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METHOD DEVELOPMENT, VALIDATION, AND ESTIMATION OF AZILSARTAN KAMEDOXOMIL IN SPIKED HUMAN PLASMA

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ABSTRACT

Azilsartan Kamedoxomil (AK) was estimated from spiking human plasma using a quick and easy reverse phase high-performance liquid chromatography (RP-HPLC) technique. The internal standard Amlodipine Besylate (AB) and the analyte AK were extracted using diethyl ether. A Hypurity C18 column (50 mm x 4.6 mm, particle size 5µm) was used for the chromatographic separation, and the mobile phase was composed of buffer, acetonitrile, and methanol at a ratio of 55:25:20 v/v/v at a flow rate of 0.8 ml/min. AK was extracted from human plasma using a technique called liquid-liquid extraction. A wavelength of 249 nm was used for detection. The procedure was approved in accordance with US-FDA guidelines for selectivity, precision, accuracy, recovery, and stability. The AB retention time was determined to be 1.88 min, while AK retention time was found to be 5.1 min. The calibration curve was discovered to be linear between 0.1 and 1.5 g/mL. In tests of accuracy and precision, it was discovered that % relative error was less than 15%. Finally, the suggested method is simple to apply, swift, dependable,

and faster for analyzing AK in human plasma in a number of samples over a reasonable amount of time in a cost-effective manner.

Keywords: Amlodipine besylate, Azilsartan kamedoxomil, Liquid-liquid extraction, High performance liquid chromatography, Validation

INTRODUCTION

Azilsartan Kamedoxomil (AK) is the monopotassium salt of Azilsartan Medoxomil. A precursor medication for the angiotensin receptor antagonist and antihypertensive medicine Azilsartan. Chemically, AK **(Figure 1)** is a salt of the compound (5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl2-ethoxy-1-[2'-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl)methyl-1*H*-benzimidazole-7-carboxylate **[1, 2]**.

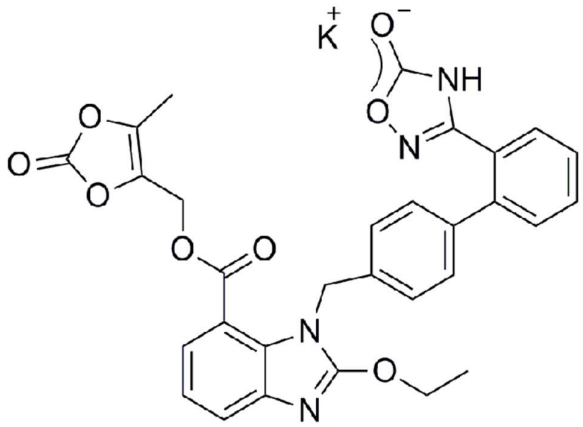

Figure 1: Structure of Azilsartan Kamedoxomil

According to literature reviews, only a few analytical techniques have been published for the quantitative analysis of AK in human plasma. These techniques include liquid chromatography and tandem mass spectrometry for stability indicating assays **[3-5]**, reverse phase high-performance liquid chromatography (RP-HPLC) method for quantification in human plasma by solid-phase extraction method **[6, 7]**, and liquid chromatography and tandem mass spectrometry for quantification in human plasma **[7, 8]**.

As a result, the goal of the current medication was to create and validate a quick, easy, and accurate RP-HPLC method with ultra-violet (UV) detection for AK quantification in human plasma utilizing the liquid-liquid extraction methodology. In order to overcome potential matrix effect-related issues and variability in recovery between analyte AK and internal standard (IS) Amlodipine Besylate (AB), we

discussed creation and validation of selectivity, sensitivity, and quick RP-HPLC techniques for quantifying AK in human plasma. The methodology provides an effective extraction process based on a liquid-liquid extraction technique with little use of organic solvents during the reconstitution step and evaporation. The technique also made sure that AK was quantified accurately and precisely as intended.

MATERIALS AND METHODS

A Shimadzu (UV2450) spectrophotometer used for measured the wavelengths. Agilent 1260 Infinity I HPLC with a UV-Vis detector and Open-LAB CDS software were used for the chromatographic analysis. The separation was performed using a Hypurity C18 (50 x 4.6mm, 5µm) column. Glenmark Pharmaceuticals Ltd., Sinnar, was provided gift sample AK. Core Analytical Pvt. Ltd., Ozar, provided the facilities AB utilized as an IS. Dr. Vasantrao Pawar Medical College, Hospital and Research Center, provided gift sample of human plasma. Core Analytical Pvt. Ltd., Nashik, provided acetonitrile, methanol, diethyl ether, ortho-phosphoric acid, HPLC grade water, n-hexane, ethyl acetate, potassium phosphate, and dichloromethane.

Preparation of standard stock solution:

AK stock solution was made by dissolving 10 mg in a 10 mL volumetric flask and then adding methanol to the mixture to bring the volume up to 10 mL.

Preparation of IS stock solution:

The stock solution of AB was prepared by dissolving 10mg in a 10 mL volumetric flask and then make volume upto 10mLwith methanol.

Preparation of working solution of AK and IS:

The AK and IS were prepared as 25µg/mL working solutions using 100µg/ mL of each standard stock solution. Buffer and acetonitrile diluent were used to adjust the volume in proportion of 55:45v/v.

Method development:

The mobile phase, that included buffer, acetonitrile, and methanol in different ratios and a pH change, was tried. Finally, it was determined that the ratio of 25:55:20v/v (pH-3.2 adjusted with ortho phosphoric acid) gave excellent separation for the peaks of AB and AK **(Figure 2)**. In addition, AK UV spectra at wavelengths between 200 and 400 nm were recorded. The selection of 249nm **(Figure 3)** as the wavelength allowed for the detection of AK with sufficient sensitivity **[9]**.

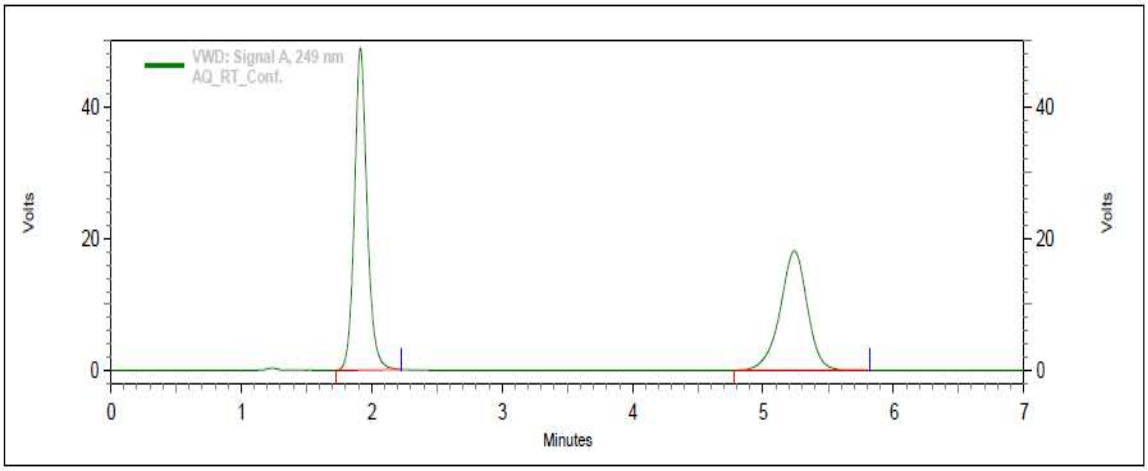

Figure 2: Chromatogram for Retention Time Confirmation of Amlodipine Besylate and Azilsartan Kamedoxomil at 249nm

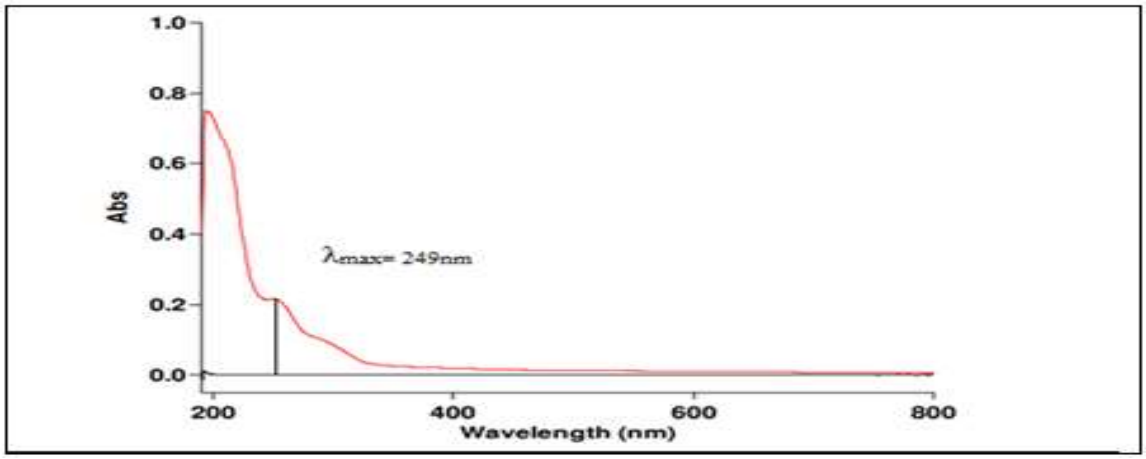

Figure 3: UV Spectrum of Azilsartan Kamedoxomil

Preparation of calibration curve (CC) standards and quality control (QC) samples:

In 20 mL stoppered glass tubes, aliquots of 475µL of pooled plasma were collected. In addition, 25µL of a 25µg/mL working standard solution of AB and 25µL of a 25µg/mL standard stock solution of AK were added to each tube. AK CC standards of 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, and 1.5µg/mL were obtained by vortexing the resultant solutions for 1min. The three concentration levels for the QC samples were 0.4 µg/mL for low quality control (LQC), 0.8µg/mL for middle quality control (MQC), and 1.2µg/mL for high quality control (HQC).

Liquid-Liquid extraction technique:

In 20 ml stoppered glass test tubes, aliquots of pooled human plasma (475µL) were taken. 25µL of 25µg/mL AB and 25µL of 25µg/mL working standard solution of AK were added to each of these test tubes. Then 3mL of diethyl ether organic solvent were added to each test tube before the test tubes were placed on an inclined shaker and agitated at 100 strokes per minute for 3 min.

Additionally, these test tubes underwent a 10 min, 3000rpm centrifugation. Each tube's organic layer was separated, transferred to a different glass tube, and evaporated to dryness while being sprinkled with nitrogen. Under ideal chromatographic conditions, the residue produced after evaporation to dryness was reconstituted with 250µL of mobile phase and 20µL was fed into the HPLC system. The chromatograms of blank plasma extracted with diethyl ether **(Figure 4)** as well as the chromatograms of AB and AK **(Figure 5)** were recorded **[10-13]**.

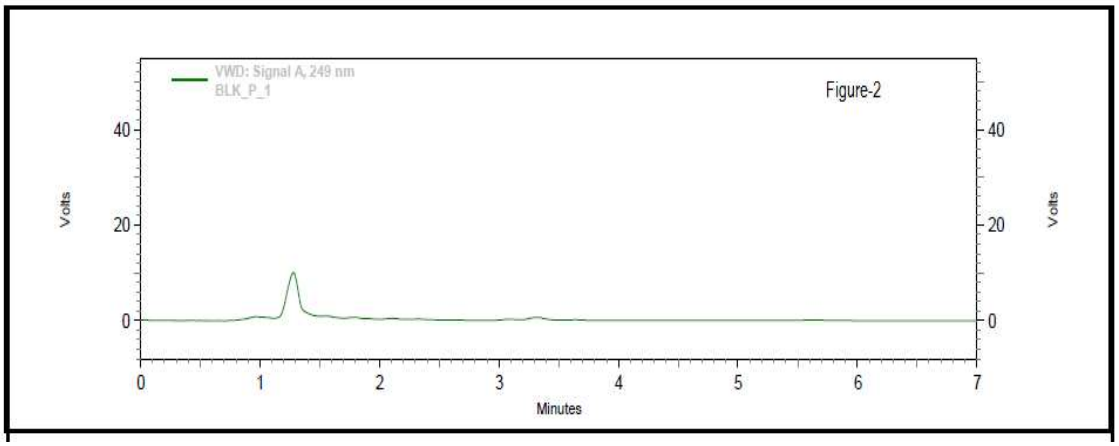

Figure 4: Chromatogram of Blank Plasma Extracted with Diethyl Ether

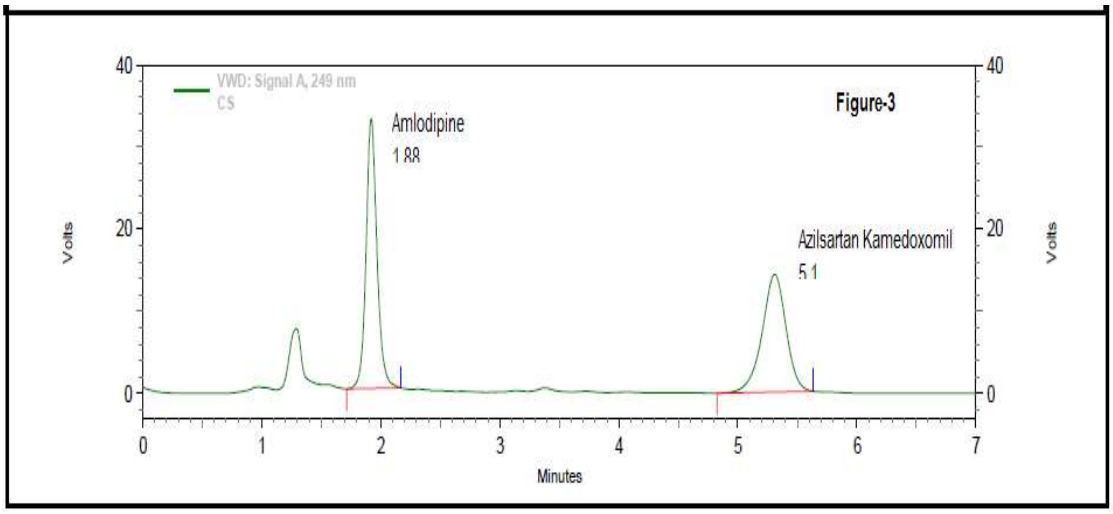

Figure 5: Chromatogram of Amlodipine Besylate and Azilsartan Kamedoxomil Extracted in Diethyl Ether

RESULTS AND DISCUSSION

Method Development:

For the development of this approach, we ran several TRIALs **(Table 1)** until the outcomes were satisfactory. Based on these tests, we used an acetonitrile, buffer, and methanol (25:55:20v/v) solution with an ortho phosphoric acid-adjusted pH of 3.2. Because it was discovered to provide excellent AB (RT- 1.88 min) and AK (RT- 5.10 min) separation **(Figure 2)**. In addition, the response for optimization was evaluated

using UV spectra of distinct medications that were captured at wavelengths between 200 and 400nm. The selection of 249nm **(Figure 3)** as the wavelength was thought to be satisfactory, allowing for the sensitivity needed to detect both drugs **[9, 12]**.

Chromatographic Conditions:

Stationary Phase: Hypurity C18 (50 x 4.6mm, 5µm)

Mobile phase: Acetonitrile: buffer: methanol (25:55:20v/v) adjusted to pH 3.2 using ortho-phosphoric acid,

Flow rate: 0.8mL/min

Wavelength: 249nm

Method Validation:

The developed method was validated as per US-FDA Guidance for industry: Bioanalytical Method Validation **[10]**.

System Suitability Test:

An analysis of five replicates of a mixture including 100µg/mL of AK and 100µg/mL of IS AB was done in order to determine the appropriateness of the system. Each replicate's column efficiency, peak asymmetry, and resolution were calculated **(Table 2)**.

Selectivity:

By contrasting the peak areas provided by lower limit of quantification (LLOQ) samples with the blank responses of plasma from six different sources, selectivity at the LLOQ at 0.1µg/mL was investigated. Six separate samples of blank plasma were examined for selectivity and lack of interference with endogenous components at the time of AK and IS retention. The results demonstrated zero percentage interference.

Sensitivity:

The LLOQ value of 0.1µg/mL, which displayed adequate precision and accuracy as indicated in **Table 3**, was obtained after the sensitivity of the method was identified.

Linearity:

Through the construction of calibration curves utilizing eight calibration standards that covered the concentration range of 0.1-1.5µg/mL, linearity was examined. The three calibration curves were run in duplicate. As demonstrated in **Table 4**, **5**, and **6**, peak height ratios of AK to AB IS versus the nominal concentration were used to measure calibration curves. The calibration curves for the intraday validation, interday validation, and stability of AK were displayed in **Table 4**, **5**, and **6**, respectively.

Accuracy and Precision:

The plasma six replicates of five different QC sample concentrations such as LLOQ, low QC1, middle QC2, high QC3, and upper limit of quantification (ULOQ) level were used to investigate intraday validation. The test was repeated the next day to ensure the validity of the inter-day measurement. As indicated in **Table 7** and **8**, the guidelines requirements for precision are a percentage CV value and accuracy percentage recovery less than or

equal to 15% (or 20% in LLOQ) is in the range of 85 to 115% (in 80 to 120% LLOQ), respectively.

Extraction Recovery:

The absolute recovery in the plasma using three concentrations of the AK and IS was determined (QC1, QC2 and QC3). The final recovery value is the average of the six divergent replicates for each individual concentration, as shown in **Table 9**. The values were obtained by calculating the ratio between the peak areas from the extracted QC samples and the peak areas corresponding to the direct injection of non-extracted solutions (Aqueous QC samples) at the same theoretical concentration **[13-15]**.

Stability:

To compare the stability of raw and processed samples of AK in plasma. After being subjected to various experimental circumstances that mimic sample handling and storage, QC1 and QC3 samples were evaluated in duplicate (n=6). A study on the stability of stock solutions was first carried out. After three freeze-thaw cycles at -20 °C, aliquots of the QC1 and QC3 samples were stored there for 24 hours, thawed naturally at ambient temperature for roughly 30 minutes, and then refrozen again for another 24 hours under the same conditions to complete the three cycles. Wet extract reconstituted samples were stored for 6 hours at 2-8^{0}C before being injected. Using the bench-top stability approach, spiked plasma samples were taken out of the freezer and held at room temperature for 6 hours. Spiked plasma samples were injected after 6 hours. AK auto sampler stability was also investigated in replicate (n=6) in processed samples at room temperature. **Table 10** presented every stability result.

Table 1: HPLC Trials for Selection of Mobile Phase

No. of Trials	Mobile Phase	Ratio (V/V)	pH	Flow Rate (ml/ min)	Observation
Trial 1	Acetonitrile: Buffer	70:30	3.2	1.4	It shown bad peak shapes and less plates count
Trial 2	Acetonitrile: Buffer	70:30	3.2	1.0	It shown good shape but run time is 30min
Trial 3	Acetonitrile: Buffer	60:40	3.2	0.8	It shown less theoretical plate count
Trial 4	Acetonitrile: Buffer	70:30	3.2	0.8	Retention time is below 1min and less plate count
Trial 5	Acetonitrile: Buffer	50:50	3.2	0.8	The trial show bad peak
Trial 6	Acetonitrile: Buffer	55:45	3.2	1	The trial show bad peak shapes
Trial 7	Acetonitrile: Buffer: Methanol	20:60:20	3.2	0.5	The trial show bad peak shapes
Trial 8	Acetonitrile: Buffer: Methanol	25:55:20	3.2	0.8	Two peaks was observed with good resolution

Table 2: System Suitability Data

Parameters	Azilsartan Kamedoxomil Mean ± SD (n = 5)	Amlodipine Besylate Mean ± SD (n = 5)
Retention Time	5.02	1.88
Area	2089252	5452523
Theoretical Plate	3473	2239
Peak Asymmetry	0.96	1.11

Table 3: Results of Sensitivity of Azilsartan Kamedoxomil

Sr. No.	Conc. (µg/mL)	Area of Drug	Area of IS	Area Ratio	Plasma Conc.	Accuracy	Limit	Precision (RSD)	Limit (%)
1	0.1	31431	2295801	0.01369	0.105	105	80-120	5.26	<20
2	0.1	33108	2191702	0.01510	0.090	90			
3	0.1	31980	2148723	0.01488	0.101	101			
4	0.1	30576	2269873	0.01347	0.102	102			
5	0.1	34400	2291701	0.01501	0.085	85			
6	0.1	29543	2145684	0.01376	0.096	96			

Table 4: Linearity Results of Azilsartan Kamedoxomil Intraday Validation

Sr. No.	Conc. (µg/mL)	Area of Drug	Area of IS	Area Ratio	Plasma Conc.	Slope	Intercept	Correlation Coefficient (r^2)
1	0.1	311550	2291701	0.014	0.08	0.1608	0.0019	0.9995
2	0.2	726960	2277778	0.032	0.020			
3	0.4	145704	2266291	0.064	0.042			
4	0.6	209198	2240282	0.093	0.060			
5	0.8	287487	2276788	0.126	0.79			
6	1.0	351112	2229003	0.158	1.0			
7	1.2	428116	2262982	0.189	1.21			
8	1.5	535527	2216587	0.242	1.49			

Table 5: Linearity Results of Azilsartan Kamedoxomil Interday Validation

Sr. No.	Conc. (µg/mL)	Area of Drug	Area of IS	Area Ratio	Plasma Conc.	Slope	Intercept	Correlation Coefficient (r^2)
1	0.1	381550	2291701	0.017	0.10	0.1509	-0.00021	0.9998
2	0.2	726960	2277778	0.032	0.20			
3	0.4	142704	2266291	0.063	0.40			
4	0.6	209198	2240282	0.093	0.60			
5	0.8	277487	2276788	0.122	0.79			
6	1.0	341112	2229003	0.153	1.00			
7	1.2	418116	2262982	0.185	1.21			
8	1.5	525527	2316587	0.227	1.49			

Table 6: Linearity Results of Azilsartan Kamedoxomil Stability Studies

Sr. No.	Conc. (µg/mL)	Area of Drug	Area of IS	Area Ratio	Plasma Conc.	Slope	Intercept	Correlation Coefficient (r^2)
1	0.1	311500	2291701	0.014	0.09	0.1471	0.0001	0.9996
2	0.2	696960	2277778	0.031	0.21			
3	0.4	535704	2266291	0.060	0.40			
4	0.6	199198	2240282	0.089	0.60			
5	0.8	263697	2276788	0.116	0.78			
6	1.0	325112	2229003	0.146	0.98			
7	1.2	398116	2262982	0.176	1.19			
8	1.5	515527	2316587	0.23	1.50			

Table 7: Data of Intraday Precision and Accuracy Obtained for Azilsartan Kamedoxomil Human Plasma

Sr. No.	Nominal conc. (µg/mL)	Mean peak area ratio (n=6)	Intraday		
			Measured (µg/ml)	Precision (%CV)	Accuracy (%)
LLOQ	0.1	0.01623	0.11	3.67	110.0
QC1	0.4	0.07201	0.39	11.20	97.5
QC2	0.8	0.12956	0.802	5.23	100.3
QC3	1.2	0.19936	1.05	9.21	87.5
ULOQ	1.5	0.21202	1.51	3.07	100.7

Table 8: Data of Interday Precision and Accuracy Obtained for Azilsartan Kamedoxomil Human Plasma

Sr. No.	Nominal conc. (µg/mL)	Mean peak area ratio (n=6)	Interday		
			Measured (µg/mL)	Precision (%CV)	Accuracy (%)
LLOQ	0.1	0.015106	0.09	0.04	90.0
QC1	0.4	0.066066	0.41	0.12	102.5
QC2	0.8	0.13336	0.81	0.05	101.3
QC3	1.2	0.189142	1.109	0.10	92.4

ULOQ	**1.5**	**0.222181**	**1.46**	**0.03**	**97.3**

$$\%Absolute\ Recovery = \frac{Response\ of\ analyte\ spiked\ into\ matrix\ (processed)}{Response\ of\ analyte\ of\ pure\ standard\ (unprocessed)} \times 100$$

Table 9: Recovery of Azilsartan Kamedoxomil at Three Different Quality Control Level

Sr. No.	Nominal conc. (µg/mL)	Mean area before extraction	Mean area after extraction	Recovery (%)	Mean Recovery (%)
QC1	**0.4**	**144254.7**	**120838.4**	**83.8**	**85.3**
QC2	**0.8**	**308133.0**	**235133.2**	**76.3**	
QC3	**1.2**	**448030.3**	**439697.3**	**95.9**	

Table 10: Results of stability study of Azilsartan Kamedoxomil

Sr. No	Type of sample	Stability	Stability Value (% nominal)	
			LQC (QC1)	HQC (QC2)
1	**Unprocessed stability**	**Stock Solution**	**100.5**	**102.5**
		Freeze-thaw stability	**97.2**	**101.6**
		Wet extract	**97.25**	**100.8**
		Bench top stability	**99.7**	**100.8**
2	**Processed stability**	**Auto sampler stability**	**102.5**	**102.0**

CONCLUSION

The present research work describes a straight forward, speed, precise, and liquid-liquid extraction method for quantifying Azilsartan Kamedoxomil in spiking human plasma. The suggested approach effectively measures Azilsartan Kamedoxomil in spiked human plasma at concentrations between 0.1 and 1.5µg/mL and complies with US-FDA regulations. The calibration curve was discovered to be linear between 0.1 and 1.5 g/mL. It was discovered that % relative error was less than 15%. Finally, the suggested method is simple to apply, swift, dependable, and faster for analyzing Azilsartan Kamedoxomil in human plasma in a number of samples over a reasonable amount of time in a cost-effective manner.

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