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Research Article

DEVELOPMENT AND VALIDATION OF AN RP-HPLC METHOD FOR THE DETERMINATON OF OLMESARTAN IN HUMAN PLASMA

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ABSTRACT

A highly sensitive, rapid and accurate HPLC method was developed for the determination of Olmesartan in human plasma using eprosartan as internal standard. 70:30 % 0f methyl- t – butyl ether and dichloromethane was used as solvent in the liquid – liquid extraction process. A non-polar Phenomenex Prodigy ODS-2, C_{18} column (150 X 4.6 mm id) was chosen as the stationary phase and a binary mixture of 10 mM mixed phosphate buffer (pH 3.0 ± 0.05) and methanol in a ratio of 40:60 v/v was used as mobile phase. The drug and the internal standard were eluted under isocratic condition at a flow rate of 1.0 mL/min of the mobile phase and the wavelength of detection is 255 nm (UV – detector). The injection volume is 20 µL and the runtime of the method is 11 minutes. A good linearity was observed for the method in the range of 59.94 to 5514.48 ng/mL. The recovery of olmesartan is 59.91 % with a standard deviation of 0.535 and recovery of internal standard was 73.84 %. The LOD of Olmesartan is 59.94 ng/mL. Matrix effects were not observed.

Keywords: Antihypertensive, Isocratic elution, Olmesartan, HPLC etc.

INTRODUCTION

Olmesartan medoxomil [OLM], (Molecular formula is $C_{23}H_{24}N_2O_4S$), (5-methyl-2-oxo-1,3-dioxolen-4-yl),methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl)-phenyl]phenyl}methyl imidazol-5-carboxylate) [Figure1(A)] is a potent antihypertensive works by blocking the binding of angiotensin II to the AT1 receptors in vascular muscle.[1,2] It is used in the treatment of hypertension in the United States, Japan, and European countries. A number of analytical and bioanalytical methods were reported for the determination of Olmesartan alone and in combination with several other drugs.[3-16] The earlier methods on HPLC based bioanlaytical estimation of Olmesartan resulted in low sensitivity and high noise in the base line. The present investigation aims at developing a more efficient, rapid, sensitive and simple method with suitable chromatographic conditions for the determination of Olmesartan using Eprosartan as an internal standard(IS) in human plasma. The present method was developed as per FDA Guidelines.[17]

MATERIALS AND METHODS

Solvents and chemicals

The reference sample of OLMESARTAN (Purity 99.90 % w/w) was gifted by M/s Glenmark Pharma Ltd., Mumbai. EPROSARTAN (used as an internal standard, Purity 99.80 % w/w) is gifted by M/s Hetero Drugs Pvt Ltd. The chemicals like methyl-t-butyl ether, dichloromethane and methanol of HPLC Grade and Potassium Dihydrogen Phosphate and Orthophosphoric acid (GR grade) were used. Deionized water was processed through a Milli-Q water purification

system. All other chemicals and reagents were of analytical grade.

Chromatographic System

The chromatographic system consisted of Shimadzu HPLC equipment comprising of binary LC 10AT vp pumps, SIL 10AD vp autosampler, CTO 10A vp column oven, SPD 10Avp UV-Visible detector. All the components of the system are controlled using, SCL-10A vp System controller. Data acquisition was done using LC Solutions ver.1.23 SP 1 software. The detector is set at a wavelength 255nm.Chromatographic separation were accomplished using a Phenomenex C_{18} column (150 X 4.6 mm id, 5 µm, ODS 2). The mobile phase consists of a mixture of 40 parts of 10mM mixed phosphate buffer and 60 parts of methanol operated using a binary HPLC. The mobile phase was pumped isocractically at a flow rate of 1.0ml /min during analysis at ambient temperature. The rinsing solution consists of mixture of 50:50 % v/v of acetonitrite: Milli-Q –Water.

Preparation of Standard Solutions

The stock solution of olmesartan was prepared in methanol such that the final concentration is apprtoximately 2.0 mg/ml. Stock solution of eprosartan containing 1.0 mg/ml was prepared in HPLC grade methanol. The stock solutions were stored below 10^0C in a refrigerator and these solutions were stable for at least two weeks. Aqueous stock dilution of Olmesartan was prepared in diluent solution (mixture of 70:30%v/v of methanol: HPLC grade water). About 1.36 grams of potassium dihydrogen phosphate was weighed accurately and transferred into a 1000 mL reagent bottle and dissolved in 200mL of Milli-Q water. The above solution was sonicated for 5 min and its pH was adjusted to (3.0 ± 0.05) with ortho phosphoric acid solution and made up to volume with Milli-Q water to obtain 10 mM mixed phosphate buffer (pH 3.0 ± 0.05). The solution was stored at room temperature and used within 3 days from the date of preparation.

Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K_2EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no Olmesartan added), 59.94, 119.88, 799.20, 1598.40, 2397.60, 3596.40, 5034.96 and 5514.48 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.7 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 63.94, 199.80, 2597.40 and 4795.20ng/ml and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

Extraction Procedure

Liquid-Liquid Extraction process was involved in the extraction of plasma samples. The stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature for processing. An aliquot of 500 µL was then transferred into a pre-labelled 2.0 mL polypropylene centrifuge tubes. 25 µL of internal standard dilution (101.80 µg/mL) was then added and mixed. To extract the drug and internal standard, 1.2 mL of extraction solvent was then added. The samples were allowed to mix for 20 minutes by placing them on a reciprocating shaker. Centrifugation of samples was then done at 5000 rpm for 5 minutes at 4^oC. The resulting supernatant liquid of 1 mL was then transferred into prelabelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40^oC. In an aliquot of 200 µL of mobile phase, the dried residue was dissolved and transferred into shell vials. 20 µL of the sample was then injected into the system for analysis. Throughout the analysis, the auto sampler temperature was maintained at 4^oC. The column temperature was maintained at ambient temperature.

Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, freeze–thaw, and auto sampler stability. The initial assay was fully validated for Olmesartan analysis in human plasma according to FDA guidelines.

Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K_2EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

Calibration curve
Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of Olmesartan(OLM) in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for Olmesartan were represented by the plots of the peak-area ratio (OLM/IS) versus the nominal concentration of the OLM in calibration standards. The regression line was generated using 1/concentration ^{2} factor as the mathematical model of best fit. OLM concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve (figure 5).

Accuracy and Precision
Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates. The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

Accuracy (%) = mean observed concentration/nominal concentration × 100
The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:
CV (%) =standard deviation/mean observed concentration× 100

Stability Studies
Autosampler, and freeze–thaw stability of LAN was determined at low, medium and high QC concentrations. To determine the impact of freeze–thaw cycles on Olmesartan concentration, samples were allowed to undergo 3 freeze (−70^{o}C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the OLM concentrations were analyzed in triplicates and compared to the control sample that had been stored at −70^{o}C. Autosampler stability of extracted samples was determined by comparing OLM concentration in freshly prepared samples and samples kept in autosampler at 4^{o}C for 24 h.

Recovery
Recovery was determined by comparing the area under the curve of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of OLM as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 101.80 µg/mL.

Data analysis
HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of Olmesartan were constructed using a 1/concentration^2 weighted linear regression of the peak area ratio versus Olmesartan concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

RESULTS AND DISCUSSION

Method Development
The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of OLM in Human Plasma. Since both Olmesartan and internal standard are highly non-polar[18, 19] we employed the usage of liquid-liquid extraction process with a mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane. To get a better response the pH of the mobile phase is set to the acidic side. During our observation, a pH value around 3 resulted in better peak shape for the internal standard while that of the drug is not acceptable. Also, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica[20] Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore experiments were performed using Potassium Dihydrogen phosphate in a limited pH range of 3.0 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of 3.0 ± 0.05 gave maximum response for the analyte at 255 nm. A similar response was observed with the usage of 10mM mixed phosphate buffer. Therefore the final mobile phase consisted of 60: 40 % v/v methanol and 10mM mixed phosphate buffer. The run time of analysis is higher when a longer normal phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the experiment is performed using a shorter column (50 X 4.6 mm id). However better

resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C18 150 X 4.6 cm 5 µm column.
The flow rate of the method is 1.0 ml/min. The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard. There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 60:40 v/v methanol and 10mM mixed phosphate buffer leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused Eprosartan to elute early. A mobile phase containing aqueous portion greater than 60 % led to very late elution and very poor peak shape for Olmesartan. The peaks were also broad with unacceptable asymmetry factor.
Extraction methods were initially attempted using Protein precipitation technique. Precipitation technique was adopted using Acetonitrile and or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma: Organic solvents. The recovery of the Olmesartan is poor while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.
Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, we have done the final analysis using liquid-liquid extraction (LLE).SPE methods although render a neat sample for final analysis, polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique.
Various solvents such as Ethyl acetate, Diethyl Ether, 100 % t-butyl methyl ether and combinations of t-butyl methyl ether and Dichloromethane were used for extraction. The recovery of Olmesartan and internal standard was poor when Ethyl acetate or Diethyl ether was used individually. The highest recovery from the plasma samples is obtained with a 70: 30 % v/v of t-butyl methyl ether: Dichloromethane.

Detection and chromatography
The typical chromatogram in figure 2 was obtained when human plasma was not spiked with sample and IS. The chromatogram in figure 3 represents a peak corresponding to IS alone when human plasma was spiked with IS and not with sample. Figure 4 was the chromatogram of human plasma spiked with IS and OLM in its ULOQ. The retention times for Olmesartan and IS were 5.15 and 2.87 minutes respectively.

Method validation
Selectivity
The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for Olmesartan in any of the six independent blank plasma extracts evaluated.

Calibration curves
A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV for the retention times of Olmesartan and internal standards is less than 2 %. The results are tabulated in Table 1. Calibration curve standards were represented in Table 2. Calibration curves for Olmesartan in human plasma were fitted by weighted 1/concentration ^{2} quadratic regression, with the r^2 values of >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma is presented in Table 3 demonstrating that measured concentration is within ± 15% of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r^2 value is depicted in Figure – 5.

Accuracy and Precision
A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in Table 4. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines.

Limit of Detection and Limit of Quantification
LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 25 ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard

deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (50 ng/mL) met the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of OLM in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.

Carryover test

A critical issue with the analysis of many drugs is their tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

Stability studies

The results of short-term, long term and freeze–thaw stability are presented in Table 5. Determination of OLM stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the OLM concentration.

Recovery

Percentage recovery of OLM was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of OLM from plasma spiked samples at LQC, MQC and HQC levels was 69.018%, 60.26 % and 59.29 % respectively. The overall recovery is 59.91 % with a Coefficient of variation of 0.89 %, respectively. IS recovery at 101.80 µg/mL was 73.84 % with a Coefficient of variation of 3.31 %.

CONCLUSION

A HPLC method was developed and validated for the determination of Olmesartan in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of 70:30 % v/v of t-butyl methyl ether and dichloromethane. LLE method is usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500 µL). There is no carryover effect. Due to the LLE method of extraction, baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of 59.94 ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 11.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of OLM in human subjects. This method has been successfully applied to analyze Olmesartan concentrations in human plasma. Future work is aimed to carry out quantitative determination of the same and several other classes of drugs in human plasma.

ACKNOWLEDGEMENTS

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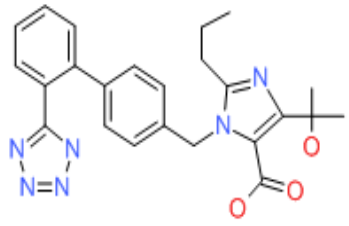
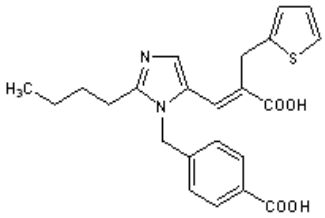


A) Structure of Olmesartan



B) Structure of Eprosartan

Fig. 1: Structure of Olmesartan (A) and Eprosartan (B)

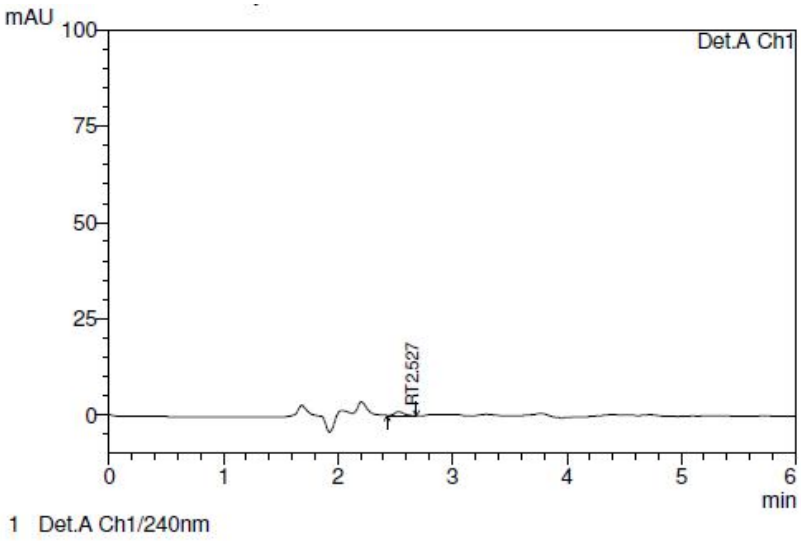


Fig. 2: A chromatogram of the extracted blank plasma sample

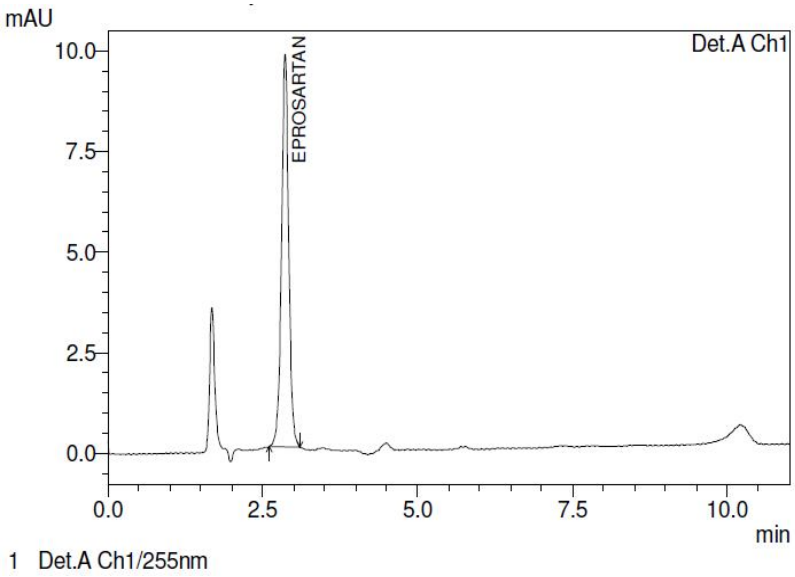


Fig. 3: A Chromatogram of Eprosartan (IS) spiked blank plasma

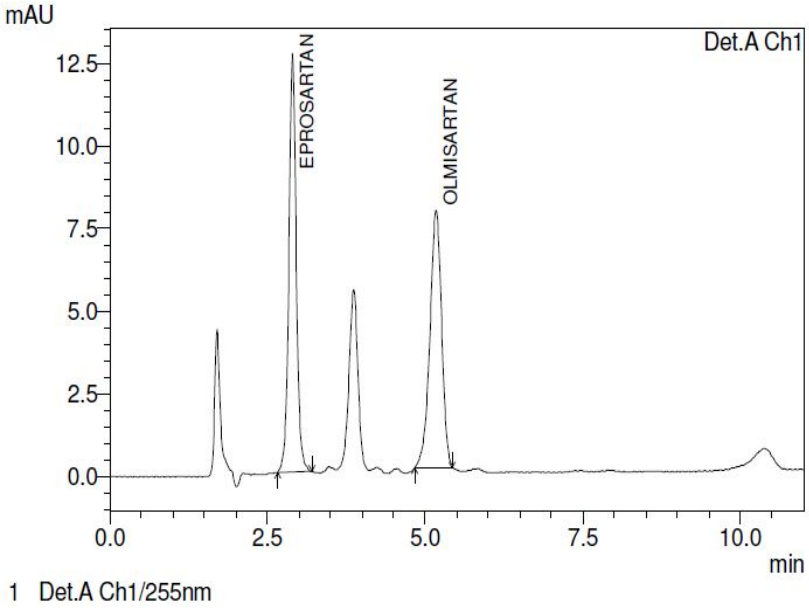


Fig. 4: A chromatogram of Olmesartan (drug) and Eprosartan (IS) spiked blank plasma sample

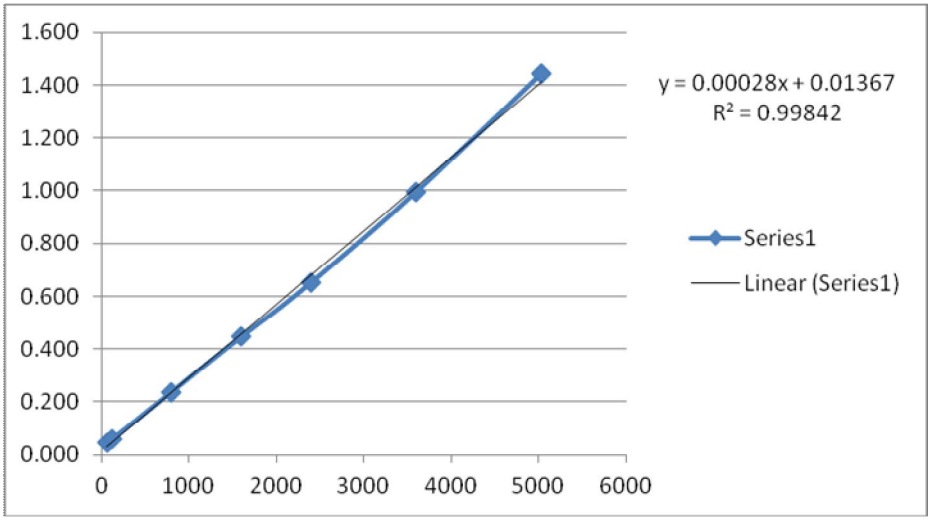


Fig. 5: Calibration curve for Olmesartan

Table 1: System Suitability Study

	Eprosartan Internal Standard (101.80 µg/mL)		Olmesartan (4795.20ng/ml)	
	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
Mean (n = 6)	2.87	151055	5.15	239788
S. D.	0.01	32488	0.01	30977
% CV	0.64	2.44	0.20	3.21

Table 2: Calibration Curve Details

Spiked Plasma Concentration (ng/mL)	Concentration measured(ng/mL) mean	SD	CV (%) (*n* = 3)	Accuracy %
59.94	59.838	1.6624	2.78	99.83
119.88	122.843	6.1484	5.01	102.47
799.20	824.437	24.7194	3.00	103.16
1598.40	1587.207	17.6567	1.11	99.30
2397.60	2332.276	49.052	2.10	97.28
3596.40	3599.182	93.4857	2.60	100.08
5034.96	4805.309	210.944	4.39	95.44
5514.48	5847.053	46.717	0.80	106.03

Table 3: Results of Regression Analysis of the Linearity Data

Linearity parameters	Mean ± SD (n = 6)
Slope	0.000280
Intercept	0.001760
Correlation coefficient (r)	0.99443

Table 4: Intra and Inter Day Accuracy and Precision of HPLC Assay

	Nominal Concentration (ng/mL)			
	63.94	199.80	2597.40	4795.20
DAY 1				
Mean	63.49	197.35	2570.93	4651.55
S.D.	2.449	16.030	83.773	181.237
% CV	3.86	8.12	3.26	3.90
DAY 2				
Mean	63.79	198.16	2583.33	4673.38
S.D.	1.55	13.26	61.50	123.67
% CV	2.43	6.99	2.38	2.65
DAY 3				
Mean	63.33	196.80	2564.84	4640.50
S.D.	1.687	14.112	61.430	146.651
% CV	2.66	7.17	2.40	3.16

Each mean value is the result of triplicate analysis

Table 5: Short Term, Long Term and Freeze Thaw Stability of Olmesartan

	Nominal Concentration (ng/mL)	
	199.80 (LQC)	**4795.20 (HQC)**
Bench Top Stability (9 Hours)		
Mean Accuracy (%)	97.32	96.95
S.D.	15.65	182.39
% CV	7.74	3.81
Long-term stability (20 Days)		
Mean Accuracy (%)	96.78	98.80
S.D.	14.29	243.75
% CV	7.39	5.14
Freeze – Thaw stability (3 Cycles)		
Mean Accuracy (%)	97.94	97.54
S.D.	13.45	125.90
% CV	6.69	2.65

Each mean value is the result of triplicate analysis

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