WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 15, 467-478. Research Article ISSN 2277– 7105

# ANALYTICAL METHOD VALIDATION AND QUANTIFICATION OF AZILSARTAN KAMEDOXOMIL DRUG SUBSTANCE AND ITS RELATED SUBSTANCES BY USING REVERSE PHASE - LIQUID CHROMATOGRAPHIC METHOD

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Article Received on
03 June 2018,

Revised on 23 June 2018,
Accepted on 14 July 2018,

DOI: 10.20959/wjpr201815-12834

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## ABSTRACT

An efficient and selective RP-HPLC method was developed and validated for the determination of process and degradation impurities in Azilsartan kamedoxomil (AKM) drug substances which were identified and characterized by LCMS, FTIR, 1H NMR, C NMR techniques. Chromatographic separation was achieved on Sunfire C18, (250 mm x 4.6 mm, 5 µm) column thermostated at 20°C under gradient elution by a binary mixture of potassium dihydrogen orthophosphate (pH-3.0) and ACN at a flow rate of 1.0 ml/min. A photodiode array (PDA) detector set at 254 nm was used for detection. Stress testing (forced degradation) was carried out under acidic, alkaline, oxidative, photolytic, thermal and humidity conditions. AKM drug substance is susceptible to degrade under acid, alkaline, oxidative hydrolysis and humidity stress condition. The developed method is validated with respect to sensitivity (LOD and LOQ), linearity, precision, accuracy, and robustness, and can be implemented for routine quality control analysis and stability testing of AKM.

**KEYWORDS:** Azilsartan medoxomil, Stability indicating, Method development and Validation, Degradation impurities.

## INTRODUCTION

Azilsartan kamedoxomil(Figure 1) is an angiotensin II receptor antagonist which has the chemical names (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 monopotassium salt and 1H-Benzimidazole-7-carboxylic acid, 1-[[2'-(2,5-dihydro-5-oxo-1,2,4-oxadiazol-3-yl)[1,1'-biphenyl]-4-yl]methyl]-2-ethoxy-,(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester.[1]

Azilsartan kamedoxomil is rapidly hydrolysed to the active moiety azilsartan by esterases in the gastrointestinal tract and/or during drug absorption. The enzyme carboxymethylenebutenolidase is a recently discovered hydrolysis mechanism for azilsartan kamedoxomil in the intestine and liver.[2-4] Azilsartan is an inverse agonist of the AT1 receptor is a highly potent, selective and competitive antagonist of the angiotensin II type 1 receptor. Molecular basis of Azilsartan kamedoxomil reveals that may be responsible for its clinical Efficacy.[5]

Both the United states Pharmacopoeia (USP) and the European Pharmacopoeia (EP) have not published monographs for this drug substances. Literature survey reveals that Azilsartan medoxomil can be estimated by RP-HPLC in combination with other drugs. Stability indicating RP-HPLC method and one more reported method in human plasma by solid phase extraction procedure.[6,7,8] U.V. Spectrophotometric method reported for the estimation of Azilsartan medoxomil in bulk and pharmaceutical dosage forms.[9] But no literature has been reported for the determination of Azilsartan kamedoxomil impurities.

Hence the method described in this paper was developed to determine impurities of Azilsartan kamedoxomil and has the ability of good separation of each impurity from Azilsartan medoxomil.

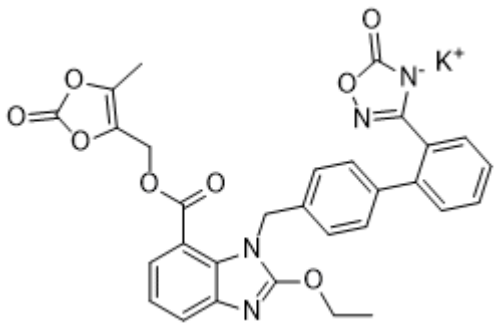



**Fig. 1: Chemical structure of Azilsartan kamedoxomil.**

## Experimental

### Chemicals, Reagents, Standards, Samples and Impurities

Pure drug Azilsartan kamedoxomil and its impurities was provided by our APL Research Centre-II. (A Division of Aurobindo Pharma Ltd). All the reagents and chemicals used were of analytical grade. Potassium dihydrogen orthophosphate, orthophosphoric acid, acetonitrile, tetrahydrofuran were procured from Merck (India) limited and pure Milli-Q water was prepared with help of Millipore purification system.

### High performance liquid chromatography (HPLC)

Chromatographic separations were performed on HPLC system with Waters alliance 2695 separation module equipped with 2996 photodiode array detector with Empower software handling system (Waters Corp., Milford, MA01757, USA). The analysis was carried out on Sunfire C 18, 250 mm x 4.6 mm, 5 µm particle size column.

Mobile phase A was phosphate buffer Ph-3.0 (prepared by dissolving 1.0 g of potassium dihydrogen orthophosphate in 1000 ml of water, and pH was adjusted to 3.0 ± 0.05 using orthophosphoric acid). Mobile phase B was acetonitrile. Diluent was prepared by mixing of phosphate buffer pH-3.0 and acetonitrile in the ratio of 1: 1 v/v. Injection volume 20 µl, flow rate was 1.0 ml/min, column oven temperature was 20 °C and autosampler temperature was 6°C. UV detection was carried out at 254 nm and data acquisition time 55 min. The gradient programme was as follows:

Time $_{(min)}$/A(v/v):B(v/v); $T_{0.01}$/65:35, $T_{35}$/40:60, $T_{45}$/20:80, $T_{55}$/20:80, $T_{57}$/65:35, $T_{65}$/65:35

### Preparation of solutions

### Standard solution

A stock solution of Azilsartan kamedoxomil (300 µg/ml) was prepared by dissolving appropriate amount of substance in the diluent. Working solution of 1.5 µg/ml was prepared from this stock solution for the related substances determination.

### Sample solution

Prepared a Concentration of 1000 µg/ml of sample solution with diluent.

### Method validation

**Specificity:** Specificity is the capability of the method to measure the analyte response of its potential impurities. The specificity of developed HPLC method for Azilsartan kamedoxomil

was carried out in the presence of its impurities I to V and also verified the blank interference for the accurate measure of impurities.

As a part of specificity, stress studies[10,11] were carried out for Azilsartan kamedoxomil drug substance to prove that stability in degradation carried out in Acid (0.5M/85°C), Base(0.5M/85°C), 30% hydrogen peroxide solution, photolytic (white fluorescent light, 1.2 million LUX hours and UV light, 200 watt-hours/$m^2$), thermal (105°C) and humidity ( 90% RH/25°C) according to ICH option 2 of Q1B. These stress samples were analysed by HPLC using proposed method at test concentration to exhibit the ability of the method to separate individual impurities and its degradation impurities at a quantification level. The peak purity test was carried out for the Azilsartan medoxomil (AM) peak by using PDA detector in the stress samples.

**Linearity / LOD & LOQ:** The limit of detection (LOD) and limit of quantification (LOQ) for impurities were determined based on the residual standard deviation of a regression line and slope method by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at about LOQ level by injecting impurity-I, impurity-II, impurity-III, impurity-IV and impurity- V calculating % RSD of the areas of each impurity. Linearity solutions were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at six concentration levels from LOQ to around 150% of impurity specification level (i.e 0.15%).

**Accuracy:** The accuracy study of the impurities were carried out in triplicate at LOQ, 50%,100%, and 150% specification level (0.15%). The sample available for validation work, do not show the presence of impurity-1, impurity-2, impurity-3, impurity-4 and impurity-5. Standard addition and recovery experiments were conducted to determine the accuracy of the related substance method for the quantification of all five impurities in the drug substance sample. The study was performed out by spiking each impurity at LOQ, 0.075, 0.15, and 0.225% in the sample solution (1000µg/mL). The percentages of recoveries for impurity-I, impurity- II, impurity-III, impurity-IV and impurity-V were calculated from amount added and amount found values.

**Precision:** The method precision of the related substances method was performed by two analyst by injecting six individual preparations of azilsartan kamedoxomil with test

concentration spiked with 0.15% level of impurities on different days, different columns and on different instruments.

**Robustness:** To establish the robustness of the method, experimental conditions were deliberately changed, and the resolution between these impurities was evaluated. System suitability solution and sample solution spiked with known related substances at specification level were prepared as per test method, and injected into HPLC at different deliberately varied conditions to evaluate system suitability and method's ability to remain unaffected. The flow rate was 1.0 mL/min. To study the effect of flow rate on the resolution, flow rate was changed by ±10% from 0.9 to 1.1 mL/min. The effect of pH on resolution of impurities was studied by varying ±0.2 pH units (between 2.8 and 3.2). The effect of the column temperature on resolution was studied by varying ±5°C (15°C and 25°C). The effect of the percent organic strength on resolution was studied by varying % of organic in mobile phase gradient composition by -2% and +2% absolute by keeping the remaining method conditions constant as mentioned in the method.

**Stability of standard and sample solutions**

Standard solution and sample solution spiked with related substances at specification level were prepared as per test method and analysed initially and different time intervals by keeping the solution at room temperature(~25°C) and at refrigerator temperature(~6°C).

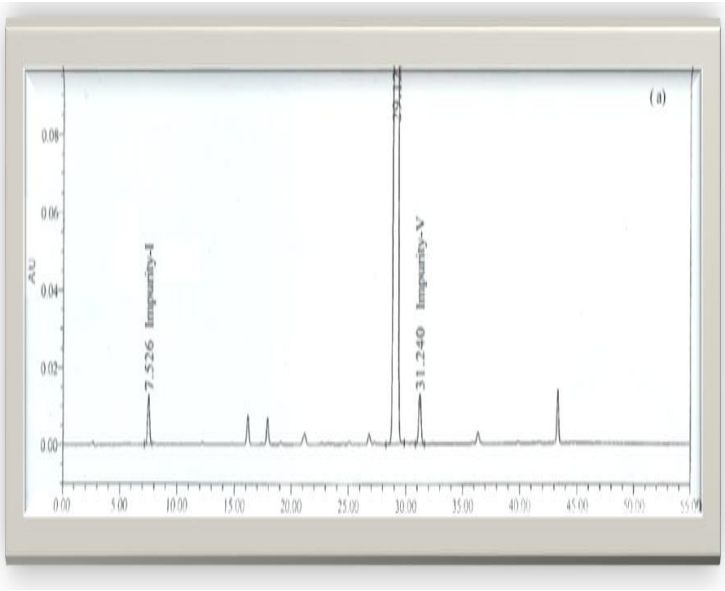
## RESULTS AND DISCUSSION

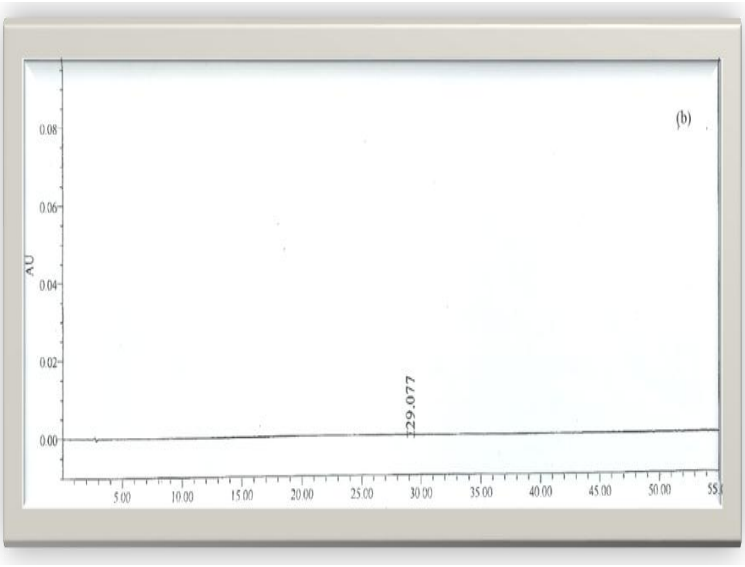
**Optimization of HPLC conditions:** The important aspect of HPLC method is to separate azilsartand medoxomil from its five impurities. These Impurities were co eluted using different stationary phases such as C8, C18, phenyl, and cyano columns tried with different mobile phases which containing buffers such as phosphate and acetate with different pH(between 2-6) and using organic modifiers like acetonitrile, methanol in the mobile phase.

Finally the HPLC method was achieved with good separation of azilsartan medoxomil from impurities using Sunfire C18, 250mm x 4.6mm, 5µm column. The mobile phase consists of phosphate buffer with pH-3.0 (Mobile phase A) and acetonitrile (Mobile phase B) with gradient program Time $_{(min)}$/A(v/v):B(v/v); $T_{0.01}$/65:35, $T_{35}$/40:60, $T_{45}$/20:80, $T_{55}$/20:80, $T_{57}$/65:35, $T_{65}$/65:35. The flow rate of the mobile phase is 1.0 mL/min at 20°C column oven temperature.

Impurities IV and V achieved better resolution with respect to azilsartan medoxomil by the above chromatographic conditions. Hence resolution of 3 was kept between azilsartan medoxomil and impurity V in the system suitability criteria. The retention time of azilsartan medoxomil was about 29 min and remaining impurities are as around relative retention of 0.26, 0.56, 0.62, 0.92 & 1.07 respectively.

The typical HPLC chromatograms of spiked sample, system suitability and diluent are shown in Fig 2. The system suitability results are also given in the (Table 1) and the developed HPLC method was found to be specific for azilsartan medoxomil and its five impurities.



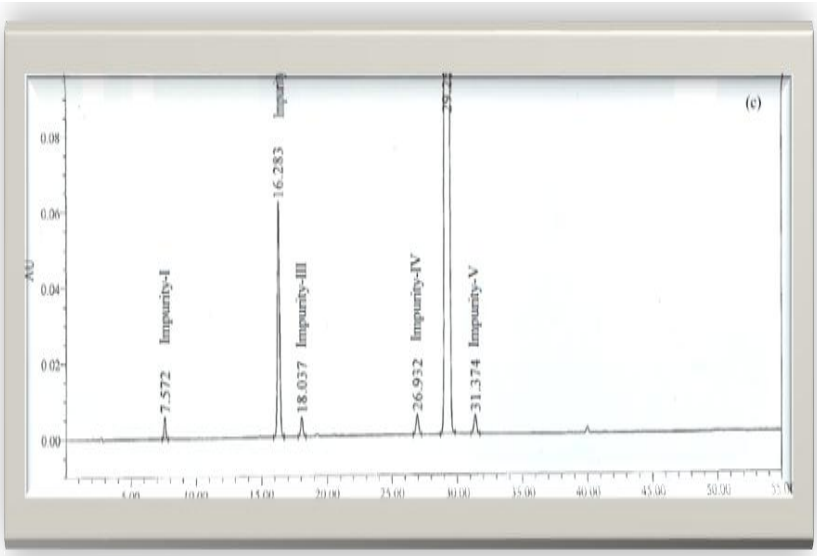
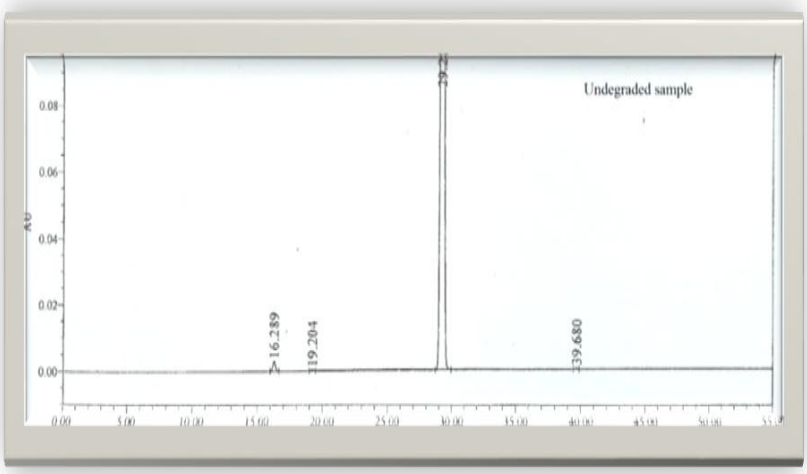
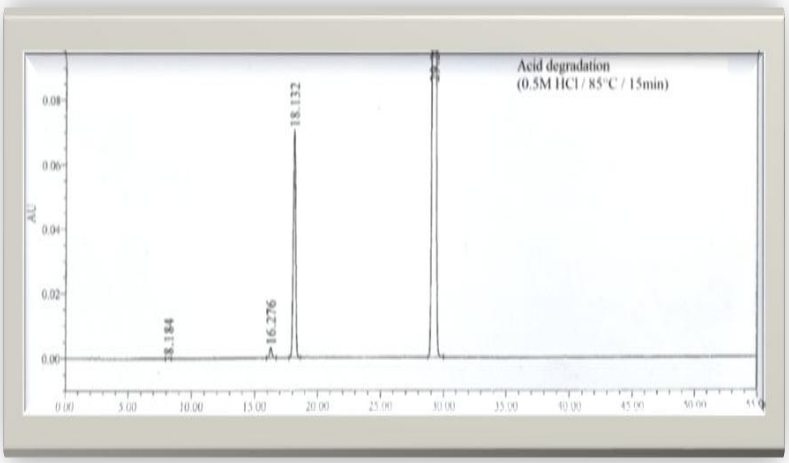


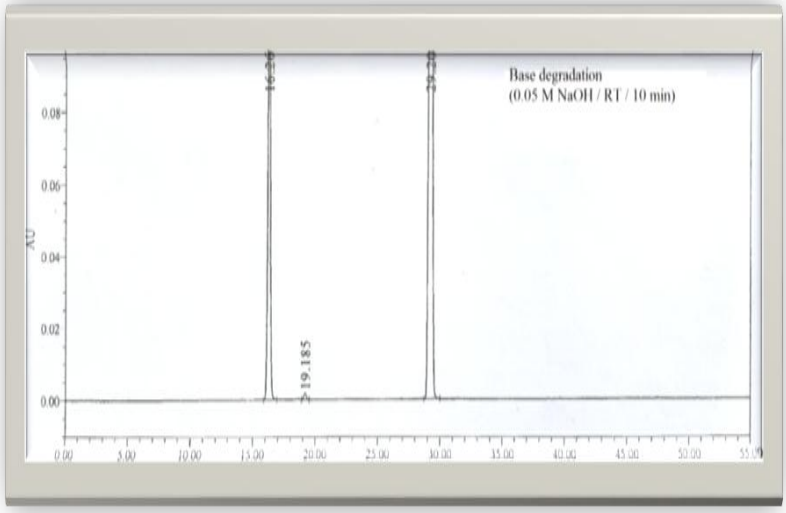

**Fig. 2: A Typical HPLC chromatogram of (a) System suitability, (b) Diluent and (c) Azilsartan medoxomil and its impurities.**

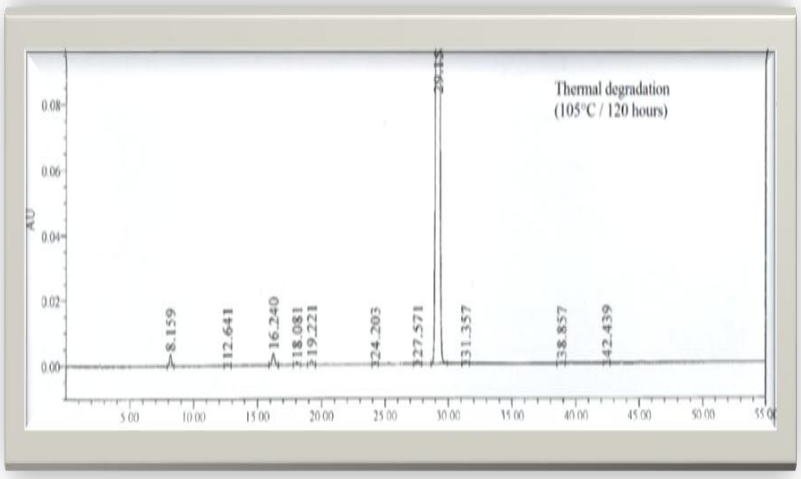
**Results of forced degradation**

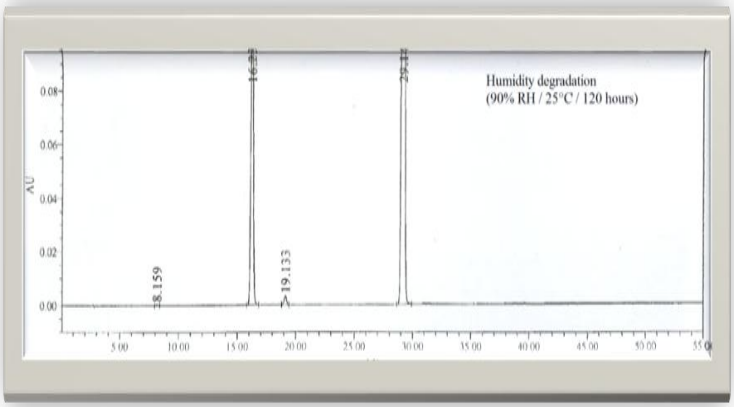
Azilsartan kamedoxomil drug substance is susceptible to degrade under acid, alkaline, oxidative hydrolysis and humidity stress conditions whereas, it is found to slightly degradation in photolytic and thermal stress condition. Based on the above observations, it can be concluded that the origin of impurity II and III are degradant. Further, the peak purity data (Table. 1) of Azilsartan medoxomil peak from every degradation sample showed that it is homogeneous, and there are no co-eluting peaks. This shows the stability-indicating power of the developed HPLC method. The typical HPLC chromatograms of forced degradation studies are shown in Fig 3.




Acid degradation
(0.5M HCl / 85°C / 15min)
18.132
16.276
18.184
AU


Base degradation
(0.05 M NaOH / RT / 10 min)
19.185
AU


Thermal degradation
(105°C / 120 hours)
8.159
12.641
16.240
18.081
19.221
24.203
27.571
31.357
38.857
42.439
AU



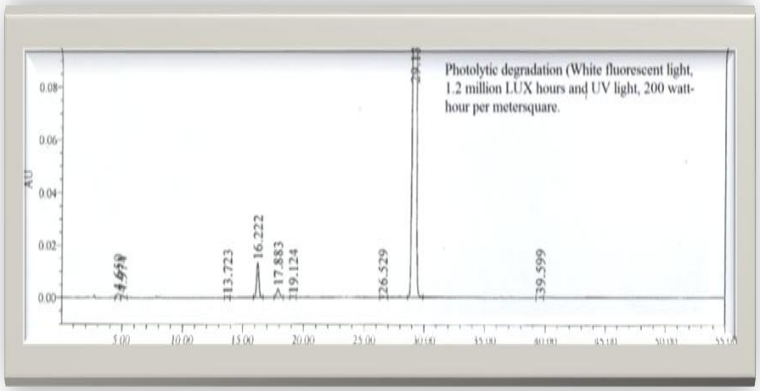




**Table 1: Specificity Data And Stress Conditions Of Azilsartan Medoxomil And Its Impurities.**

| Name | RT(min) | RRT | Peak purity | |
|---|---|---|---|---|
| | | | Purity angle | Purity threshold |
| Impurity I | 7.572 | 0.26 | 0.290 | 0.601 |
| Impurity II | 16.283 | 0.56 | 0.074 | 0.239 |
| Impurity III | 18.037 | 0.62 | 0.240 | 0.636 |
| Impurity IV | 26.932 | 0.92 | 0.249 | 0.622 |
| Azilsartan medoxomil (AM) | 29.254 | 1.00 | 0.033 | 0.243 |
| Impurity V | 31.374 | 1.07 | 0.268 | 0.658 |

| Degradation mechanism | Degradation condition | Area | Degradation (%) | Peak purity | |
|---|---|---|---|---|---|
| | | | | Purity angle | Purity threshold |
| - | Undegraded sample | 8002952 | - | 0.012 | 0.238 |
| Acid | 0.5M HCl / 85°C / 15 min | 7180251 | 10.2 | 0.013 | 0.236 |
| Base | 0.05M NaOH / RT /10min | 6399040 | 20.0 | 0.013 | 0.233 |
| Peroxide | 30% $H_2O_2$ / RT / 5min | 6875012 | 14.0 | 0.015 | 0.259 |
| Thermal | 105°C / 120 hours | 7987192 | 0.2 | 0.013 | 0.238 |
| Photolytic | White Fluorescent light, 1.2 million LUX hours and UV light, 200 watt-hours / $m^2$ | 7798098 | 2.5 | 0.014 | 0.245 |
| Humidity | 90%RH / 25°C / 120hours | 6351202 | 20.5 | 0.013 | 0.232 |

**Table 2: Summary Of Linearity / Lod-Loq Experiments.**

| Name | Response factor | Linearity range(µg/ml) | Correlation Coefficient | LOD (%w/w) | LOQ (%w/w) | LOD (%RSD) | LOQ (%RSD) |
|---|---|---|---|---|---|---|---|
| Impurity I | 1.01 | 0.150-2.292 | 0.9999 | 0.005 | 0.016 | 11.8 | 5.4 |
| Impurity II | 0.80 | 0.142-23.241 | 0.9999 | 0.005 | 0.015 | 15.5 | 5.1 |
| Impurity III | 0.93 | 0.152-2.284 | 0.9999 | 0.005 | 0.016 | 10.3 | 3.6 |
| Impuirty IV | 0.80 | 0.141-2.296 | 0.9999 | 0.005 | 0.016 | 22.5 | 5.8 |
| Azilsartan medoxomil | 1.00 | 0.153-22.696 | 0.9999 | 0.005 | 0.016 | 14.1 | 8.2 |
| Impurity V | 0.82 | 0.151-2.233 | 0.9999 | 0.005 | 0.016 | 15.1 | 2.4 |

**Table 3: Precision Data.**

| Method precision (Analyst – I) (n=6) | | | | |
|---|---|---|---|---|
| **Name** | **Mean (n)** | **SD** | **% RSD** | **95% Confidence interval (±)** |
| Impurity I | 0.146 | 0.002 | 1.4 | 0.002 |
| Impurity II | 1.655 | 0.008 | 0.5 | 0.008 |
| Impurity III | 0.146 | 0.001 | 0.7 | 0.001 |
| Impuirty IV | 0.155 | 0.001 | 0.6 | 0.001 |
| Impurity V | 0.158 | 0.001 | 0.6 | 0.001 |
| **Intermediate precision (Analyst – II) (n=6)** | | | | |
| Impurity I | 0.145 | 0.001 | 0.7 | 0.001 |
| Impurity II | 1.715 | 0.031 | 1.8 | 0.033 |
| Impurity III | 0.143 | 0.001 | 0.7 | 0.001 |
| Impuirty IV | 0.148 | 0.002 | 1.4 | 0.002 |
| Impurity V | 0.151 | 0.001 | 0.7 | 0.001 |
| **Overall statistical analysis data(n=12)** | | | | |
| Impurity I | 0.145 | 0.002 | 1.4 | 0.001 |
| Impurity II | 1.685 | 0.038 | 2.3 | 0.024 |
| Impurity III | 0.145 | 0.002 | 1.4 | 0.001 |
| Impuirty IV | 0.152 | 0.004 | 2.6 | 0.003 |
| Impurity V | 0.154 | 0.004 | 2.6 | 0.003 |

**Table 4: Accuracy Data.**

| Name | (%)Level | Amount added (n=3) (%w/w) | Amount found (n=3) (%w/w) | % Recovery (n=3) |
|---|---|---|---|---|
| Impurity I | LOQ | 0.0156 | 0.0159 | 101.9 |
| | 50 | 0.073 | 0.072 | 98.6 |
| | 100 | 0.147 | 0.146 | 99.3 |
| | 150 | 0.220 | 0.217 | 98.6 |
| Impurity II | LOQ | 0.0158 | 0.0160 | 101.3 |
| | 50 | 0.740 | 0.778 | 105.1 |
| | 100 | 1.481 | 1.489 | 100.5 |
| | 150 | 2.221 | 2.241 | 100.9 |
| Impurity III | LOQ | 0.0161 | 0.0163 | 101.2 |
| | 50 | 0.074 | 0.074 | 100 |
| | 100 | 0.148 | 0.146 | 98.6 |
| | 150 | 0.222 | 0.220 | 99.1 |
| Impurity IV | LOQ | 0.0150 | 0.0152 | 101.3 |
| | 50 | 0.073 | 0.075 | 102.7 |
| | 100 | 0.146 | 0.147 | 100.7 |
| | 150 | 0.219 | 0.219 | 100 |
| Impurity V | LOQ | 0.0159 | 0.159 | 100 |
| | 50 | 0.072 | 0.074 | 102.8 |
| | 100 | 0.145 | 0.145 | 100 |
| | 150 | 0.217 | 0.218 | 100.5 |

**Table 5a: Robustness Data of System Suitability.**

| Condition | Variation | System suitability | | |
|---|---|---|---|---|
| | | USP resolution NLT 3.0 btw AM and Imp-V | USP plate count NLT 30000 for AM | USP tailing NLT 1.5 for AM |
| STP | - | 5.7 | 107500 | 1.0 |
| Flow | -10% | 5.8 | 112616 | 1.0 |
| | +10% | 5.5 | 100675 | 1.0 |
| Wave length | -3nm | 5.7 | 107570 | 1.0 |
| | +3nm | 5.7 | 110997 | 1.0 |
| % of Organic in gradient composition | -2% absolute | 5.6 | 124414 | 1.0 |
| | +2% absolute | 5.7 | 90441 | 1.0 |
| Column oven temperature | -5°C | 5.0 | 93228 | 1.0 |
| | +5°C | 6.0 | 105008 | 1.0 |
| pH | -0.2 unit | 5.3 | 89195 | 1.0 |
| | +0.2 unit | 5.4 | 92758 | 1.0 |

**NLT: Not less than**

**Table 5b: Robustness Data of Spiked Sample.**

| Condition | Variation | RRT | | | | | |
|---|---|---|---|---|---|---|---|
| | | Imp-I | Imp-II | Imp-III | Imp-IV | AM | Imp-V |
| STP | - | 0.25 | 0.56 | 0.62 | 0.92 | 1.00 | 1.07 |
| Flow | -10% | 0.26 | 0.57 | 0.63 | 0.92 | 1.00 | 1.07 |
| | +10% | 0.24 | 0.55 | 0.61 | 0.92 | 1.00 | 1.07 |
| Wave length | -3nm | 0.25 | 0.56 | 0.62 | 0.92 | 1.00 | 1.07 |
| | +3nm | 0.25 | 0.56 | 0.62 | 0.92 | 1.00 | 1.07 |
| % of Organic in gradient composition | -2% absolute | 0.28 | 0.58 | 0.64 | 0.92 | 1.00 | 1.07 |
| | +2% absolute | 0.23 | 0.54 | 0.60 | 0.92 | 1.00 | 1.08 |
| Column oven temperature | -5°C | 0.23 | 0.55 | 0.62 | 0.92 | 1.00 | 1.07 |
| | +5°C | 0.26 | 0.54 | 0.59 | 0.92 | 1.00 | 1.07 |
| pH | -0.2 unit | 0.21 | 0.56 | 0.62 | 0.92 | 1.00 | 1.07 |
| | +0.2 unit | 0.29 | 0.56 | 0.62 | 0.92 | 1.00 | 1.07 |

**Standard and sample solution stability**

Standard solution is stable for atleast 24 hours at room temperature (~25°C) while sample solution is not stable at room temperature as the area of Azilsartan is continuously increasing. Sample solution is stable for atleast 13 hours at refrigerator condition (~6°C) as after 13 hours the area of Azilsartan is increasing.

## CONCLUSION

In this paper a simple validated and well defined specific stability indicating HPLC method for the determination of Azilsartan kamedoxomil drug substance as well as its related substances was described, and the behaviour of Azilsartan kamedoxomil drug substances

under various stress conditions was studied and presented. All the degradation products and process impurities were well separated from the main analyte, which demonstrates that the method is stability indicating. The information presented here in could be very useful for quality monitoring of bulk drug samples, and also employed to monitor the quality of the drug substances during stability studies.

**ACKNOWLEDGEMENT**

The authors are thankful to the Dr. B. Gowtham Prasad, SVV University, for providing necessary facility to conduct the Laboratory experiment.

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