



Research article

Bioanalytical RP-HPLC method development and validation for estimation of azelnidipine and Olmesartan medoxomil in human plasma

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ABSTRACT

An accurate, rapid and simple reversed-phase high performance liquid chromatography (RP-HPLC) bioanalytical method was developed for estimation of Azelnidipine (AZP) and Olmesartan medoxomil (OLM) in human plasma. The AZP, OLM and internal standard (IS) were extracted by liquid-liquid extraction technique. Chromatographic separation was accomplished using BDS Hypersil C18, 250 mm X 4.6 mm, 5 μ analytical column. The mobile phase consisted of Acetonitrile: Water, pH adjusted with ortho-phosphoric acid in the ratio 60:40 with flow rate of 1ml/min. Detection and quantification were performed by UV detector at 256 nm. The retention times were found to be 10.05 \pm 0.02, 4.3 \pm 0.02 and 6.9 \pm 0.02 for AZP, OLM and IS respectively. The linearity range was found to be 0.5 to 12 μ g/ml and 1 to 15 μ g/ml for AZP and OLM respectively. The method was validated as per CDER guideline, was found to be suitable for analysis in biological fluid.

Keywords: Azelnidipine, Olmesartan medoxomil, RP-HPLC, Bioanalytical method, Human plasma..

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INTRODUCTION

Azelnidipine (AZP) (Fig. 1) is a recently launched dihydropyridine calcium channel blocker having selective effect on L-type calcium channels. It has been approved in Japan recently for treatment of hypertension [1-3]. Olmesartan Medoxomil (OLM) (Fig. 2) is imidazole derivative prodrug with the antihypertensive property. OLM is converted to Olmesartan after hydrolysis and it binds to the AT1 receptor in vascular smooth muscle and competes with angiotensin II. This prevents vasoconstriction and decreases aldosterone production, which prevents aldosterone-stimulated retention of sodium and excretion of potassium. Thus, OLM is used to treat the hypertension and it relaxes blood vessels to allow the flow of blood more easily [4,5].

Figure 1: Structure of AZP

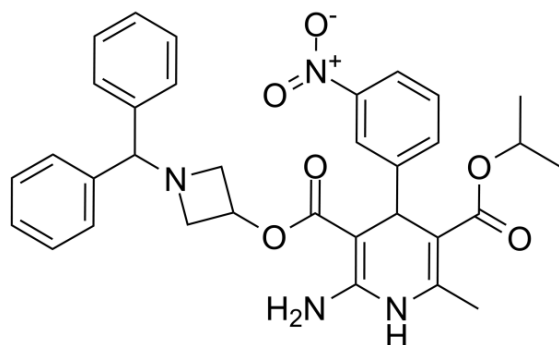
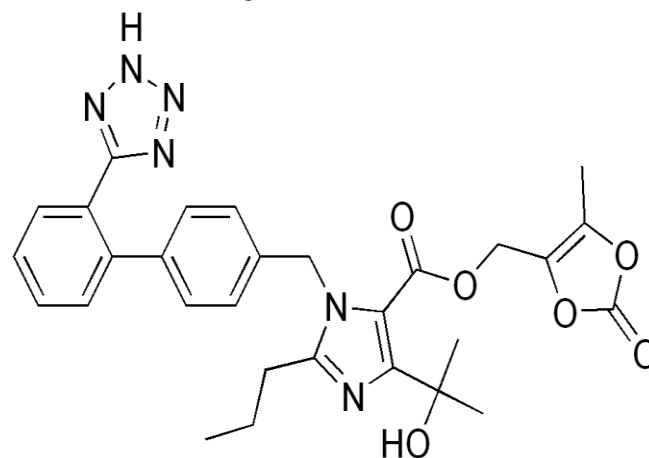


Figure 2: Structure of OLM



AZP/OLM combination is recently launched combination and is marketed in Japan as Rezaltas® by Daiichi Sankyo Company [6]. This combination of azelnidipine, a dihydropyridine calcium channel antagonist and Olmesartan medoxomil, an orally active angiotensin receptor antagonist, is proving to be better choice than other combinations used to treat hypertension [7,8]. The clinical studies on this combination revealed that it helps to reduce the possible side effects and control the heart rate with minimum or no

changes in normal heart rate^[9,10]. Hence, we targeted this novel combination for the analytical study.

Extensive literature search was done through various online sources like PubMed, Elsevier, and Web of Science etc. From the complete survey, some recent and relevant articles related to chromatographic methods for estimation of AZP/OLM have been studied. Stability-Indicating Reverse-Phase High Performance Liquid Chromatographic (RP-HPLC) method^[11] and rapid reversed phase Ultra-Fast liquid chromatography (UFLC) method for simultaneous determination of AZP and OLM in pharmaceutical dosage form has been reported with greater precision and accuracy^[12]. Ramesh et al., (2017) developed novel, rapid, specific and sensitive liquid chromatography tandem mass spectrometry (LCMS/MS) method for the simultaneous determination of AZP and OLM in human plasma. The mobile phase acetonitrile: 5mM ammonium formate pH-3.00 (80:20 V/V) with flow-rate of 1.0 mL/min, total run time of 3.0 minutes^[13].

Prabhakar et al., (2017) developed RP-HPLC method for AZP with retention time of 6.130 min in mobile phase methanol: water containing 0.1% glacial acetic acid (75:25)^[14]. Modi et al., (2016)^[15] and Gore et al., (2016)^[16] optimized mobile phase consisting of sodium dibasic phosphate buffer: acetonitrile: methanol (10:50:40) and methanol: water (80:20%v/v) respectively for estimation of AZP by RP-HPLC method. Ubale et al., (2021) reported estimation of AZP using mobile phase acetonitrile: water (90:10)^[17], while Bharti et al., (2016) demonstrated Reverse Phase Ultra performance Liquid Chromatography (RP-UPLC) technique for the analysis of OLM using mobile phase of 5mM ammonium acetate in water and acetonitrile^[18]. Chavda et al., published review on analytical methods for estimation of AZP and telmisartan^[19], while recently developed HPLC methods for AZP and telmisartan have also been reported^[20,21].

Literature review revealed that there are few analytical methods i.e., RP-HPLC, stability indicating RP-HPLC method established for estimation of AZP and OLM. Also, LC-MS methods were reported in human plasma, but the use of LC-MS method is less economic. Thus, the aim of present research study was to develop a simple, economic and robust RP-HPLC method for estimation of AZP and OLM in human plasma.

MATERIAL AND METHOD

Materials

AZP and OLM was provided as gift samples by Precise pharma and Intas pharma, Mumbai. Methanol and ortho-phosphoric acid HPLC grade were procured from Fisher Scientific, India. Acetonitrile HPLC grade was procured from SD Fine chem.Ltd,

Mumbai, India. Human plasma was collected from Arpan Blood Bank, Nashik. Agilent 1260HPLC model was used.

Selection of Solvent and Internal Standard (IS)

Various solvents like distilled water, ethanol, ACN, methanol and water-methanol combinations were tried and methanol was used for stock solution preparation. Further dilutions were prepared by adding mobile phase to the required quantity of stock solutions in separate volumetric flasks. IS plays important role for maintaining the reproducibility of the developed method. Various trials were done to decide IS for this combination and finally Azilsartan (AZL) was selected as IS.

Standard Stock Solutions and Working Standard Solutions Preparation

Accurate 25 mg of AZP, OLM and IS were weighed, transferred to separate volumetric flasks of 25 ml with respective labels of AZP, OLM and IS. 10 ml methanol was added, sonicated for 10 minutes, volume was adjusted to 25 ml using methanol and standard stock solutions 1000 µg/ml of AZP, OLM and IS each was obtained. From these standard stocks, 2.5 ml of each was poured to another 25 ml volumetric flasks, volume was adjusted up to 25 ml using methanol and working standards 100 µg/ml of AZP, OLM and IS each was obtained.

Selection of Wavelength

UV spectra of AZP, OLM and IS were compared and the appropriate wavelength showing absorbances of all APIs was selected. Thus, 256 nm was the selected wavelength for bioanalytical method development of AZP and OLM.

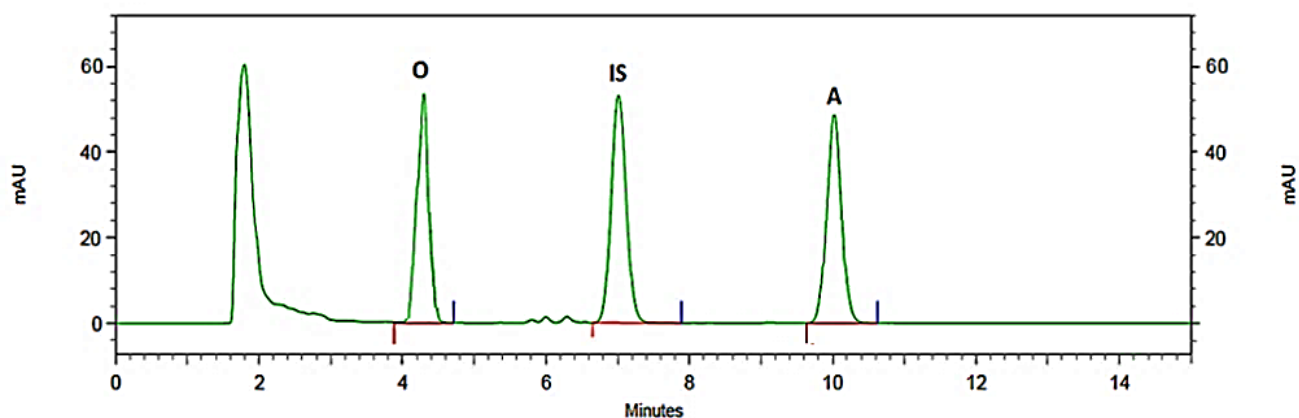
Mobile Phase and Optimization of Chromatographic Conditions

Various mobile phase combinations were tried using polar solvents. The combination of ACN: Water, pH adjusted to pH 3 with OPA in the ratio 80:20, 70:30 and 60:40 as tried and finally the ratio of 60:40 was selected as mobile phase. The other chromatographic conditions such as column, column dimension, detector and injection volume were decided according to the model available at lab and respective specifications of the HPLC system.

Extraction of APIs from Human Plasma

Depending upon literature search and physicochemical properties of analytes, variety of extracting solvents like toluene, ethyl acetate, chloroform and acetonitrile were tried. The combination of Ethyl acetate: Acetonitrile: 0.5% Formic acid (5:2.5:2.5) was found to be best extracting solvents with good peak shape as shown in Fig.3 with recovery of 85%, 91% and 88% of AZP, OLM and IS respectively. Also, the addition of 0.5% formic acid enhanced the recovery due to precipitation of protein. The results obtained using the finalized combination was reproducible and showing higher recoveries as compared to other solvents.

Figure 3: Chromatogram of AZP and OLM with IS after LLE



The liquid-liquid extraction was carried out in following manner:

- Pooled human plasma frozen at below -25°C were thawed at RT followed by vortexing to confirm the homogeneity.
- 1 ml of pooled human plasma was taken in 15 ml centrifuge tubes and respective μL of stock solutions of $100\ \mu\text{g}/\text{ml}$ of AZP, OLM and IS were added.
- The spiked samples were kept for 1 hr to reach equilibrium concentration.
- The tubes were vortexed for 2 minutes and ethyl acetate: ACN: 0.5% formic acid in the ratio 5:2.5:2.5 were added.
- It was then vortexed for 1 minute and centrifuged for 10 min at 5000 rpm. The supernatant was collected and evaporated on hotplate.
- Then it was reconstituted with 1ml mobile phase and was transferred into pre-labelled auto sampler vials and injected to HPLC system with optimized chromatographic conditions.

Calibration standards (CS) and quality control (QC) samples preparation

As per CDER guidelines, the calibration curve must contain at least six dilutions, which are known as calibration standards (CS). Quality control standards (QC) has critical role to measure the performance of optimized method and these should be selected in such a way that it should cover the whole range of calibration line. Thus, lower limit of quantitation (LLOQ), low QC (LQC), mid QC (MQC), high QC (HQC) and the upper limit of quantitation (ULOQ) were prepared. 5, 15, 30, 60, 90 and $120\ \mu\text{l}$ from $100\ \mu\text{g}/\text{ml}$ working standard solutions of AZP and 10, 30, 60, 90, 120 and $150\ \mu\text{l}$ from $100\ \mu\text{g}/\text{ml}$ working standard solutions of OLM were spiked in 1 ml pooled plasma and processed further with optimized liquid-liquid extraction method. After reconstitution the concentrations of 0.5, 1.5, 3, 6, 9 and $12\ \mu\text{g}/\text{ml}$ of AZP and 1, 3, 6, 9, 12 and $15\ \mu\text{g}/\text{ml}$ of OLM were obtained. Similarly, $50\ \mu\text{l}$ from $100\ \mu\text{g}/\text{ml}$ of IS (working standard) was spiked and $5\ \mu\text{g}/\text{ml}$ was obtained.

Validation

Calibration Curve and Linearity

Selection of LLOQ

Initially, $100\ \mu\text{g}/\text{ml}$ of both drugs, AZP and OLM were analysed in combination using optimized conditions and peak areas and peak heights were observed. Accordingly, the dilution for LLOQ was prepared so that it can be detected with a minimum height of 2 to 3 units. Thus, $0.5\ \mu\text{g}/\text{ml}$ AZP and $1\ \mu\text{g}/\text{ml}$ OLM were selected as LLOQ.

Linearity

Similarly, other dilutions were analysed by making serial dilutions and the dilutions having peak area values in linear fashion were selected for calibration curve. Preparation of calibration curve was done by plotting peak area ratio on y-axis and respective concentrations in $\mu\text{g}/\text{ml}$ on x-axis. The slope, y-intercept, linear equation and regression coefficient (r^2) were calculated for each drug.

Quality Control Samples (QCs)

LLOQ, LQC, MQC, HQC and ULOQ were determined from linearity range of each drug.

Selectivity

Selectivity for the developed bioanalytical assay is normally performed by analyzing blank samples of the blood plasma from multiple sources. Thus, 6 normal blank plasma lots from different sources were processed as per the developed method. It is also mentioned in the guidelines that the IS should be analyzed to avoid interference. Thus, 6 different zero calibrators were also processed as per method.

Sensitivity

As per the CDER guidelines, method sensitivity is better determined by LLOQ thus it becomes necessary to perform it during method development. Thus, LLOQ was spiked in plasma and six different LLOQ dilutions were processed. The CV for the area of six LLOQ samples was calculated.

Carry Over

Carry over between two runs was studied by injecting blank

plasma initially, followed by ULOQ and then two blank runs back to back.

Accuracy and Precision (A and P)

A and P determines the readiness of developed method for validation as well as its application to marketed products or biological samples. It was performed by preparing fresh dilutions of all QCs in triplicate and analysing them at specific intervals.

Within Run

The QCs were analysed in triplicate for three times on same day at the interval of 2 hours and recovered concentrations were calculated from linear equations of respective drugs at respective wavelengths. Percentage accuracy and relative percentage of nominal concentration (%RE) were calculated.

Between Run

The QCs were analysed in triplicate for one time on three consecutive days and recovered concentrations were calculated from linear equations of respective drugs at respective wavelengths. Percentage accuracy and relative percentage of nominal concentration (%RE) were calculated of each QC.

Recovery

Determination of recovery was done by calculating percentage of drug obtained from un-extracted (extracts of blanks that were spiked with analyte after extraction) and extracted plasma samples of QCs. Un-extracted samples were prepared by taking 1 ml of pooled plasma, vortexed for 2 minutes, added extracting solvent. Then, it was mixed thoroughly by vortex for 1 minute and centrifuged for the period of 10 min at 5000 rpm, supernatant was collected. It was then evaporated on hotplate and respective QC concentrations of AZP and OLM in combination were added and reconstituted with 1 ml mobile phase and was transferred into pre-labelled auto sampler vials, injected to HPLC system. Similarly, extracted plasma samples were prepared by adding respective QC concentrations of AZP and OLM in combination at initial stage and then extraction process was carried out and percentage recovery was computed by using equation given below:

Recovery = Peak area of extracted QC sample/

$$\frac{\text{Peak area of un-extracted QC sample} \times 100}{\text{Peak area of extracted QC sample}} \text{ ----- Eq.(1)}$$

Stability

Validation of stability of API in a biological fluid is critical parameter for determination of the storage conditions as well as the physicochemical properties of APIs in matrix and in container system. Freshly extracted QCs of AZP and OLM were subjected to various stability conditions and RSD were calculated.

Freeze Thaw Stability

For determination of freeze thaw stability, 3 freeze and thaw cycles were performed on extracted LQC and HQC of AZP and OLM in triplicate. Spiked samples were kept in freezer for 24 hrs,

withdrawn to thaw it completely and again kept in freezer. Again they were withdrawn after 24 hrs to thaw it completely and again placed in freezer. The samples were withdrawn after 24 hrs and analysed them by injecting on HPLC system. Percentage recovery was calculated.

Autosampler Stability

LQC and HQC samples of AZP and OLM were re-injected in triplicate after keeping the spiked stability samples in auto-sampler at 25°C for 4 hrs and percentage recovery was calculated.

Processed Extracted Samples at R.T.

Processed LQC and HQC of AZP and OLM in triplicate were kept on bench for 6 hours and then injected. The percentage recovery was calculated.

Stock Solution Samples at R.T.

Stock solutions of AZP and OLM were kept as such for 24 hours and after that new 3 LQC and 3 HQC were prepared, processed and injected. The percentage recovery was calculated.

Bench Top

Spiked plasma samples of AZP and OLM were kept on bench for 4, 6 and 24 hrs. LQC and HQC were prepared in triplicate and processed after 4, 6 and 24 hrs and injected immediately after the processing was completed. The percentage recovery was calculated.

Long Term

Spiked plasma samples were kept at intended temperature for 10, 20 and 30 days. LQC and HQC were prepared and processed after 10, 20 and 30 days and injected immediately after processing completed. The percentage recovery was calculated.

RESULTS AND DISCUSSION

Solvent and IS Selection

In accordance with the trials for solubility and stability of analytes in various solvents, methanol was found to be very convenient solvent for bioanalytical method development of AZP and OLM and thus methanol was finalized as a solvent for preparation of dilutions. The drugs like telmisartan, valsartan, irbesartan and azilsartan (AZL) were tried as IS and analysed on HPLC in similar chromatographic conditions as that of developed bioanalytical method of AZP and OLM. It was found that telmisartan and valsartan had effect of peak tailing. Irbesartan was showing very less resolution. While, AZL showed proper peak shape, distinguished retention time as well as resolution. Thus, AZL was selected as IS for bioanalytical method of AZP and OLM.

Mobile Phase Selection

Exhaustive literature search, some trials and physicochemical properties of AZP and OLM resulted into final trials of three mobile phase combinations as listed in Table 1.

ACN is hydrogen bond donor, has a higher dipole moment, low UV cut-off, higher boiling point and lower viscosity.

Table 1: Mobile phase trial output for HPLC analysis of AZP and OLM

Mobile	Phase Observations
Acetonitrile : Water, pH adjusted with ortho-phosphoric acid (80:20)	AZP, OLM and IS eluted. But peak shape of OLM is not acceptable.
Acetonitrile : Water, pH adjusted with ortho-phosphoric acid (70:30)	AZP, OLM and IS eluted properly. But peak distortion was observed for OLM.
Acetonitrile : Water, pH adjusted with ortho-phosphoric acid (60:40)	All the peaks were eluted with proper separation with good peak shape.

.Water with adjusted pH is always a good solvent for RP-HPLC and water with specific pH monitors the elution properties of the analytes by maintaining the ionization characteristics of drugs. Depending upon the observations of chromatographic runs, the combination of ACN: water pH adjusted with OPA (60:40) was finalized as mobile phase for the bioanalytical method of AZP and OLM. AZP and OLM were showing well separated peaks along with proper peak shapes, sufficient number of theoretical plates, good symmetry and good resolution.

Chromatographic Conditions

There were some fixed chromatographic conditions depending upon the availability of instrument type at analytical lab. These include the parameters like type of column was BDS hypersil C18, column dimension was (250 mm X 4.6 mm i.d.) 5 μ m, detector was U.V. detector and injection volume was 20 μ l. Flow rate also had a significant effect on the retention times of analytes when it was varied as 0.8, 0.9, 1, 1.1 and 1.2 ml/min. Thus, 1.0 ml/min was finalized as it showed good chromatography in the selected mobile

phase. 256 nm was finalized as detection wavelength for bioanalytical method of AZP and OLM, because both the drugs and IS were showing optimum absorbances at 256 nm. The retention times obtained were 10.05 \pm 0.02, 4.3 \pm 0.019 and 6.9 \pm 0.017 minutes for AZP, OLM and IS resp. after extraction from human plasma.

Extraction of APIs from Human Plasma

Selection of solvents for extraction of drugs from human plasma relates to several factors like solubility, volatility, selectivity and miscibility with other solvents. Selection of proper pH is also important factor for extracting the drugs depending upon their pka values. Liquid-liquid extraction method was selected as the sample preparation method. The extracted sample obtained using liquid-liquid extraction is clearer than the precipitation extraction technique. It does not interfere with column composition and also increases the column life. To achieve better recovery, the extraction method was repeated with variations in solvents, variations in centrifugation time and vortexing time. Simultaneously, the peak shapes and peak areas were observed for each trial. The combination of ethyl acetate: ACN: 0.5% formic acid in the ratio 5:2.5:2.5 proved to be the best extracting solvents with good peak shape and better recovery in the range of 85 -91%.

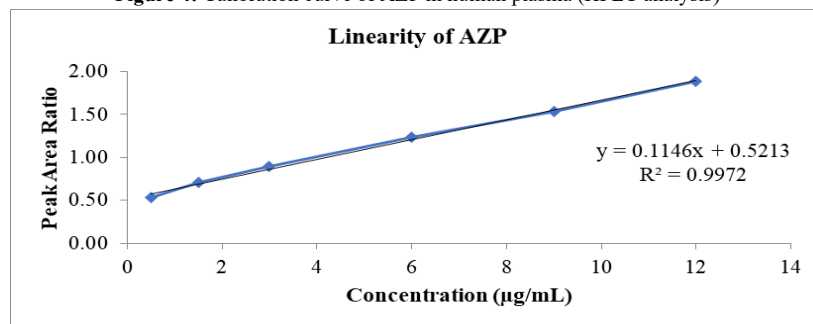
Validation

The validation of developed bioanalytical method was performed as per CDER guidelines.

Calibration Curve

The linearity was observed in the range 0.5, 1.5, 3, 6, 9 and 12 μ g/ml for AZP and 1, 3, 6, 9, 12 and 15 μ g/ml for OLM (Table 2).

Figure 4: Calibration curve of AZP in human plasma (HPLC analysis)



The calibration graphs were obtained with regression equations, $y = 0.1146x + 0.5213$ and $y = 0.1028x + 0.4342$ having r^2 values 0.9972 and 0.9986 for AZP and OLM respectively (Fig 4 and 5).

Figure 5: Calibration curve of OLM in human plasma (HPLC analysis)

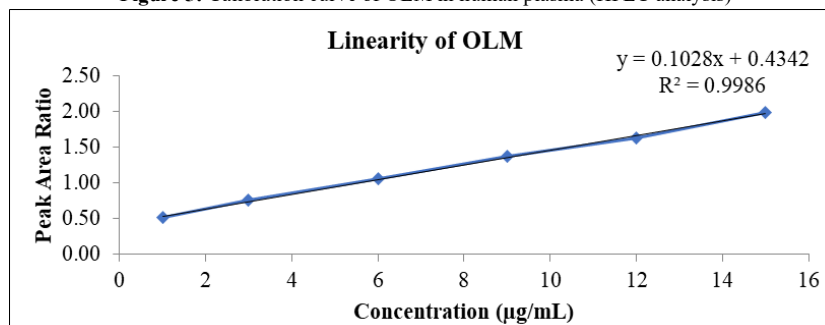


Table 2: Linearity data after LLE for HPLC analysis

Standards	AZP			OLM		
	Conc. ($\mu\text{g/mL}$) (n= 6)	Peak area ratio	Recovered concentration ($\mu\text{g/mL}$)	Conc. ($\mu\text{g/mL}$) (n= 6)	Peak area ratio	Recovered concentration ($\mu\text{g/mL}$)
STD A	0.5	0.54	0.12	1	0.51	0.78
STD B	1.5	0.71	1.64	3	0.76	3.16
STD C	3	0.89	3.23	6	1.06	6.13
STD D	6	1.23	6.21	9	1.37	9.14
STD E	9	1.53	8.84	12	1.64	11.71
STD F	12	1.89	11.95	15	1.98	15.07

Selectivity

Selectivity has to be performed to check the interference of biological fluids or matrices in the chromatograms of analytes. For this purpose, normal blank human plasma collected from 6 different sources was combined and analysed under the developed chromatographic conditions. As per blank chromatogram there was no intervention of plasma at the Rts of analytes. Also the analysis of IS was conducted to find any interferences with the analyte. 6 different zero calibrators were analysed and it was found that there is no any interference of IS at the retention times of analytes.

Sensitivity

The detection ability, to observe all analytes at all concentrations including a very small quantity of analyte is tested in the validation parameter called sensitivity. It can be defined as the lowest concentration of analyte that can be measured with tolerable precision and accuracy (i.e., LLOQ).

Thus, sensitivity was determined by analyzing 6 replicates of LLOQs of AZP and OLM along with IS (Table 6). It was observed that AZP at 0.5 $\mu\text{g/ml}$ and OLM at 1 $\mu\text{g/ml}$ were detectable with the height of 3 and 2 units respectively. The RSD of peak areas was 3.75, 3.88 and 2.27 for AZP, OLM and IS respectively, which shows the sensitivity of the method.

Similarly, sensitivity of method was tested by observing system suitability parameters of the chromatographic runs. Acceptance limit of asymmetry factor is 1, theoretical plates should be more than 1500 and resolution should be more than 2. It was observed that the values of asymmetry factor, theoretical plates and resolution of AZP, OLM and IS (Table 3) were within acceptance

limit indicating that the method is selective.

Table 3: System suitability parameters for HPLC analysis of AZP and OLM

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)	Resolution
OLM	4.34	82816	0.93	3982	--
IS	7.13	173810	1.07	6348	5.2
AZP	9.92	86197	1.09	5219	5.9

Carry Over

Carry over effect should be determined to observe the left over effect of chromatographic runs one after another. It was observed that the blank run after the ULOQ was having peaks of area not exceeding 20% of LLOQ at Rt of AZP, OLM and IS. This indicated that there was no any carry over effect of chromatographic runs.

Accuracy and Precision (A and P)

QCs were obtained according to calibration curves plotted for both analytes. LLOQ, LQC, MQC and HQCs of both analytes were prepared freshly and analysed for A & P parameters. As per the guidelines, acceptance criteria for accuracy is that in each validation run, the analysed QCs should be $\pm 15\%$ of nominal concentrations, whereas at LLOQ, they should be $\pm 20\%$ of nominal concentrations.

Within Run

Table 4 shows the recovered concentrations, percentage accuracy, SD and RSD or CV (%) of 6 replicates of QCs of AZP and OLM analysed for three times on same day at the interval of 2 hours.

Between Run

The values of recovered concentrations, percentage accuracy, SD and RSD or CV (%) of QCs of AZP and OLM resp. analysed in triplicate for one time on three consecutive days are listed in Table 4.

Table 4: A and P data for HPLC analysis of AZP and OLM

LEVEL	Mean Recovered conc. of AZP ($\mu\text{g/mL}$) (n=6)	% accuracy	SD	CV (%)	Mean Recovered conc. of OLM ($\mu\text{g/mL}$) (n=6)	% accuracy	SD	CV (%)
Within run A and P data								
LLOQ	0.46	92.39	0.054	11.80	1.00	99.88	0.103	10.35
LQC	1.58	105.08	0.134	8.50	3.12	103.98	0.309	9.90
MQC	6.16	102.67	0.369	5.98	9.16	101.79	0.527	5.76
HQC	8.94	99.38	0.381	4.26	12.05	100.43	0.552	4.58
Mean		99.88		7.637		101.52		7.646
Between run A and P data								
LLOQ	0.47	94.89	0.062	13.05	1.00	100.33	0.126	12.61
LQC	1.54	102.83	0.168	10.87	2.92	97.40	0.320	10.97
MQC	5.92	98.74	0.473	7.80	9.17	101.93	0.730	7.95
HQC	9.00	100.00	0.519	5.77	11.82	98.50	0.785	6.64
Mean		99.54		9.422		99.11		9.544

It was found that, percentage accuracy was within the percentage assay limit of the analytes and all the CV (%) values were within acceptance criteria as per the guidelines.

combination was done, analysed and percentage recovery was computed. The recovery was within assay limit of respective drugs as shown in Table 5.

Recovery

Extraction of respective QC concentrations of AZP and OLM in

Table 5: Recovery data for HPLC analysis of AZP, OLM and IS

	QC	Mean area of Un-extracted drug	Mean Recovery	CV (%)	Mean Recovery	Mean CV (%)
AZP	LQC	105101	85.07	2.765	85.12	3.02
	MQC	193287	85.21	2.991		
	HQC	240838	85.08	4.468		
OLM	LQC	122127	91.33	1.525	91.04	2.32
	MQC	214883	91.23	3.024		
	HQC	252455	90.55	3.084		
IS	5 µg/mL	157362	88.04	1.793	88.04	1.79

Stability

An acceptance criterion for stability study is that accuracy or % nominal should be $\pm 15\%$ at selected QC levels.

Freeze Thaw Stability

3 freeze and thaw cycles were conducted on extracted LQC and HQC of AZP and OLM in triplicate. Percentage recovery of AZP was found to be 89.27% and 88.55% for LQC and HQC resp. and that of OLM was 91.57% and 90.48% for LQC and HQC resp.

Autosampler Stability

The percentage recovery for autosampler for AZP was found to be 96.75% and 97.80% for LQC and HQC resp. and that of OLM was 98.24% and 97.08% for LQC and HQC resp.

Processed Extracted Samples at R.T.

The percentage recovery for processed extracted samples stability for AZP was 95.56% and 96.68% for LQC and HQC resp. and that of OLM was 97.39% and 97.75% for LQC and HQC resp.

Stock Solution Samples at R.T.

The percentage recovery for stock solution samples stability for AZP was found to be 97.75% and 97.66% for LQC and HQC resp. and that of OLM was 97.04% and 98.04% for LQC and HQC resp.

Bench Top Stability

The percentage recovery for bench top stability AZP was found to be 96.02% and 97.87% and that of OLM was 98.86% and 97.91% for LQC and HQC resp. after 4 hrs. The percentage recovery for bench top stability after 6 hrs for AZP was found to be 97.71% and 96.40% for LQC and HQC resp. and that of OLM was 97.76% and 97.94% for LQC and HQC resp. The percentage recovery for bench top stability after 24 hrs for AZP was found to be 95.98% and 95.43% for LQC and HQC resp. and that of OLM was 95.67% and 94.77% for LQC and HQC resp.

Long Term Stability

The percentage recovery for long term stability was found to be 89.10% and 90.44% for LQC and HQC resp after 10 days for AZP and that of OLM was 90.32% and 90.37% for LQC and HQC

resp. after 10 days. The percentage recovery for bench top stability after 20 days for AZP was found to be 87.00% and 88.31% for LQC and HQC resp. and that of OLM was 88.49% and 87.43% for LQC and HQC resp. The percentage recovery for bench top stability after 30 days AZP was found to be 86.96% and 84.79% for LQC and HQC resp. and that of OLM was 86.16% and 85.65% for LQC and HQC resp.

CONCLUSION

The proposed research work presents development of novel, precise, accurate and robust chromatographic method for determination of AZP and OLM in human plasma. The developed chromatographic method was employed for validation as per CDER guidelines and the obtained results of each validation parameter were within the standard limit which ensures optimum method performance over the life cycle of the drugs. The proposed method can be used to analyze AZP and OLM in human plasma by RP-HPLC as well as it will be extremely useful for conducting pharmacokinetic research.

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Conflict of interest Authors declare that there is no any conflict of interest.

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