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ORIGINAL ARTICLE

Development and Validation of LC-MS/MS Method for Determination of Azilsartan Medoxomil from the Human Plasma and Its Application to Pharmacokinetic Study

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ABSTRACT
Azilsartan Medoxomil (AZTM) is potent angiotensin-II receptor antagonist drug used for the treatment of Hypertension. Simple, accurate precise and reproducible reverse phase high-performance liquid chromatography (RP-HPLC) hyphenated with Tendom mass spectroscopy method was developed and validated using protein precipitation technique for the determination of AZT in human plasma; detection was carried out by photo diode array detector. Chromatographic separation of the analyte AZTMand Internal Standard were achieved within 12.0 min by Younglin (S.K) isocratic system using C_{18} (4.6×250mm, 5µm) column as stationary phase and methanol: Phosphate Buffer [60:40, v/v] with pH adjusted 2.5 was used as mobile phase at a working temperature of 25°C, flow rate was 1.0 mL/min at detection value at 250 nm. Calibration curve was linear (r^2> 0.998) in the range of 10-2000 ng/mL, while intraday and interday coefficient of variation shows11.31% and 7.31for LLOQ and 7.95 to 3.82% for MQC while 3.77 to 5.46% for HQC. The overall mean recovery of AZTM was 89.49%. No any co-administered drugs were found to interfere at retention time of the analyte. LC was hyphenated with API 4000 triple quadrupole mass spectrometer (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX).The LC-MS-MS method was simple, reproducible and can be effectively used for determination of AZT in human plasma to conduct bioavailability and bioequivalence studies.
Keywords: Azilsartan Medoxomil, protein precipitation, bioanalytical, validation, LC-MS-MS.

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INTRODUCTION

Azilsartan Medoxomil (Figure 1) is a prodrug [1] which acts as Angiotensin-II receptor antagonist (ARB) having chemical entity [2] (5-Methyl-2-oxo-1,3- dioxol-4-yl) methyl 2-ethoxy-1-{ [2'-(5-oxo-4,5-dihydro 1,2,4-oxadiazol-3-yl) biphenyl-4-yl] methyl}-1H-benzimidazole-7- carboxylate with Cmax 888 ng/mL after single dose, Vd of 16 L, 99% plasma protein bound, Renal clearance is 2.3 L/minute and half-life is 11 hours.

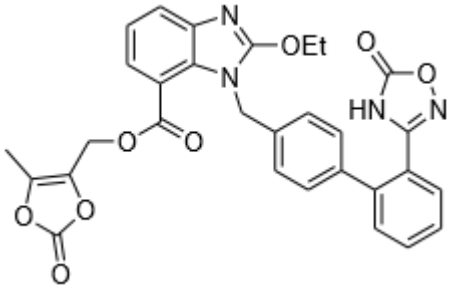

Figure 1: Structure of Azilsartan

It is a white crystalline powder practically insoluble in water but freely soluble solvents like methanol, dimethylsulfoxide and dimethylformamide, soluble in acetic acid, slightly soluble in acetone and Acetonitrile and very slightly soluble in Tetrahydrofuran and 1-octanol. It is available in dosages of 40 mg and 80 mg, the recommended dosage is 80 mg per day. By hydrolysis of medoxomilester in the gastrointestinal tract, the active moiety of Azilsartan Medoxomil can be released. It is an active Angiotensin Receptor Blockers (ARBs) (AT1) type which is more active in lowering blood pressure within 24 hours when compared to other ARBs.

Clinical studies shown that Azilsartan Medoxomil is a produrg which hydrolyzed to its active metabolite Azilsartan (AZT) in the gastrointestinal tract as the inactive AZTM is rarely found in the plasma [3].Literature Survey reveals various experimental works related with estimation of AZTM RP-HPLC and HPTLC methods for formulation in alone or combination at exaggerated condition [4, 5] further bioanalytical method for determination of AZTM on RP-HPLC- PDA [6, 7]are reported. However, these methods were describing multiple and time consuming steps of sample preparation in LLE and SPE along with application of this method for analysis of AZT in clinical samples might be difficult.

Further protein precipitation is a commonly used and faster technique for sample analysis in bioanalytical method [8]. A simple protein precipitation UPLC-MS/MS method for determination of AZT in dog plasma is reported [9] along with other LC-MS/MS methods under bioanalytical methods [10-12].

The literature study revealed only a single method for simultaneous estimation of AZT and CTD from rat plasma and human plasma, where the extraction method was liquid-liquid extraction [13].

Hence above stated methods were deficient in simple sample preparation techniques, high volume of plasma and time consuming steps with less resolved peaks in combination. Thus the present study was majorly focused to study the analytes in presence of co-administered drugs with metabolites which now a days has become an integral part of drug research in the discovery process in order to lessen the risk posed by development of drugs that form large amounts of reactive intermediates.

MATERIAL AND METHODS

Azilsartan Medoxomil was obtained as a gift sample from Hetero Drugs Ltd, India and Ropinirole used as Internal Standard (IS) was provided as a gift sample by IPCA Laboratories Ltd, Mumbai, India.LC-MS grade Acetonitrile and Methanol was purchased from Lobachemie, Mumbai, India. Human plasma was procured from Malegaon Blood Bank, Malegaon, Nashik India. Ultrapure water was obtained in-house using a Millipore with 0.45 micron membrane water purifying system. Further, other reagents and solvents were of analytical grade and purchased from standard chemical suppliers.

Instrument and Chromatographic conditions

Instruments employed wasYounglin (S.K) gradient system, UV Detector with Autochro-3000 database software, connected with API 4000 triple quadrupole mass spectrometer (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray interface in positive ionization mode was used. Data processing was performed on Analyst 1.4.1 software package (SCIEX). Eppendorf, Hamburg, Germany, Finn pipett, Biofuge Centrifuge, Sayno deep freezer, Labline ultrasonicatore. Resolution was carried out on RP C_{18} column (250mm length × 4.6 mm internal diameter), particle size 5µ, using Methanol: Phosphate buffer [60:40, v/v] with pH adjusted to 2.5 as Mobile phase at a working temperature of 25°C.

Biological matrix employed in bioanalysis:

The biological media containing analytes are usually blood, plasma, urine, serum, fecal matter, sweat etc [12]. Through GIT absorption after oral administration, drug reachesto plasma thus human plasma was the first matrix choice for analysis. After selection of biological matrix, sample preparation technique was chosen.

Various methods have been employed for extraction of drug from plasma which includes
A. Liquid-liquid extraction (LLE),
B. Solid phase extraction (SPE) and
C. Protein Precipitation method.
Plasma contains about 90% water. The remaining 10% is inorganic or organic ions (2%) and proteins (about 8%). The inorganic ions are dominated by sodium and chloride, whereas the main part of the proteins is albumin. A minor but not less important part of the proteins is the enzymes (e.g., esterases) that may still be more or less active in collected plasma [13].
Principle of Protein Precipitation is based on precipitation (denaturation) of the proteins by zwitter ion formation by using various reagents like acid (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation the sample is centrifuged, which results in extraction of analyte in the precipitating solvent. It is less time consuming, smaller amounts of organic modifier or other solvents are used. Methanol and Acetonitrile are generally preferred solvents amongst the organic solvent as it can produce clear supernatant which is appropriate for direct injection into LC-MS/MS [14-15].
Protein precipitation is the simple method of extraction as compared to the LLE and SPE. This can be carried out by using the suitable organic solvents, Acids which has good solubility of the analyte and protein precipitating properties. Methanol is the first choice of solvent for protein precipitation due to its complete flocculent precipitation of proteins which settles down after centrifugation at high speed and gives clear supernatant. The aim of Protein precipitation is to minimize the protein interferences form plasma and serum and subsequently reduces the deterioration of HPLC columns from proteins, salts, endogenous macromolecules, small molecules and metabolic byproducts.

Sample preparation Technique

Primary standard stock solution was prepared by dissolving 10 mg of AZTM in 10 mL volumetric flask and volume was made up to mark with Methanol. Shake and ultrasonicate the resultant solution till clear solution obtained (Primary stock solution with 1000 PPM). From the resultant solution, pipette out accurately 1 mL solution and transfer to 10 mL volumetric flask and make up the volume up to the mark with methanol (Secondary stock solution with 100 PPM). Working standard were prepared by diluting 20, 40, 100, 200, 400, 1000 and 2000 µL from secondary stock solution, which was diluted upto 10 mL with suitable solvent.
In a 1.5 mL eppendrofs tube, Drug Free Human Plasma (250 µL), test drug (25 µL) and internal standard (25 µL) were spiked and resultant blend was vertex for 03 min. After presumed time, sample was subjected to sample preparation using chilled Methanol (200 µL), again mixture was subjected to vertex for 03 minutes followed by centrifugation for 10 minutes at 10000 RPM. This results in protein precipitation and clear supernatant solution. About 20 µL was injected into the HPLC system.

Bioanalytical method validation

The developed HPLC conditions were validated as per the FDA-CDER guideline for bioanalytical method validation. Typical method development and establishment of a bioanalytical method include determination of selectivity, accuracy, precision, recovery, calibration curve and stability of analyte in spiked samples.

Selectivity:

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at thelower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

Calibration/standard curve:

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample

(matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ.

Lower limit of quantification (LLOQ):

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

1. The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
2. Analyte peak (response) should be identifiable, discrete and reproducible with a %CV of 20% and accuracy of 80-120%.

Calibration curve/standard curve/concentration-response:

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighing and the use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

1. 20% deviation of the LLOQ from nominal concentration
2. 15% deviation of standards other than LLOQ from nominalconcentration

At least four out of six non-zero standards should meet the above criteria including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

ACCURACY:

The accuracy of a bioanalytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value for coefficient of variation (CV) should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

PRECISION:

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% CV except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, andlaboratories.

RECOVERY:

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

STABILITY:

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

FREEZE AND THAW STABILITY:

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, then analysed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70^{0}C during the three freeze and thaw cycles.

SHORT-TERM TEMPERATURE STABILITY:

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analysed.

LONG-TERM STABILITY:

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

STOCK SOLUTION STABILITY:

The stability of stock solutions of the drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen 8 for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

POST-PREPARATIVE STABILITY:

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

RESULTS AND DISCUSSION

Development and Optimization of Chromatographic Conditions:

Various mobile phase compositions of Acetonitrile, Methanol with phosphate buffer, ortho phosphoric acid or ammonium acetate buffer were tried in an isocratic mode with variable pH.Based on these investigations methanol with Phosphate buffer [60:40] at 2.5 pH, mobile phase flow rate 1.0 mL/Min, at working temperature 25^{0}C gave good peak shapes of the analytes. The resultant chromatogram (**Figure 2**) was Gaussian with decreased peak width indicated optimised chromatographic condtions. This resolution was made on Younglin (S.K) isocratic system using C_{18} (4.6×250mm, 5µ) column as stationary phase at detection value 250nm with total run time 12 mins.

Development and Optimization of Mass Conditions:

During the optimization of the MS/MS parameters, we aimed to develop a selective and sensitive method. Standard solutions were directly infused into the mass spectrometer and the operating conditions were optimized to monitor the analytes. Positive ion mode tuning was used to find the parent and daughter ions, thus, to achieve maximum response for the analyte as well as the IS. AZTM with IS was detected by triple- quadrupole tandem mass spectrometric detection with an ESI (Electrospray Ionization) running in positive mode. The MRM transition of m/z 456.84 278.93 was chosen for AZTM, with a dwell time of 100 milliseconds.

Development of protein precipitation (PP) Procedure:

For PP procedure, different organic solvents were studied. Reasonable, Clear and reproducible recoveries were obtained by using Methanol as the PP solvent and this makes the experiment more simple, fast and easier in handling the samples.

Bio-Analytical Method Validation:

Lower Limit of Quantitation:

LLOQ (lower limit of quantitation) is the lowest concentration level of an analyte that can be quantitatively determined with good precision and accuracy and a coefficient of variation less than 20%. LLOQ value in the developed method was 10 ng/mL for AZTM having the coefficients of variation 12.39.

Sample Preparation technique and internal standard selection:

Methanol and Acetonitrile was considered for the protein precipitation. Chilled Methanol gave reproducible and consistent recovery at each QC level. Thus, Methanol was chosen as an appropriate

solvent for protein precipitation. Ropinirole was used as an IS as it has reproducible recovery and belongs treat Parkinson's disease.

Selectivity:

Selectivity of analytical method was checked by processing and injecting six different sources of Drug Free Human Plasma with concentration of LLOQ (10ng/mL) to check for endogenous components or associated drugs which might interfere with analytes or IS. All the plasma samples were free from any significant interference at retention times of analyte **(Figure 3).**

Linearity:

Responses from calibrators were recorded for AZTM. This was created by plotting the area ratio (area of each analyte/area of IS) versus each analyte concentration. The ranges of linearity were chosen with respect to C-max (888.3 ng/mL) of each analyte which was 10-2000 ng/mL for AZTM. Linear regression was applied and linearity was indicated by the high correlation coefficients of 0.998 **(Figure 2)** for the analyte and by evaluating the back-calculated concentrations of the calibration standards. **Table 1** show that results obtained was less than 20% deviation at LLOQ level from nominal concentration and less than 15% deviation at other levels from nominal concentrations.

Accuracy:

Accuracy of the developed method shows %CV within the ±15% with four QC samples including LLOQ to HQC. Table 2 comprises the concentration of QC samples selected from the calibration curve along with range of %CV allotted as per the FDA-CDER guideline for accuracy under bioanalytical method validation.

Precision:

The Precision at Interday and Intraday for the selected concentration based on calibration curve is summarized in **Table 3**. It can be concluded that the precision of established bioanalytical method shows the % CV value within the ±15% at each level other than LLOQ which have ±20%.

Extraction Recovery:

Extraction recoveries were calculated by comparing the mean peak areas obtained from extracted plasma quality control (QC) samples (low, medium and high) to mean peak areas of the bulk standard solution of equivalent concentration. The mean recovery was 89.49% indicates good sample recoveries for the analyte **(Table 4).**

Stability:

Measuring the stability of analytes in human plasma was done by analyzing LLOQ to HQC comprises the calibration range points for the analyte after applying different conditions that must be evaluated. Concerning short term stability, four replicates were kept at room temperature (25^0C) then processing and analyzing samples was established and compared with nominal concentrations after 24 Hrs. For long term stability, four replicates of low and high QC plasma samples were stored in a freezer at -70ºC ±15 then processing and analyzing samples was established after 30 days and compared with nominal concentrations. Low and high QC samples were subjected to three freeze and thaw cycles, samples then analyzed after the third cycle and results compared with nominal concentrations. Stock solution stability of drug and IS was estimated at room temperature (25 ^{0}C) for about 6 h and compared with freshly prepared solutions. Results of stability are shown in **Table 4**, **5** and **6** which reveal good stability.

Pharmacokinetic application:

The above fully developed LC-MS/MS method was used to study the metabolic pathway of Azilsartan in presence of IS with good recovery rate. Mass spectrum of AZT in human plasma shows intense peak at 279 describes metabolic pathway of AZT. Thus above proposed method can be applied for In vitro studies with precise determination of metabolites. Further toxicological studies can be done by exctraction the metabolites along with pharmacokinetic studies in human plasma.

Linearity of Azilsartan			
concng/ml	**DR**	**IS**	**DR/IS**
10	1035	33178	0.031195
20	2509	34178	0.07341
50	5565	35239	0.157922
100	13043	32562	0.400559
200	24876	31981	0.777837
500	55298	30918	1.788537
1000	110825	34178	3.242583
2000	223934	32178	6.959227

Table No. 1: Calibration Curve for AZTM

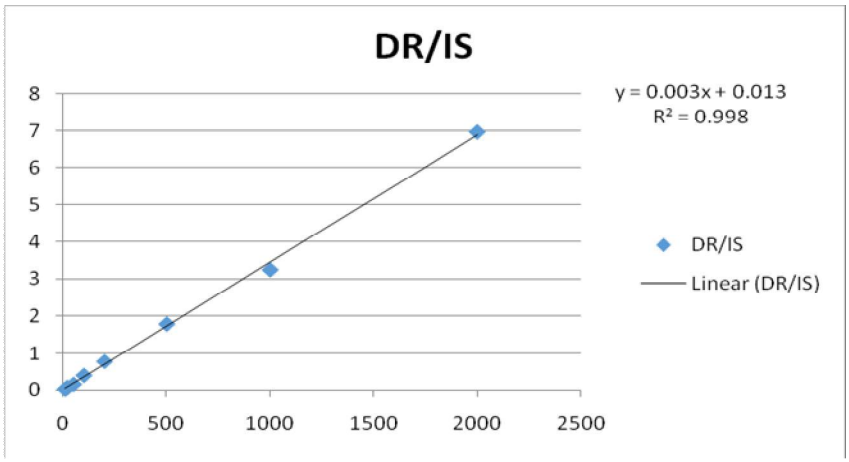

Figure No. 2: Calibration Curve for AZTM

Concentration ng/mL	Average DR/IS	SD	%RSD	conc found ng/ml	% accuracy	Range of % accuracy	Average DR/IS
10	0.0416	0.0028	6.7191	9.5401	95.4011	80-120	0.0416
50	0.1573	0.0079	5.0471	48.1046	96.2092	85-115	0.1573
500	1.3900	0.0906	6.5189	459.0103	91.8021	85-116	1.3900
1000	3.0319	0.1290	4.2546	1006.3019	100.6302	85-117	3.0319

Table No. 2: Accuracy of the method

Azilsartan Interday Precision				Azilsartan Intraday Precision		
concng/ml	Average DR/IS	SD	%RSD	Average DR/IS	SD	%RSD
10	0.038586367	0.004364	11.31058	0.035100914	0.002568	7.314668
500	1.624617437	0.12927	7.956922	1.54621529	0.059219	3.829946
1000	3.812884059	0.144109	3.779516	4.242490684	0.231812	5.464061

Table No. 3: Intraday and Interday Precision

Conc. ng/mL	Average DR/IS	SD	%RSD	% recovery	Avg recovery
10	0.03376	1.07844	12.35999	87.25225225	89.49898
50	0.16661	1.84054	4.05345	90.81338144	
500	0.12087	19.54703	4.29256	91.07401713	
1000	0.38522	46.04034	5.18144	88.85625338	

Table No. 4: Extraction Recovery of AZTM

Conc. Spiked ng/mL	Short term Room temp 24hrs			Freez Thaw Stability			Long Term Stability		
10	0.035718	0.004405	12.3327	0.035718	0.004405	12.3327	0.035718	0.004405	12.3327
50	0.171056	0.013155	7.690352	0.171056	0.013155	7.690352	0.171056	0.013155	7.690352
500	0.123655	0.008359	6.759648	0.123655	0.008359	6.759648	0.123655	0.008359	6.759648
1000	0.391944	0.027735	7.076241	0.391944	0.027735	7.076241	0.391944	0.027735	7.076241

Table No. 5: Short-term and long-term stability data

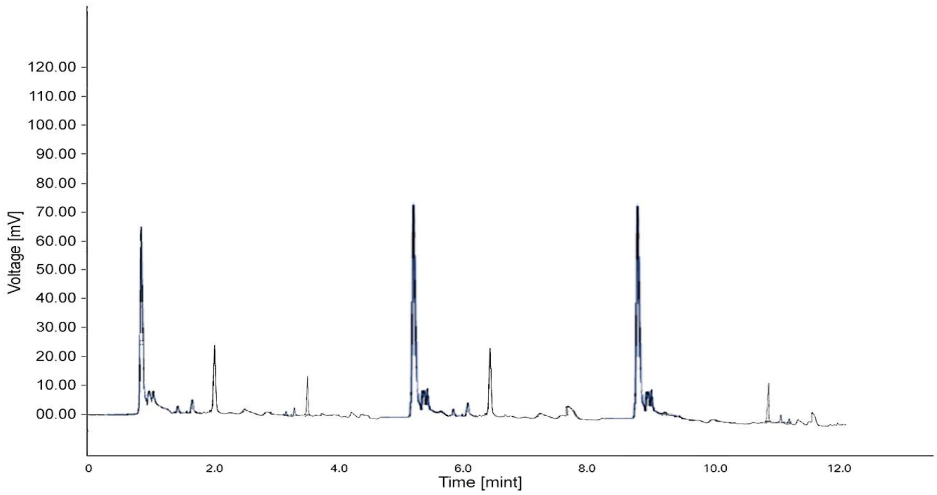

Figure 3: Typical chromatogram of Azilsartan with IS and co-administered drugs

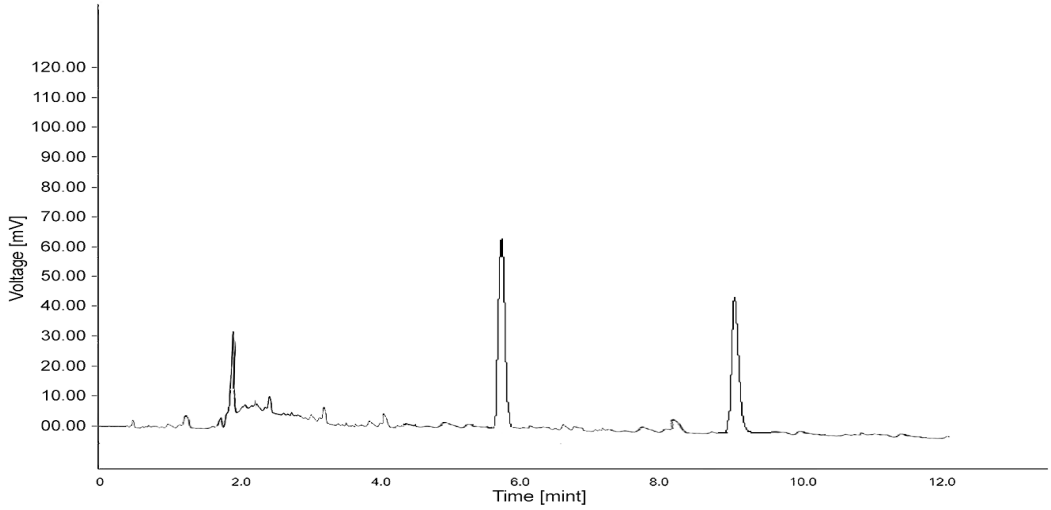

Figure 4: Typical chromatogram of Azilsartan and Ropinirol with retention time 5.68 and 9.18 minutes respectively

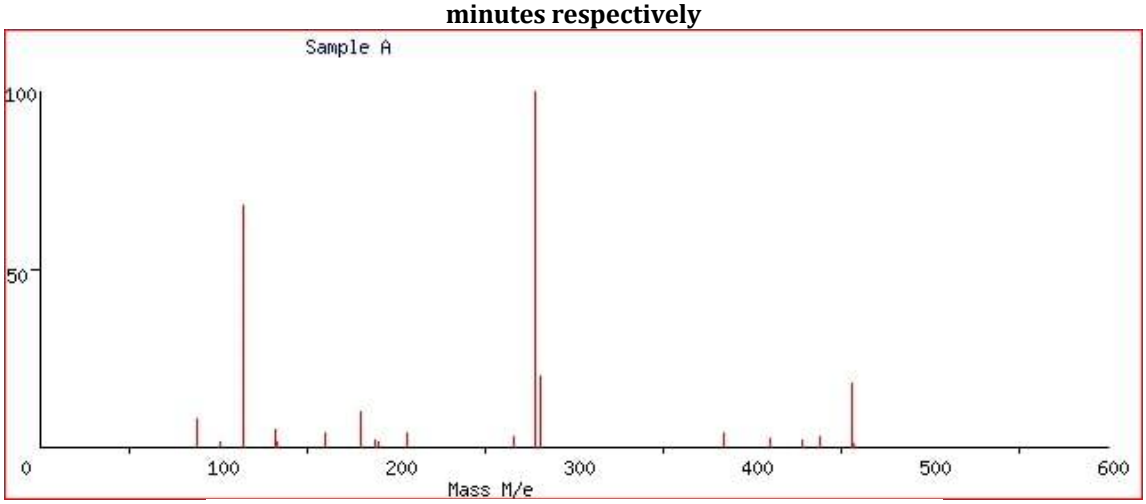

Figure 5: Mass Spectra of Azilsartan and IS in Human Plasma

CONCLUSION

A selective, sensitive, precise and accurate LC-MS/MS method for the determination of Azilsartan Medoxomil can be validated for in vitro studies in human plasma. The developed extraction procedure

was fast, simple with reproducible recoveries; isocratic elution and short run time permit fast analysis. So, the assay allows and facilitates their therapeutic monitoring when needed in a short time at a low cost.

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