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BIOANALYTICAL METHOD FOR QUANTITATIVE DETERMINATION OF OLMESARTAN IN HUMAN PLASMA BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Balaji Kodali*[1], Surya Prakasarao Kovvasu[2], Priyanka Kunamaneni[2], Kalyan Chakravarthy Janjanam[3] and Pappula Nagaraju[4]

[1]University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur- 522510, Andhra Pradesh, India.

[2]College of Pharmacy, Western University of Health Sciences, Pomona, CA 91766, United States.

[3]University College of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad–500 085, Telangana, India.

[4]Department of Pharmaceutical Analysis, Hindu College of Pharmacy, Guntur-522002, Andhra Pradesh, India.

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***Corresponding Author**
Balaji Kodali
University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur- 522510, Andhra Pradesh, India.

ABSTRACT

A selective, rapid and highly sensitive liquid chromatography–tandem mass spectrometry (LC -MS/MS) method was developed and validated for the quantification of olmesartan in human plasma with valsartan as internal standard (IS), sample pretreatment followed by a simple solid phase extraction using HLB cartridges. The analysis was carried out on a Chromolith, speed ROD column (50 mm×4.6 mm, 5 µ) with flow rate of 0.6 mL/min. The mobile phase contains 0.1% formic acid buffer–acetonitrile in the ratio of 30:70 v/v. The detection was performed on a triple quadrupole mass spectrometer operated with MRM (multiple reaction monitoring) mode. Linear calibration curves were obtained in the concentration range of 25 to 1800 ng/mL. The intra- and inter-day precision (%RSD or CV.) values were below 15% and accuracy (RE) was −10.09% to 5.69% at all QC levels.

INTRODUCTION

Olmesartan belongs to the class of medicines called angiotensin II receptor antagonists, which are used to treat the patients with high blood pressure. Olmesartan works by blocking the binding of angiotensin II to the AT1 receptors in vascular muscle; it is therefore independent of angiotensin II synthesis pathways, unlike ACE inhibitors. By blocking binding rather than synthesis of angiotensin II, olmesartan inhibits the negative regulatory feedback on renin secretion. Because of this blockage, olmesartan restricts vasoconstriction and the secretion of aldosterone. This reduces blood pressure by causing vasodilation and reducing peripheral resistance.[1-3] The previously published LC–MS and LC–MS–MS methods[4-9] for the analysis of olmesartan in biological fluids such as human plasma and urine were suffering from lack of sensitivity, use of more sample volume, longer chromatographic run time. As per literature,[10-21] an efficient bioanalytical method should rapid, simple and consumes less sample volume for analysis. Also, it should be specific and selective to avoid possible inferences at mass transitions of analyte and the internal standard.

In view of above, we have developed and validated a simple, reliable and rapid LC-MS/MS method for the determination olmesartan in human plasma. The present method is having run time of 2 min and utilized very low plasma of 200 µL for sample preparation. Also, the sample extraction by solid phase technique was very efficient in obtaining high recovery for analyte with no or minimal matrix effect.

EXPERIMENTAL

Reagents and chemicals

Working standards of olmesartan (99.5%) and valsartan (99%) was obtained from Sigma Aldrich (Bangalore, India). Chemical structures of olmesartan and valsartan were shown in Fig. 1(A) & 1(B). LC-MS grade methanol was obtained from J.T. Baker (Phillipsburg, USA). Analytical grade ammonium formate and formic acid were procured from Merck Ltd (Mumbai, India). Blank human plasma was obtained from Deccan's Pathological Lab's (Hyderabad, India).

Instrument conditions

Liquid chromatography

The chromatography was performed on HPLC system (Shimadzu, Japan) with cooling autosampler and column oven which enables temperature control for analytical column. A Chromolith, speed ROD column (50 mm×4.6 mm, 5 µ) maintained at 40 °C was used to

chromatograph the analyte and internal standard. The mobile phase consists of 30% of 0.1% formic acid buffer and 70% acetonitrile (v/v) pumped at flow of 0.6 mL/min. The auto-sampler temperature was maintained at 10 °C and 5 µL of the reconstituted sample was injected. The total chromatographic run time was 2.5 min.

Mass spectrometry

The detection was carried out on tandem quadrupole mass spectrometer (API 4000, Applied Biosystems/MDS Sciex, USA). The turbo ion spray source was operated under optimized conditions mentioned as follows: ionization mode, positive; curtain gas, 20 psi; nebulizer gas, 35 psi; turbo gas, 55 psi; ion spray voltage, 5500 V; temperature, 450 °C. The molecular ions of Olmesartan and valsartan were monitored with voltage values of declustering potential (DP-52 V and 51 V), entrance potential (EP-10V and 10V) respectively. The product fragments were monitored by applying collision energy (CE) of 25 V and 20 V respectively. The collision-activated dissociation (CAD) gas at a pressure of 8 psi was set on the instrument. Multiple reaction monitoring (MRM) mode was employed for the quantification: m/z 447.1 → 207.0 for olmesartan and m/z 436.4 → 235.0 for valsartan. Chromatographic peak area was automatically integrated using analyst software, version 1.4.2.

Preparation of standards and quality control samples

Standard stock solutions of olmesartan and IS were prepared in methanol at a concentration of 1.0 mg/mL. The internal standard working solution was diluted with 60% methanol to get a concentration of 6 µg/mL. The olmesartan solution was then serially diluted with 60% methanol to provide working standard solutions of desired concentrations. All the solutions were stored at below 10 °C. Calibration standards were prepared by spiking (2%) the working dilutions to get olmesartan standard plasma samples of 25, 50, 100, 200, 400, 600, 900, 1400, 1800 ng/mL. Calibration standards and quality control samples (QCs) were prepared freshly. The prepared QC concentrations for lower limit of quantification, low, middle and high level were 25, 75, 850, 1300 ng/mL respectively. The standards and quality controls were extracted on each day analysis as per procedure described below.

Plasma sample preparation

200 µL of blank plasma and 25 µL of internal standard working solution was vertex mixed for proper mixing. The samples were subjected to solid phase extraction technique, includes conditioning and equilibration of cartridge (Oasis HLB, 30 mg/1 cc) with 2 mL of methanol and 2 mL of milli-Q water followed by loading the prepared plasma sample. Washing step

includes 2 mL of milli-Q water followed by elution with 2 mL of methanol and evaporated to dryness under nitrogen at 50 °C. The residues were dissolved in 500 µL of reconstitution solution (mobile phase) by vertexing for 40 seconds, then transferred into vials, and 5 µL of sample was injected for LC–MS/MS analysis.

Method validation

Validation of the method included the assessment of calibration curve performance, accuracy and precision of the method (determined by QC performance), stability of the analytes at various test conditions, specificity, recovery and matrix effect.

Selectivity

During the validation, blank plasma samples from (8) different lots were evaluated. During the selectivity run, an LLOQ standard was extracted and injected. The responses for the blank plasma from the eight (8) different lots were compared with the response of LLOQ standard. The method is also validated for specificity; specificity is the ability of the bio-analytical method to measure and differentiate the analytes in the presence of other components that may be expected to be present. In this experiment, interference at analyte retention time due to IS and vice versa in six screened blank plasma lots was evaluated.

Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by making serial dilutions of the working stock and assaying standard plasma samples at nine concentrations of olmesartan ranging from 25–1800 ng/mL. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of olmesartan/IS versus the nominal concentration (x). The calibration curves were constructed by weighted ($1/X^2$) least square linear regression. The lower limit of quantification should at least have 5 times more response compared to interference in blank plasma. The validation of LLOQ was conducted in at least six different batches of blank plasma. The results should be acceptable for accuracy and precision.

Precision and accuracy

For determining the intra-day accuracy and precision, a replicate analysis of QC plasma samples of olmesartan was performed on the same day. The run consisted of a calibration curve and six replicates of each lower limit of quantification, low, mid, and high quality control samples. The inter-day accuracy and precision were assessed by analysis of three

batches on different days. The precision was expressed as the relative standard deviation (% RSD) and the accuracy as the relative error (RE).

Extraction recovery and matrix effect

The extraction recovery of olmesartan was determined by comparing the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. As per guidance of USFDA,[22] recovery experiments should be performed at three concentrations of 75, 850, 1300 ng/mL, each with 5 replicates. To evaluate the effect of matrix on the ionization of analyte, i.e. the potential ion suppression or enhancement, olmesartan at two concentration levels was added to the post spiked extract of blank plasma. The corresponding peak areas were compared with those of the olmesartan standard solutions. The calculated IS normalized matrix factor values were should be within 0.85-1.15.

Stability

Freeze -thaw stability

The effect of freeze - thaw cycles on the stability of analyte in plasma was determined by subjecting six aliquots of low and high QC samples to four freeze–thaw cycles. After completion of four cycles, the samples were analyzed and the back calculated concentrations were compared with the nominal values.

Long-term stability

Six aliquots of QC samples at low and high concentration were stored at −70 °C and −20 °C for 30 days. Then, the samples were processed and analyzed against the fresh calibration curve and the back calculated concentrations were compared with the nominal values.

Bench top stability

Six aliquots of QC samples at low and high concentration were kept at ambient temperature (25 °C) for 12 h to determine the bench top stability of olmesartan in human plasma. Then the samples were processed and analyzed, the concentrations obtained were compared with the nominal values.

Post-preparation stability

To estimate the stability of olmesartan in the prepared samples, six aliquots of processed QC samples at low and high concentration were kept in autosampler maintained at 10 °C for

about 24 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values.

Stock solution stability

The stock solution stability of olmesartan and IS was assessed by comparing mean response of stability solutions left at 2-10 °C for 30 days was compared with freshly prepared stock solutions.

RESULTS AND DISCUSSION

Optimization of the chromatographic separation and MS/MS working conditions

The separation and ionization of olmesartan and IS were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. Practically, different ratios (50:50, 40:60, 30:70 and 20:80 v/v) of water/acetonitrile were tried as mobile phase. Finally, 30% water and 70% acetonitrile (v/v) in mobile phase was believed to be suitable in view of retention time and peak shape of analyte. Formic acid was employed to increase the ionic strength in mobile phase. It was found that a mixture of 0.1 % formic acid buffer– water/acetonitrile could preferably improve peak shape and sensitivity, so adopted as mobile phase. The selection of MRM transitions and associated acquisition parameters (collision energy) were evaluated for best response under positive mode by infusing a standard solution, via a syringe pump along with the mobile phase. The corresponding full-scan MS/MS spectra for olmesartan and valsartan are shown in Fig. 2 (A&B). As MRM technique provides inherent selectivity and sensitivity, hence we decided to apply MRM procedure for the present study. The very narrow chromatographic peaks produced indicate an increase in chromatographic efficiency. Both olmesartan and IS were rapidly eluted with retention times less than 2.0 min (Fig. 3). The shorter analysis time and reproducibility of the results makes the method suitable for high sample throughput in bioanalysis.

Selection of IS

The best internal standard in LC–MS assay is a deuterated form of the analyte. In our laboratory, no deuterated compound was available, therefore, a compound having almost equivalent physico-chemical properties of the analyte was considered. In LC–MS/MS the IS should mimic the analyte behavior in any sample preparation steps. Valsartan was chosen as the internal standard for the assay because of its similarity in structure, retention time and ionization properties as that of olmesartan.

Method validation

Selectivity

Selectivity was assessed by comparing the chromatograms of eight different batches of blank human plasma with the spiked plasma. As shown in Fig. 3, no significant response (≤20% for the analyte response and ≤5% of the internal standard response) was observed at the retention time of the analytes and internal standard in the blank plasma as compared to the LLOQ standard and all plasma lots were found to be selective.

Linearity and LLOQ

The standard calibration curves for olmesartan were linear over the concentration range of 25–1800 ng/mL (r^2 > 0.99) by using weighted least square linear regression analysis with a weigh factor of $1/x^2$. A typical equation for the calibration curves was: y = 0.00106X+ 0.00363, r^2 = 0.9976. The lower limit of quantification for olmesartan was 25 ng/mL (S/N≥5) with 5 µL injected into the HPLC column with RE within ±20% and RSD lower than 20%.

Precision and accuracy

The data results for intra-day and inter-day precision and accuracy of the method are presented in Table 1. The intra- and inter-day precision was found to be less than 7.43%. The %RE was in the range of 1.62-10.09% across the four QC levels. The overall results indicated that method was reproducible and conform to the criteria required for the analysis of biological samples as per the guidance of USFDA bioanalytical method validation.

Extraction recovery and matrix effect

The extraction recoveries of olmesartan from human plasma were 72.70%, 75.25%, and 76.12% at concentration levels of 75, 850 and 1300 ng/mL, respectively, and the mean extraction recovery of IS was 80.02%. The calculated IS normalized matrix factor values were between 85% and 115%, which means that there was no significant effect of matrix on quantification of olmesartan.

Stability

The olmesartan in plasma was found to be stable at room temperature for 12 h, at 10 °C for 24 h, and at −70 °C and −20 °C for 30 days. The analyte was stable after four continues freeze and thaw cycles. The stability results were represented in Table 2. The stock solutions of olmesartan and IS were found to be stable after 27 days.

Table 1: The mean results of precision and accuracy for olmesartan.

Quality control Run	Concentration found Mean ± SD (ng/mL)	RSD (%)	RE
Intra–day (*n*=12)			
LLOQ	24.8 ± 1.4	5.81	5.83
LQC	70.7 ± 3.5	5.01	5.69
MQC	924.0 ± 32.6	3.53	8.71
HQC	1375.5 ± 43.7	3.18	5.81
Inter–day (*n*=18)			
LLOQ	24.6 ± 1.8	7.43	1.62
LQC	69.0 ± 4.0	5.80	7.97
MQC	935.8 ± 27.8	2.97	10.09
HQC	1370.2 ± 49.3	3.60	5.40
Nominal concentrations of LLOQ, LQC, MQC and HQC are 25, 75, 850 and 1300 ng/mL, respectively.			

Table 2: Stability data for olmesartan in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Stability (%)
Auto-sampler (24 h)	75	74.2 ± 2.5	3.33	98.93
	1300	1356.0 ± 55.6	4.10	104.31
Bench top (12 h)	75	72.7 ± 5.3	7.29	96.93
	1300	1291.8 ± 38.8	3.01	99.37
Freeze and thaw (4 Cycles)	75	76.8 ± 8.2	10.68	102.40
	1300	1310.8± 49.2	3.75	100.83
Long term (-20^{0}C) (30 days)	75	77.5 ± 5.8	7.48	103.33
	1300	1280.6 ± 65.2	5.09	98.51
Long term (-80^{0}C) (30 days)	75	72.5 ± 8.9	12.28	96.66
	1300	1276.2 ± 68.2	5.34	98.17

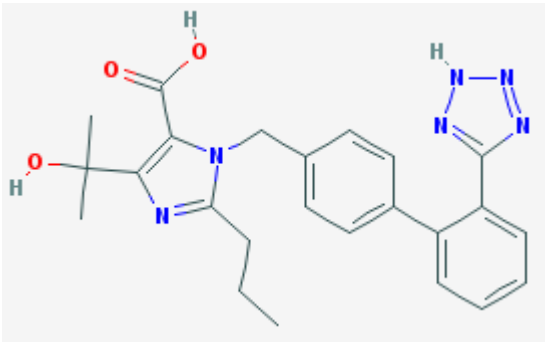

Figure 1(A): Chemical structure of olmesartan.

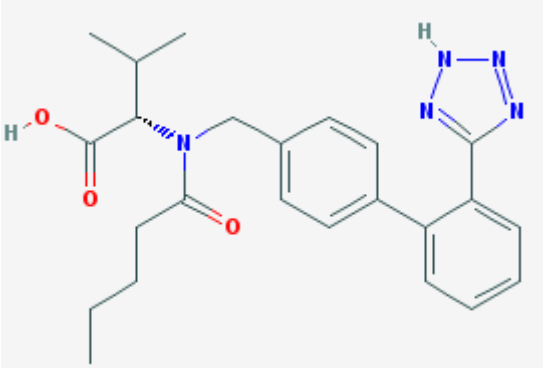

Figure 1(B): Chemical structure of valsartan.

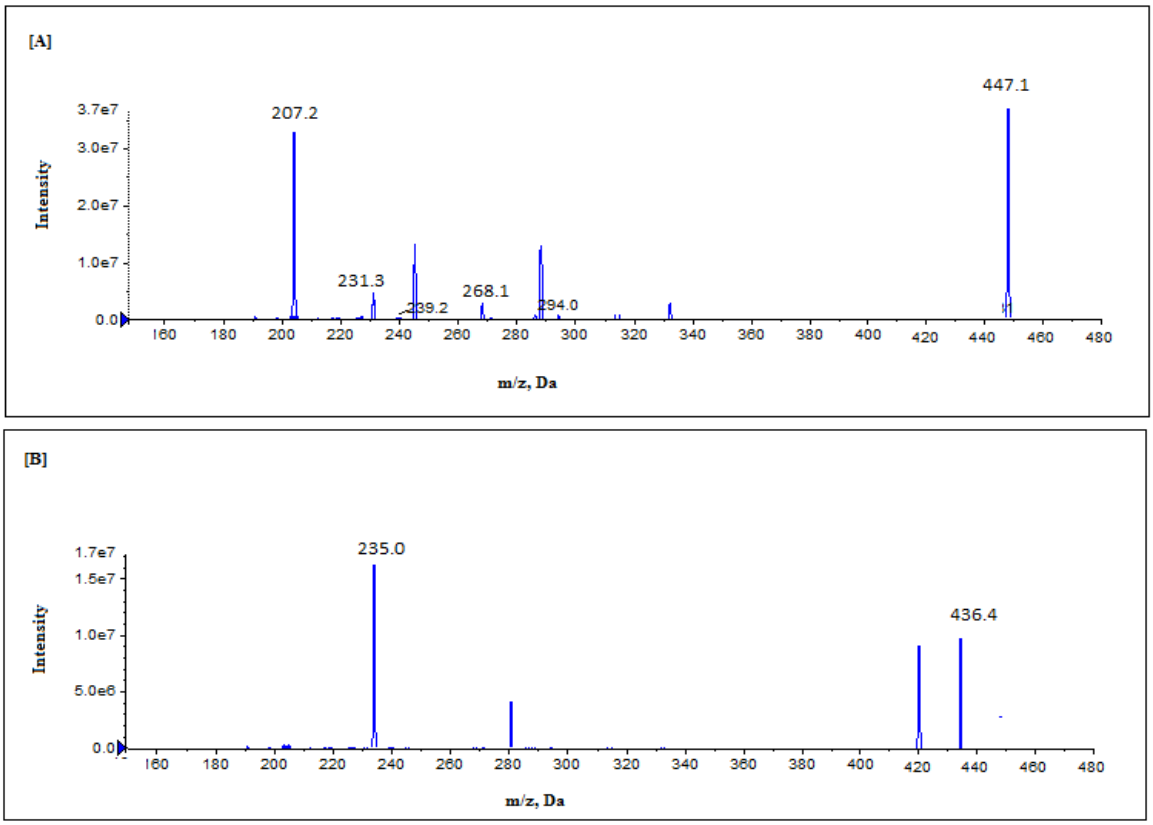

Figure 2: Mass spectra of olmesartan [A]; and valsartan [B].

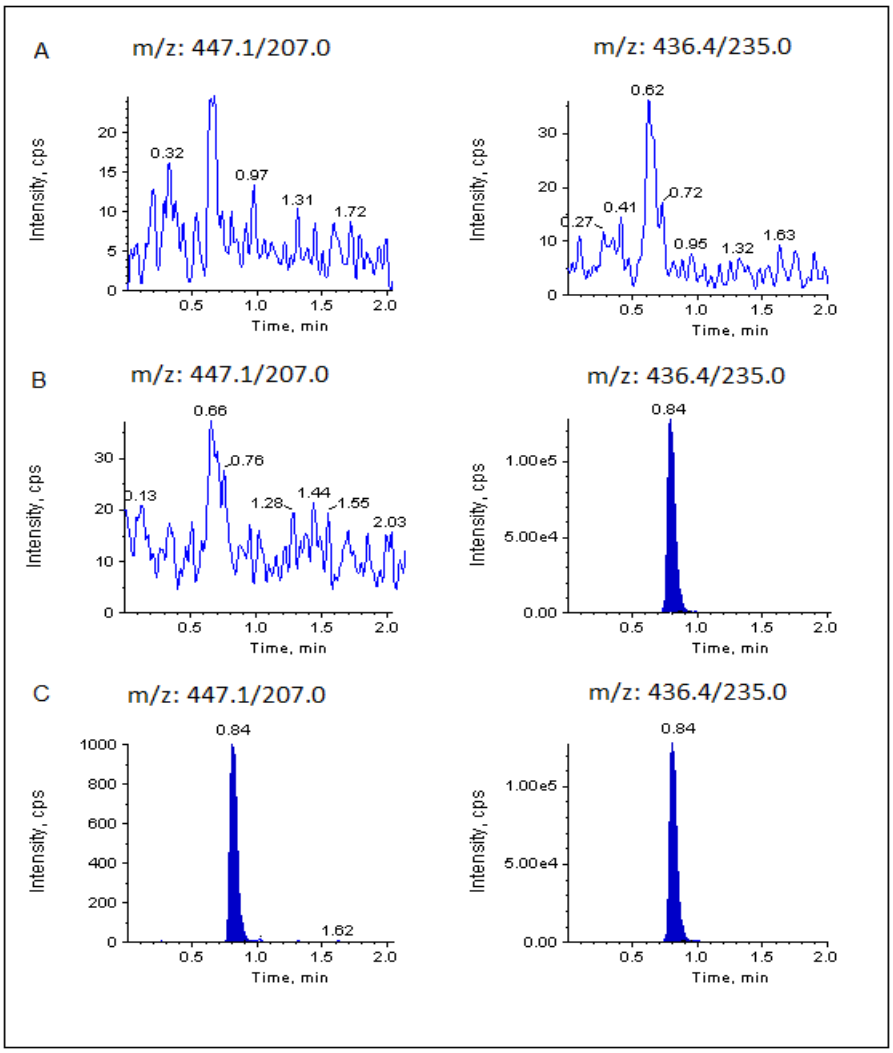

Figure 3: Representative chromatogram of [A] blank plasma; [B] blank plasma with internal standard; [C] LLOQ sample.

CONCLUSION

The highly sensitive liquid chromatography–tandem mass spectrometry (LC -MS/MS) method for the determination of olmesartan in human plasma has been established. The method was selective, specific and validated fully as per USFDA bioanalytical method validation guidelines. The method can be directly applied for determination of olmesartan concentration in patient or healthy volunteer samples from clinical studies.

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