ISSN 2581 – 4346

Available Online at www.ijms.co.in
Innovative Journal of Medical Sciences 2021; 5(2):31-39

RESEARCH ARTICLE

Bioanalytical method development and validation of azilsartan medoxomil potassium in human plasma by reversed-phase-high-performance liquid chromatographic with solid-phase extraction and its application in bioequivalence study

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Received on: 10 April 2021; Revised on: 20 May 2021; Accepted on: 25 June 2021

ABSTRACT

Azilsartan medoxomil potassium (AMP) is an antihypertensive medication used to treat adult hypertension. This study aimed to validate for the 1st time a fast and simple high-performance liquid chromatographic (HPLC) method for AMP quantification in human plasma to aid future pharmacokinetic studies in humans. For that, aliquots (475 µL) of plasma were spiked with known amounts of AMP and telmisartan (IS). Compounds were extracted using a solid-phase extraction. The instrument used was a Agilent HPLC with control panel software and their separation was accomplished using a Inertsil C_8 ODS column (5 µ, 150 mm × 2.5 mm) at 25°C. Mobile phase was phosphate buffer (pH 3.2):acetonitrile: methanol (60:25:15 %, v/v/v), flow rate was 1.5 mL/min with 25 µL injection volume, and detection was performed using a UV detector set at 254 nm. The bioanalytical method here in developed was validated according to bioanalytical guidelines and showed to be selective and linear (r2 ≥0.997, $n = 8$) over the concentration range of 0.1–1.5 µg/mL. Precision and accuracy are within current acceptability standards regarding quality control samples, overall RSD and accuracy of intraday precision were in the range of 3.07–13.0% and 90–102.5%, while for interday, it was found to be 0.04–13.8 and 93–109%, respectively. The results demonstrated high precision and accuracy. AMP recovery in human plasma was found to be 93.7% v/w. As a result, this method was successfully used in a preliminary pharmacokinetic study as well as for therapeutic drug monitoring.

Keywords: Antihypertensive agents, Azilsartan medoxomil potassium, Bioanalysis methods, Plasma, Reversed-phase high-performance liquid chromatography, Solid-phase extraction, Telmisartan

INTRODUCTION

Azilsartan medoxomil potassium (AMP) is an antihypertensive medication. He is a receptor antagonist for angiotensinogen II. It helps prevent strokes, heart attacks, and renal problems by balancing the renin-angiotensin-aldosterone system, regulating blood pressure, and preventing strokes, heart attacks, and kidney problems.[1]

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IUPAC name of this drug is (5-methyl-2-oxo-1, 3-dioxol-4-yl) methyl 2-ethoxy-3-{[4-[2-(5-oxo-4H-1, 2, 4-oxadiazol-3-yl) biphenyl-4-yl] methyl}-1H-benzimidazole-7-carboxylate monopotassium salt. Molecular formula is $C_{30}H_{23}KN_4O_8$. It's a crystalline white powder. Solubility of AMP is Insoluble in water and soluble in organic solvents. It is soluble in methanol, dimethyl sulfoxide, and N,N-Dimethylformamide at room temperature.[2]

The AMP brand is a tablet called "Edarbi." It was developed by Takeda in Japan.[3]

Azilsartan kamedoxomil is the potassium salt of AZM, which is extensively utilized. Hydrolysis of

the medoxomil ester reveals the active component of AZM, which transforms to azilsartan, an active angiotensin II receptor blocker that is more effective in decreasing blood pressure.[4]

Most of the previous methods are based on sophisticated instruments such as liquid chromatography–mass spectrometry (LC–MS)[5] and UPLC-MS[6] which are not available in ordinary quality control (QC) laboratories and laboratories of academic institutes. No AMP quantification method is described in human plasma, so developing such a sensitive, quick, and accurate approach that may be used to study additional bioavailability studies and bioequivalence study is inevitable.[7]

As a result, the current study was carried out with the goal of developing and verifying a simple and speedy reversed-phase-high-performance liquid chromatographic (RP-HPLC) with UV detection method for determining AMP in human plasma using a solid-phase extraction (SPE) approach that is both cost effective and efficient.

EXPERIMENTAL WORK

Material and reagent

Pharmacopoeial grade standard of AMP was gifted by Glenmark Pharmaceuticals, Sinnar, Nashik, India. Telmisartan used as an internal standard were provided from Kapynag Pharmaceuticals, Dindori, Nashik, India. Blank human plasma was provided as a gift sample from Dr. Vasantrao Pawar Medical College, Hospital and Research Centre, Nashik, India. Blank plasma was obtained from six different sources. Spectrochem Pvt. Ltd., Baroda, provided LC grade methanol, the Durapore, 0.45 µ × 47 mm, membrane filter papers were purchased from Millipore (India) Pvt. Ltd., Bengaluru, India. A Milli-Q assembly of water purified the water used, Spectrochem Pvt. Ltd. obtained the analytical grade of ammonium acetate, and Phenomenex bought SPE cartridge (Strata-X).

An HPLC system with a quaternary pump was used to conduct the chromatographic analysis (Infinity 1260, Agilent Techniques). Control panel OpenLab software was used to perform detection on a UV detector with a 100 µL injection volume capability.

Preparation of mobile phase buffer

0.5 g potassium dihydrogen phosphate dissolved in 1000 mL water, 2 mL triethylamine, orthophosphoric acid adjusted to pH 3.2. The buffer was filtered and kept at room temperature using a 0.22 µ membrane filter.

Preparation of mobile phase

Appropriate volumes of phosphate buffer (pH 3.2, adjusted with orthophosphoric acid), acetonitrile, and methanol were transferred into a reagent bottle, mixed thoroughly, sonicated for 5 min, and filtered through 0.22 µm membrane filter and used as mobile phase. The HPLC analysis was performed on RP-HPLC system with isocratic elution mode using a mobile phase of phosphate buffer: acetonitrile:methanol (60:25:15% v/v/v) on Inertsil C_8 column (150 mm × 4.6 mm, 5 µm particle size) with 1.5 ml/min flow rate at 254 nm using UV detector.

Stock solutions of AMP and Telmisartan (1000 ppm)

About 10 mg of AMP transferred into a 10 ml volumetric flask and dissolved in double-distilled water and the volume made up to the mark to obtain the AMP stock solution. About 10 mg of telmisartan (IS) was weighed accurately, transferred to a 10 ml volumetric flask, and dissolved in double-distilled water and the volume was made up to the mark to obtain a telmisartan stock solution.

Working standard solution for AMP and Telmisartan (IS)

The stock solution of 1 mg/ml (1000 ppm) of AMP was further appropriately diluted with diluent to get eight different working standard solutions with concentration 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 µg/ml. Similarly, the stock solution (1 mg/ml) of telmisartan was prepared in diluent and appropriately diluted with diluent to get working standard solution of 100 µg/ml.

Preparation of calibration curve (CC) standards and QC samples

Aliquots of 475 µl of pooled blank plasma were taken in stoppered glass tubes of capacity 20 ml. To this, 25 µl of 25 µg/ml standard stock solution of AMP (2500 ng/mL) was added and to each tube, 25 µl of working standard solution of telmisartan (as internal standard) was added. The linearity range was selected on the basis of reported peak plasma concentration (C_{max}=888.3 ng/ml) from literature, that's why the range selects in between 100 and 1500 ng/mL. The resulting solutions were vortex mixed for 1 min to get CC standards containing 100, 200, 400, 600, 800, 1000, 1200, and 1500 ng/ml of azilsartan, respectively. The QC samples were similarly prepared to contain five concentrations level (100 ng/ml lower limit of quantitation quality control, 400 ng/ml low QC [LQC], 800 ng/ml middle QC [MQC], 1200 ng/ml high QC [HQC], and 1500 upper limit of quantitation quality control).

Sample preparation using SPE procedure

There are a variety of extraction techniques available, including protein precipitation, liquid-liquid extraction, and SPE, however, we chose the SPE approach due to its high recovery rates, minimal organic solvent requirement, and lack of cross-contamination. This extraction process has a high level of selectivity and reproducibility.

Four hundred and seventy-five microliters plasma was spiked with 25 µL of each QC and calibration standard in a glass tube and vortexed thoroughly before being placed into a Strata-X cartridge that had been preconditioned with 2 mL methanol and 2 mL water before being rinsed twice with 1 mL water. After that, the cartridge was placed in a clean test tube, the analyte was eluted in 1.0 mL methanol, and a 25 L volume was injected into the HPLC system.

Bioanalytical method development

Mobile phase trials were taken on unextracted samples. Various mobile phases such as buffer: methanol (50:50 and 20:80), buffer: acetonitrile (30:70 and 55:45), phosphate buffer: acetonitrile (pH 3; 70:30), phosphate buffer: methanol:acetonitrile (55:10:35), and phosphate buffer: acetonitrile: methanol (55:25:20) were tried in which phosphate buffer: acetonitrile:methanol (60:25:15) at pH 3.2 shown by the best peak.

Furthermore, trials for the selection of appropriate IS were taken in which screening was done on the basis of structural resemblance, log *P* value, pKa value, and availability. Chromatographic trials for AMP and telmisartan were undertaken with conditions described earlier in which telmisartan gave good peak, as shown in Table 1.

Chromatographic conditions

Chromatographic analysis was carried out on a reverse phase column C_8 Inertsil column (150 × 4.6 mm, 5 µm) with mobile phase consisting of buffer: ACN:methanol (60:25:15% v/v) (pH 3.5), at a flow rate of 1.5 ml/min, retention time (RT) of analyte was 14.7 min, and the injection volume was 25 μL. The wavelength of the detector was 254 nm.

Method validation

The bioanalytical method was validated according to the US-FDA guidelines as per "Guidance for Industry: Bioanalytical Method Validation" (M10) with respect to the following parameters:[9-14]

Table 1: Chromatographic condition for the selection of IS

Drug	Mobile phase	Column used	Flow rate	Retention time	Peak shape
Azilsartan medoxomil potassium	Phosphate buffer:acetonitrile:methanol (60:25:15)	Inertsil C_8 column (150 mm×4.6 mm, 5 µm particle size)	1.5 ml/min	14.7	Good and sharp peak
Telmisartan[8]	Phosphate buffer:acetonitrile:methanol (60:25:15)	Inertsil C_8 column (150 mm×4.6 mm, 5 µm particle size)	1.5 ml/min	13.5	Good and sharp peak

System suitability test (SST)

The SST was performed before analysis of every batch of sample to ensure the reproducibility of the chromatographic system. The HPLC SST was performed by running six injections of diluted drugs and ISTD in the linear region of the **CC** and measuring the percentage coefficient of variance (%coefficient of variation [CV] or % RSD).

Acceptance Criteria: (1) RSD should not be more than 2.0% for five replicate injections of standard. (2) USP tailing factor is not more than 2.0. (3) The column efficiency as determined for plate count should be more than 2000.

Selectivity

The selectivity parameter was showed to ensure analyte separation, distinguish the analyte from other interfering substances of the biological fluid (human plasma). Selectivity was performed using six different lots of blank human plasma (four different sources human plasma, one hemolyzed plasma, and one lipemic plasma) by SPE procedure.

Sensitivity

Lower limit of quantification (LLOQ), the lowest analyte concentration of the CC s able to be measured with acceptable intraday and interday precision and accuracy, was defined by analyzing samples prepared in replicate (n = 6). The acceptance criteria considered to define AMP LLOQ was a CV that did not exceed 20%.

Calibration curves

Five standard curves of eight different concentration standards and two blank samples have been assayed. Calibration curves constructed using eight calibration standards covering the range of 0.1–1.5 μg/mL showed to be linear relationship for plasma matrix, r2 >0.997 noted between the peak height ratios and concentrations. Reason behind using this blank sample to check interference and contamination. Each CC should meet the following acceptance criteria: Deviation at LLOQ standard ≤20%, all other standards ≤15%, and at least 75% of standards should meet above criteria.

Range

It is the distance between the highest and lowest concentrations. The approach was linear, with an acceptable correlation value, and it was adequate for estimating AMP in human plasma at concentrations ranging from 0.100 to 1.500 μg/mL.

Precision and accuracy

Interday and intraday precision and accuracy were evaluated by six spiked samples at analyzing five different concentrations of AMP (0.1, 0.4, 0.8, 1.2, and 1.5 μg/mL). All the intraday and interday CV and recovery values calculated in the plasma using low, medium, and HQC samples (QC1, QC2, and QC3) and values are 0.4, 0.8, and 1.2 μg/mL. Precision and accuracy were determined within each batch by analyzing six spiked AMP samples at each QC level. Three consecutive days of investigation were used to determine intraday precession and accuracy (five series per day). Standard curves were created and analyzed on the same day to determine the concentration of each sample. Taking into account, the FDA's and the ICH's guidelines is for acceptance.

Matrix effect

Matrix effect was evaluated by comparing peak areas of extracted samples using five different lots of human plasma in triplicate at three concentration level of analyte (0.4, 0.8, and 1.2 μg/mL); percentage RSD and accuracy were calculated to check interference of matrix effect on the analyte concentration.

Recovery

Absolute recovery of analyte (AMP) was determined by comparing the AMP peak obtained from extracted plasma samples with aqueous sample of the same concentration standard. This procedure was performed for three different concentration levels of analyte low, medium, and high QC samples (QC1, QC2, and QC3) 1.5, 4.5, and 8.0 μg/mL.

RESULTS AND DISCUSSION

We found that in method development process, the SPE observed better result than the other extraction process such as protein precipitation and liquid-liquid extraction. SPE shows good separation, recovery, and efficiency.

Separation

Different mobile phases used by their different proportion in that methanol, acetonitrile, and phosphate buffer are used in various proportions; after several trials, mobile phase confirmed, buffer: ACN:methanol (60:25:15) (pH 3.2) mode of selection. Flow rate was based on peak parameters height, asymmetry, tailing, baseline drift, and run time. Flow rate was set at 1.5 mL/min. The RT for the investigated drug was found at 14.7 min, and runtime was 17 min. Different columns have been used, they show minimal effect on the resolution of the analyte, and a Waters C8 column has been finalized because of its demonstrated smoothness and reproducibility in this method. Column-to-column reproducibility was also evaluated; when injecting the sample on columns from different manufacturers and containing the same brand of packing material, only minor differences in RT were detected. The best wavelength for analyte detection was 254 nm, which resulted in significantly improved detector response. A crisp and symmetrical peak with a good baseline was obtained for the estimation of AMP, which aids the precise determination of the peak area.

SST

Number of area ratio, RT, and peak areas were also determined as a means of validation parameter. The values are shown in Table 1. The % CV of the RT calculated for the method was found to be 0.5% (acceptance limit – LT 2%) and the % CV of the area ratio was found to be 2.79% (≤5.00%), which revealed the suitability of the developed method and the optimized chromatographic conditions. These values met the requirements of USP24/NF19 46 and were, therefore, found to be satisfactory. From all five samples of AMP and IS, the parameters of system suitability are within limit and hence it is clear that the system is suitable for given method [Figure 2].

Assay Selectivity

Six blank samples were assayed to examine selectivity of the method, and there was no any interference of endogenous peak observed at the RT of AMP [Figures 3 and 4].

Sensitivity (LLOQ)

The sensitivity of the method was recognized after LLOQ value being established as 0.1 μgmL^{-1}, a value that demonstrated acceptable precision and accuracy: The overall precision did not exceed 4.05 % and in accuracy was between 85% and 110% given in Table 2. Hence, intending an easy and fast analysis capacity, this obtained LLOQ value is sufficiently low to enable the application of this bioanalytical method to several pharmacokinetic studies.

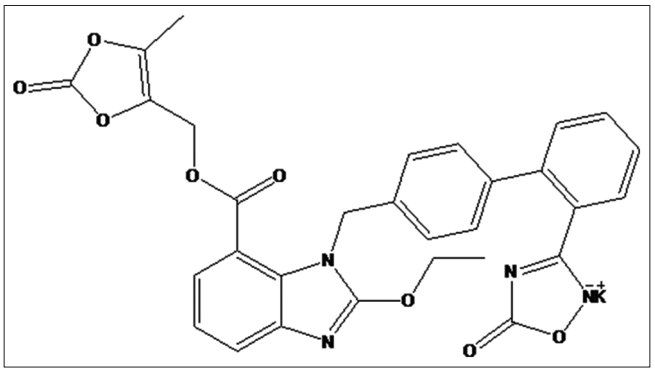

Figure 1: Structure of azilsartan medoxomil potassium

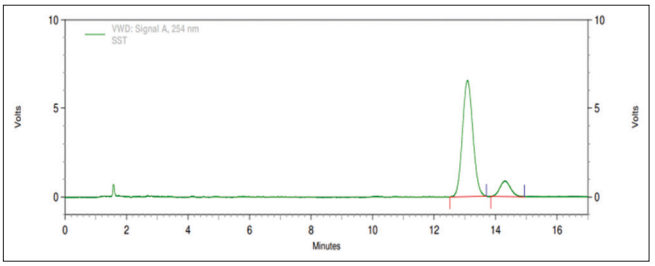

Figure 2: Chromatogram of IS and system suitability test sample

CC

Five CCs were assayed as mentioned and all of them met the acceptance criteria with good regression (r^2 = 0.997) given in Figure 2. The linearity of the method was evaluated by a CC in the concentration range of 0.1–1.5 μg/mL. CC was determined by peak area ratio versus concentration. Concentration of analyte was calculated using mean calculation formula $Y = 0.14788x + 0.00096$. Data of CCs are given in Tables 3 and 4.

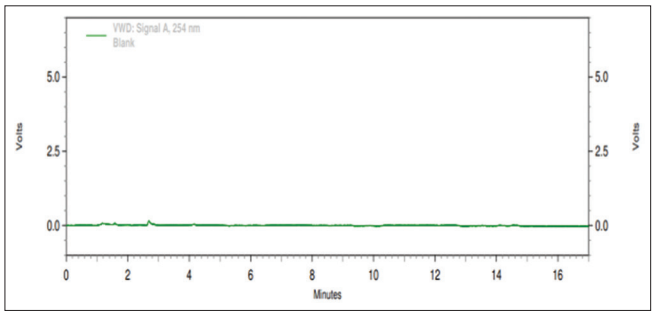

Figure 3: Chromatogram of blank plasma

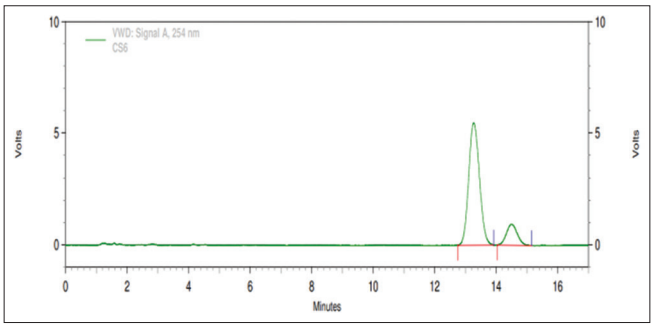

Figure 4: Chromatogram of azilsartan medoxomil potassium and IS

Precision and accuracy

RSD and accuracy of intraday precision were in the range of 3.07–13.0% and 90–102.5%, while for interday, it was found to be 0.04–13.8 and 93–109%, respectively. The results revealed good precision and accuracy given in Table 5.

Matrix effect-

Percentage of RSD and accuracy of the injected samples for LQC was 1.19–7.54 and 99.66–104.78%, for MQC 1.64–2.58 and 103.16–108.4%, and for HQC was 3.43–7.11 and 103.16–106.46%, respectively. Results indicate that there is no considerable endogenous component from blank plasma interferes in the measurement of analyte. As per result given in Table 6: {Matrix effect (values in percentage) of AMP from human plasma

Table 2: Sensitivity results of LLOQ sample (***n***=6)

S. No.	TC	Area of drug	Area of IS	Area ratio	PC	Accuracy	Limit	Precision (RSD)	Limit
LLOQ_01	0.1	32,431	2,295,801	0.014126	0.090	90	80–120%	4.05	<20%
LLOQ_02		33,108	2,191,702	0.015106	0.105	105			
LLOQ_03		32,980	2,148,723	0.015349	0.101	101			
LLOQ_04		31,576	2,269,873	0.013911	0.103	103			
LLOQ_05		33,400	2,291,701	0.014574	0.085	85			
LLOQ_06		32,543	2,145,684	0.015167	0.110	110			

LLOQ: Lower limit of quantification

Table 3: Results of five calibration curves for azilsartan medoxomil potassium determination

Added conc. (ug/mL)	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.5
Back calculated concentration (ug/mL)	0.08	0.20	0.42	0.60	0.79	1.00	1.21	1.49
	0.09	0.20	0.39	0.58	0.79	1.03	1.19	1.48
	0.09	0.19	0.38	0.58	0.82	1.00	1.19	1.48
	0.08	0.20	0.42	0.60	0.79	1.00	1.21	1.49
	0.09	0.21	0.42	0.60	0.78	0.97	1.19	1.53
Mean	0.086	0.2	0.406	0.592	0.794	1	1.198	1.494
Accuracy%	86	100	101.5	98.67	99.25	100	99.83	99.6
RSD%	6.37	3.54	4.80	1.85	1.91	2.12	0.91	1.39

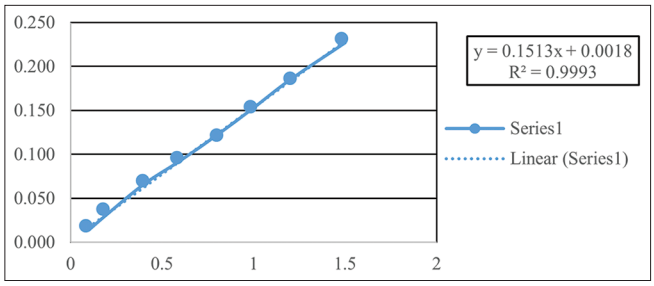

Figure 5: Calibration curve of azilsartan medoxomil potassium

Table 4: Parameter corresponding to linear regression obtained from five calibration curves of azilsartan medoxomil potassium

Calibration curve	Slope	Intercept	Correlation coefficient
1	0.1513	0.0018	0.9993
2	0.1447	0.0005	0.9992
3	0.1437	0.0006	0.9990
4	0.1513	0.0018	0.9993
5	0.1484	0.0001	0.9982
Mean	0.14788	0.00096	0.999
SD	0.003579	0.000789	0.000464
RSD%	2.4204662	0.82219	0.04641

at low (QC1), medium (QC2), and high (QC3) concentrations of the calibration range (n=3)}.

Extraction recovery

The absolute recoveries of the extraction procedure at high, medium, and low concentrations of AMP were 97.7%, 99.0%, and 99.2%, respectively, while the recovery of telmisartan was 100%. The extraction recovery determined for AMP was shown to be consistent, precise, and reproducible. As per result given in Table 7. {Recovery (values in percentage) of azilsartan medoxomil potassium from human plasma at low (QC1), medium (QC2), and high (QC3) concentrations of the calibration range (n=6) and for IS}.

Stability

Table 8 summarizes stability experiments which are the fresh sample stability, stock solution, bench top 24 h at room temperature, autosampler stability,

Table 5: Result and statistical data of intraday and interday precision (% CV) and accuracy (%) values obtained for azilsartan medoxomil potassium in human plasma at the QCLLOQ, and at the low (QC1), middle (QC2), and high (QC3) and ULOQ concentration levels representative of the calibration ranges.

S. No.	Cnominal (µg/mL−1)	Intraday				Interday			
		Mean peak are a ratio (*n*=6)	Measured (µgml^{-1})	Precision (%CV)	Accuracy (%)	Mean peak area ratio *n*=6)	Measured (µgml^{-1})	Precision (%CV)	Accuracy (%)
QC LLOQ	0.1	0.01523	0.09	3.91	90.0	0.01471	0.109	0.04	109.0
QC1	0.4	0.06201	0.41	13.00	102.5	0.06004	0.39	13.08	97.5
QC2	0.8	0.13956	0.812	4.86	101.5	0.12976	0.801	5.22	100.1
QC3	1.2	0.20936	1.21	8.77	100.8	0.19936	1.12	9.21	93.3
QC ULOQ	1.5	0.21202	1.52	3.07	101.3	0.22262	1.45	2.92	96.7

CV: Coefficient of variation, QCLLOQ: Quality control lower limit of quantification

Table 6: Matrix effect (values in percentage) of AMP from human plasma at low (QC1), medium (QC2), and high (QC3) concentrations of the calibration range (*n*=3)

LOT	0.4 (Low-quality control)			0.8 (Middle-quality control)			1.2 (High-quality control)		
	mean	%RSD	%Accuracy	mean	%RSD	%Accuracy	mean	%RSD	%Accuracy
1	0.43	10.71	107.5	0.81	6.41	101.3	1.21	4.79	100.8
2	0.39	5.2	97.5	0.85	3.4	106.3	1.26	2.3	105.0
3	0.4	1.7	100	0.89	2.8	111.3	1.28	4.1	106.7
4	0.45	2.8	112.5	0.83	4.5	103.8	1.24	3.8	103.3
5	0.41	1.2	102.5	0.86	1.1	107.5	1.29	7.2	107.5

Table 7: Recovery (values in percentage) of azilsartan medoxomil potassium from human plasma at low (QC1), medium (QC2), and high (QC3) concentrations of the calibration range (***n***=6) and for IS

S. No.	Cnominal (μgmL^{-1})	Mean area before extraction	Mean area after extraction	Recovery (%)	Mean recovery
QC1	0.4	155,254.6	140,121.3	90.3	93.7
QC2	0.8	298,133.0	275,133.0	92.3	
QC3	1.2	439,030.6	432,697.3	98.6	
IS	-	2,232,752.1	2,232,641.0	100.0	-

Table 8: Stability (values in percentage) of azilsartan medoxomil potassium in human plasma at low (QC1) and high (QC3) concentrations of the calibration range in unprocessed and processed samples under different storage conditions

S. No.	Types of sample	Stabilities	Stability value (%)	
			QC1	QC3
1		Fresh sample	102.5	103.3
2	Unprocessed sample	Stock solution stability	96.25	99.16
3		Freeze-thaw stability	104	101.25
4		Wet extract stability	99.75	100.83
5		Bench top stability	95.25	98.3
6	Processed sample	Autosampler stability	97.5	102.5

freeze-thaw stability (three cycles), and stability data of AMP. All the results showed the stability behavior during these tests and no stability-related problems occurred during the validation and stability testing [Figures 1-5].

Application

1. Bioanalytical application of AMP used in a qualitative and quantitative analysis[15]
2. This analysis important in drug discovery and development
3. This information used in a clinical, preclinical, and non-clinical studies it includes bioavailability studies, toxicology studies, pharmacological, and pharmacokinetical studies, therapeutic drug monitoring
4. Bioanalytical method validation of AMP
5. It is a documental proof of purity and identity of drug and their standards and internal standards used in daily analysis
6. It gives reproducible and reliable results and it is more precise and accurate for specific analyte interest.[16]

CONCLUSION

The RP-HPLC bioanalytical approach is a precise, specified, and simple method used for the determination by SPE of AMP in human plasma. To the best of our knowledge, this is the first description of the development and validation of AMP through the RP-HPLC process. The method involves simple extraction, separation with an internal standard, and UV detector on the reversed octylsilane column C8.The validation data have shown good accuracy and reliability, which demonstrate the reliability. For the bioavailability and bioequivalence of NDA and ANDA submission, the presently developed method could be adapted. Giving priority attention to convenient and effective economic and time aspects of the test methods used in the QC of AMP, given the methods developed for the pharmacokinetic studies of plasma samples in bioanalytical laboratories.

ACKNOWLEDGMENTS

The authors are thankful to Glenmark Pharmaceuticals Pvt. Ltd., for supplying them with the AMP; facilities were given under the Core Analyticals Pvt. Ltd., Department of Pharmaceutical Chemistry of NDMVP's College of Pharmacy, Nashik, to give a guidance facility. The authors are thankful to them for providing analytical facilities.

CONFLICTS OF INTEREST

No any conflicts of interest.

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