) obtained from the

matrix and standards prepared in neat solvent at similar concentration levels 1, 5, 50 and 500ng/mL. The ME (%) observed in the range of  $\pm 15$  to 23 in rat plasma. The stabilities of ATV and OLM in plasma at different storage condition were evaluated and the results were expressed as mean percentage accuracies. The short term stability was determined by keeping QC samples in six

replicates (n=6) at room temperature for 24 h. The auto sampler stability was evaluated by keeping the QC samples at 4°C for 24 h in auto sampler before analysis. Freeze-thaw stability of QC samples were analyzed after three freeze- thaw cycles by freezing at -80°C for 24 h and thawing at room temperature for 24 h. The stability studies of analytes were showed in Table 3.

**Table 2: The validation parameters of drugs at six replicates (n=6).**

Analyte	Concentration ng/mL	Intra day		Inter day		Extraction recovery	RSD (%)
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)		
ATV	1	90.55	2.05	91.42	3.97	72.12	10.65
	5	94.60	5.50	90.71	1.99	83.64	6.29
	50	98.65	6.50	102.55	3.19	91.83	8.32
	500	98.48	3.18	99.31	2.70	95.28	6.75
OLM	1	90.69	3.22	93.01	5.10	74.39	9.43
	5	91.58	4.77	90.40	2.86	76.28	6.48
	50	97.75	5.73	104.17	4.21	84.39	10.53
	500	104.37	5.58	100.96	4.33	94.96	8.93

**Table 3: Stability studies of ATV and OLM in rat plasma at four QC levels in six replicates (n=6).**

Storage conditions	ATV				OLM			
	Mean accuracy (%), RSD (%)				Mean accuracy (%), RSD (%)			
	1	5	50	500	1	5	50	500
Short term stability (24 h, room temperature)	102.21 (2.1)	101.87 (1.8)	100.16 (2.9)	99.54 (3.0)	99.15 (1.5)	101.52 (1.1)	99.23 (3.0)	100.17 (3.2)
Freeze/thaw stability (3 cycles each)	98.71 (1.5)	100.44 (2.4)	99.82 (3.1)	99.26 (3.8)	99.64 (1.2)	99.23 (1.6)	100.31 (2.5)	100.68 (4.9)
Pre-preparative stability at 4 °C for 12h (auto sampler)	98.12 (1.8)	100.47 (2.5)	99.35 (3.2)	98.41 (2.8)	101.32 (1.1)	101.12 (1.4)	99.76 (2.1)	100.27 (3.5)

#### Animal study

Six healthy Wistar rats with an average weight of  $200 \pm 10$  g were procured from National Institute of Nutrition, Tarnaka, Hyderabad (India). The animals were housed in BIOSAFE, under standard ( $22 \pm 2$  °C, 60–70% humidity) laboratory conditions, maintained on a 12-h natural day–night cycle, with free access to standard food and water. Animals were acclimatized to laboratory conditions before the study. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of IICT, Hyderabad (India) (CPCSEA registration number: 97/1999/CPCSEA, Dated 28.4.1999) and was conducted according to the CPCSEA guidelines on the use

and care of experimental animals. After overnight fasting of animals, ATV and OLM mixture was prepared and administered orally to the rats as a gum acacia suspension at a dose of 1 mg/kg and 2 mg/kg respectively. A volume of 0.3 mL of blood was collected in EDTA coated glass tubes at time intervals of 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24h after drug administration. Blood was centrifuged at 5000 rpm for 10 min and the plasma was separated and stored at -80 °C until analysis. A volume of 20  $\mu$ L was injected into the LC-MS/MS system. Pharmacokinetic parameters were estimated using non-compartmental analysis with WinNonlin (version 1.1) kinetic software. The pharmacokinetic parameters of drugs were presented in Table 4.

**Table 4: Pharmacokinetic parameters of atorvastatin and olmesartan in rat plasma (n = 6) after oral administration (mean  $\pm$  SD).**

Parameter	ATV	OLM
C <sub>max</sub> (ng/mL)	391.72 $\pm$ 36.14	762.02 $\pm$ 33.50
T <sub>max</sub> (h)	0.90 $\pm$ 0.22	0.70 $\pm$ 0.27
AUC <sub>0-t</sub> (ng mL/h)	864.37 $\pm$ 115.32	1778.82 $\pm$ 339.04
AUC <sub>0-∞</sub> (ng mL/h)	873.97 $\pm$ 118.15	1794.30 $\pm$ 348.26
t <sub>1/2</sub> (h)	3.89 $\pm$ 0.40	3.56 $\pm$ 0.45

C<sub>max</sub> is the peak plasma concentration, T<sub>max</sub> is the time to reach peak plasma concentration, t<sub>1/2</sub> is elimination half-life, AUC<sub>0-t</sub> is the area under the plasma concentration-time curve from time zero to the last sampling time and AUC<sub>0-∞</sub> is the area under the plasma concentration-time curve from time zero to infinity.

## RESULTS AND DISCUSSION

### Optimization of LC-ESI-MS/MS conditions

Separation of these drugs on aquasil-C<sub>18</sub> was examined using various combinations of acetonitrile and water mobile phase compositions with different percentage of modifiers. The combination of acetonitrile (A) and water containing 0.04 % glacial acetic acid (B) (80:20, v/v) was gave good separation for all the analytes with good peak shapes. The standard solutions at 10  $\mu$ g/mL respective ATV, OLM and LOS were directly infused into the ESI mass spectrometer. The analytes ATV, OLM and LOS observed as prominent protonated molecular ions [M+H]<sup>+</sup> in positive ion mode and showed the ions at m/z 559, 559 and 423 respectively. The

[M+H]<sup>+</sup> ions of respective analytes were subjected to collision induced dissociation (CID) for MS<sup>2</sup> experiment at average collision energy of 30%. Later the collision energies were optimized for each analyte to obtain the intense fragment ions. The molecules undergone fragmentation and yield the following fragment ions at m/z 440, 541 and 405 for ATV, OLM and LOS respectively. The structures of these drugs and their putative fragments were also confirmed through exactive orbitrap high resolution mass spectrometer (Thermo Scientific, CA, USA).

### Method validation

The specificity of this method was confirmed by comparing chromatograms of blank plasma, spiked plasma with analytes at a

concentration of 1ng/mL and plasma sample obtained after 1 h of oral administration shown in Fig. 2. ATV, OLM and LOS (IS) in LC-ESI experiment were well separated under the described chromatographic conditions and not observed any endogenous matrix interfering peaks around their retention times.

The calibration curves of the analytes showed good linearity over the studied concentration range of 1-1000ng/mL for ATV and OLM with correlation coefficients ( $r^2$ ) were 0.9998 and 0.9997 respectively. The LODs and the LOQs were 0.15ng/mL and 1.0ng/mL for ATV and 0.21ng/mL and 1.0ng/mL for OLM respectively.

Intra-day and inter-day accuracy of the method was found to be 90-103 % for atorvastatin and 90 - 105 % for olmesartan respectively with the precision values was less than 10 % in terms of % RSD. The mean percentage recoveries of ATV and OLM were 70 to 96 % and 74 to 95 % respectively. The results indicate the sample preparation technique is suitable for extracting the studied drugs from rat plasma. The stability studies of these drugs were performed at three QC concentration (low, medium and high) levels in six replicates ( $n=6$ ). The predicted concentrations for each analyte deviated within  $\pm 10$  % of nominal concentrations after storage of plasma samples at room temperature for 24 h, three freeze-thaw cycles and in auto sampler for 12 h at 4°C. The mean accuracies were found to be more than 90 % with relative standard deviations less than 10 %.

Stability of both the drugs was performed for three QC concentrations. The recoveries of atorvastatin and olmesartan in all stability studies were found to be more than 90 % with less than 5 % precision (% RSD).

System suitability parameters such as retention time, tailing factor and theoretical plates were taken into consideration. The system suitability was done by six replicate concentrations of 500ng/mL for atorvastatin and olmesartan. The acceptance criterion was  $\pm 2\%$  for the percent relative standard deviation (%RSD) for peak area retention time for both the drugs. The % RSD of peak area and retention time for both the drugs were within 2% indicating the suitability of system. The efficiency of the column was determined by considering parameters like tailing factor and theoretical plates. All the values for the system suitability parameters were within acceptable range.

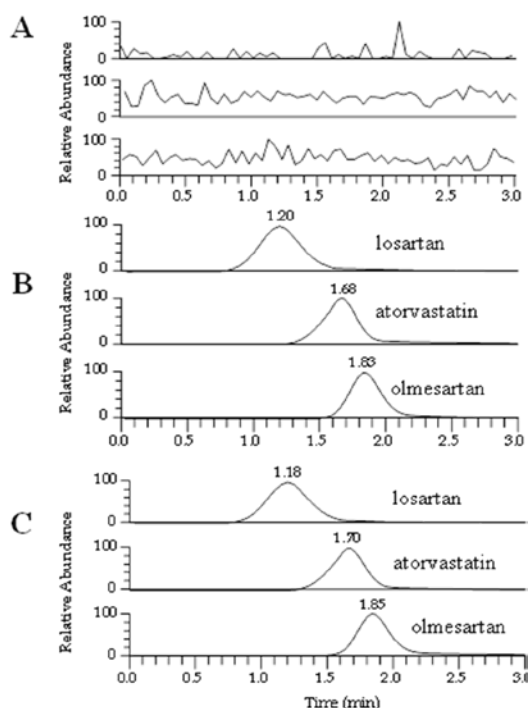


Fig. 2: LC-ESI-MS/MS extracted ion chromatograms of (A) blank plasma, (B) spiked plasma at 1 ng/mL, and (C) plasma extracted from rats after 1 h of oral administration.

### Application to the pharmacokinetic study

The established method was applied to analysis of plasma samples after an oral administration of 1mg kg<sup>-1</sup> and 2mg kg<sup>-1</sup> of atorvastatin and olmesartan simultaneously in rats. The concentrations and mean plasma concentration - time profile of atorvastatin and olmesartan are shown in Fig.3. A non-compartmental model was used to estimate the pharmacokinetic parameters of atorvastatin and olmesartan in rat plasma. After administration of the three drugs, peak plasma concentrations ( $C_{max}$ ) were reached at  $0.90 \pm 0.22$  h and  $0.70 \pm 0.27$  h ( $T_{max}$ ) with an elimination half-life ( $t_{1/2}$ ) of  $3.89 \pm 0.40$  h and  $3.56 \pm 0.45$  h for atorvastatin and olmesartan respectively. Thus the developed method was successfully applied for pharmacokinetic study in rats after administration of atorvastatin and olmesartan in combination.

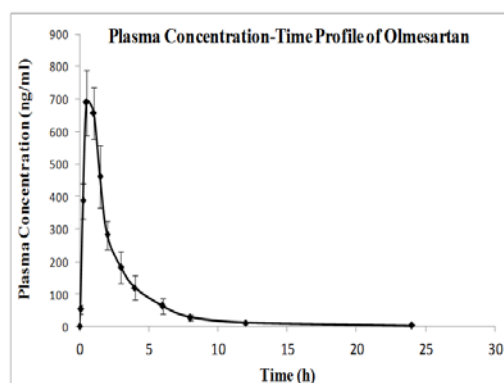
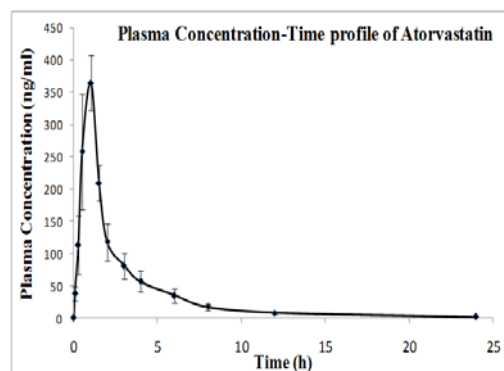


Fig. 3: Mean plasma concentration-time profile of atorvastatin and olmesartan in rat plasma after oral administration. Each point represents the mean  $\pm$  SD ( $n = 6$ ).

### CONCLUSION

A simple and reliable LC-ESI-MS/MS assay was developed for quantitative evaluation of ATV and OLM simultaneously in rat plasma. Single step protein precipitation was used to extract analytes from rat plasma. The major advantages of the assay are simple sample preparation and short run time. The obtained LODs and LOQs of the drugs were adequate to perform the pharmacokinetic study in rat plasma. Based on the results we can conclude that the present method is not only suitable for assessing the pharmacokinetics but also applicable for clinical pharmacokinetics.

### ACKNOWLEDGEMENT

Authors would like to thank Director, IICT, Hyderabad, India and Aurobindo Pharma Ltd., Hyderabad for providing gift samples. The author, Mr. Shankar Ganesh, would like to thank JNTUK, Kakinada for their kind support. The authors, Mr. Shankar Ganesh and Mr. Pragney Deme, would also like to thank CSIR, New Delhi for awarding Senior Research Fellow.

**CONFLICT OF INTERESTS**

Declared None

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**ABBREVIATIONS**

ATR, Atorvastatin calcium; OLM, Olmesartan medoxomil; LOS, Losartan; MRM, Multiple Reaction Monitoring;