Quantification of related/degraded impurities in Tolvaptan drug substance by HPLC

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ABSTRACT
A reverse phased high performance liquid chromatographic (HPLC) method was developed for the determination of Tolvaptan (TLP) drug substance and it process impurities TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05 and TLPC06 using an analytical column Unison UK-C18, (250mm x4.6mm, 5μ) maintained at 30°C ±2°C. The wavelength was set at 265 nm. Flow rate was maintained as 1.0 ml/min with injection volume of 10 μl. All the analytes could be separated with run time of 60 min. The peaks of analytes are well resolved with the resolution of 2.0. The results of all the validation parameters showed that the developed method is well confined to the limits of ICH guidelines. Degradation studies showed that the mass balance of Tolvaptan in all conditions is in the range of 98 to 102 %. The accuracy of six impurities TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05 and TLPC06 found in the range of 80-120%. The proposed method is able to quantify the related compounds present in drug substance and also able to see the assay of drug substance in presence of degraded impurities without the loss of purity of drug substance and other impurities. The method has proved to be robust by introducing miniscule changes in the chromatographic conditions.

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INTRODUCTION
Tolvaptan is chemically known as N-{{[(5R)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl]carbonyl}-3-methylphenyl}-2-methylbenzamide with the molecular formula C_{26}H_{25}ClN_2O_3 of a molecular weight of 448.941. It is the drug administered orally and act as non peptide vasopressin (VP) V2 receptor antagonist that inhibits water re-absorption in the kidney by competitively blocking VP binding, resulting in water diuresis without significantly changing total electrolyte excretion (Hauptman et al., 2005) (Hauptman., et al., 2005; Sojeong Yi., et al., 2012). Very few methods have been published for the estimation of Tolvaptan using UV – visible Spectrophotometer (Chaudhari and Patel, 2012; Murugan et al., 2013), HPLC (Chakravarthy and Shankar, 2011) (Chakravarthy, et al., 2011) in bulk, dosage forms and biological matrices, Assay under stress conditions (Gandhi et al., 2014), UPLC (Prasad et al., 2012) and LC MS (Pei et al., 2013). But till date no method was reported for Tolvaptan and its related compounds and degradation impurities in the literature. The present method is rapid, simple, sensitive and stability indicating method in which all related compounds as well as degradation impurities were well separated and the method has been validated as per ICH, current industrial trends and acceptable analytical practices (ICH Q14, 2018; Q1A(R2), 2003; Q2(R1), 1994).
MATERIALS AND METHODS

Chemicals
Tolvaptan and impurities of Tolvaptan (TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05 and TLPRC06) were received from spectra laboratories Limited, Hyderabad, India. Acetonitrile was purchased from JT Baker supplied by Avantor India. HPLC grade water (Milli-Q), Sodium dihydrogen phosphate (HPLC grade) and Orthophosphoric acid (HPLC grade) from Sigma-Aldrich. Nylon membranes were purchased from Merck (Germany).

Optimized HPLC Conditions
Waters (Model: Alliance 2695 & 2996 PDA detector) with Empower 3.0 software was employed for the analysis. The separation was performed using an analytical column Unison UK C-18, (250mm x 4.6mm, 5μ) maintained at 30°C±2°C. The wavelength was set at 265 nm. Flow rate was maintained at 1.0 ml/min with injection volume of 10 μl. Mobile Phases A and B were used in proportionate way to run this gradient programme. At the beginning (0 minute), both mobile phases were mixed at the ratio of 75:25. At 5 minute, ratio became 75:25 and at 35 minute, it changed to 20:80 and same ratio maintained up to 50 minute. Ratio became 75:25 at 52 minute. At 60 minute, ratio was returned to its initial position i.e. 75:25 and hold up to 60 minutes. So, total run time was set as 60 minutes.

Preparation of Mobile Phase and Sample Solutions

Sodium dihydrogen phosphate
Prepared 0.02M sodium dihydrogen phosphate in water and adjusted the pH to 3.0 ± 0.05 with ortho phosphoric acid. Filter through 0.45 mm and degassed.

Preparation of diluent
Prepared diluent by taking Buffer and Acetonitrile in the ratio 20:80 v/v

Preparation of mobile phase
Mobile Phase A: Sodium dihydrogen phosphate buffer, Mobile phase B: 0.1 % Orthophosphoric acid in acetonitrile.

Preparation of reference solution
Accurately weighed and transferred about 10 mg of each Tolvaptan, TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05, TLPRC06 standards into a 100 mL volumetric flask, dissolved with aid of sonication and diluted to volume with diluent. Diluted 1.0 mL of the above solution to 100 mL with diluent.

Preparation of sample solution

RESULTS AND DISCUSSION
The method was validated as per ICH guidelines. The validation parameters such as specificity, LOD, LOQ, precision, linearity, accuracy and robustness are performed using the optimized chromatographic conditions.

Validation Study Parameters for Related Substances

Specificity
### Table 1: Specificity of Tolvaptan and its related compounds

| Component | Retention time (RT) | Relative retention time (RT) | Purity angle | Purity threshold |
|-----------|---------------------|-------------------------------|--------------|-----------------|
| TLPRC01   | 17.69               | 0.70                          | 4.21         | 5.19            |
| TLPRC02   | 21.25               | 0.84                          | 2.85         | 3.73            |
| TLPRC03   | 24.20               | 0.95                          | 3.13         | 4.34            |
| Tolvaptan  | 25.38               | 1.00                          | 0.15         | 1.21            |
| TLPRC04   | 28.37               | 1.12                          | 3.22         | 4.34            |
| TLPRC05   | 33.66               | 1.33                          | 4.53         | 6.64            |
| TLPRC05   | 39.60               | 1.56                          | 3.39         | 4.67            |

### Table 2: Accuracy of Tolvaptan and its related compounds

| % Level | TLPRC01 % Recovery | TLPRC02 % Recovery | TLPRC03 % Recovery | TLPRC04 % Recovery | TLPRC05 % Recovery | TLPRC06 % Recovery |
|---------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| LOQ     | 102.8              | 104.8              | 125.0              | 111.1              | 91.2               | 100.0              |
|         | 100.0              | 109.5              | 125.0              | 105.6              | 97.1               | 105.6              |
|         | 105.6              | 104.8              | 125.0              | 111.1              | 97.1               | 100.0              |
| 25      | 92.5               | 99.5               | 98.9               | 100.0              | 95.2               | 100.5              |
|         | 93.6               | 98.9               | 100.0              | 102.1              | 95.2               | 102.1              |
|         | 94.1               | 97.9               | 98.9               | 103.2              | 94.7               | 104.3              |
| 50      | 93.3               | 98.7               | 98.7               | 102.1              | 96.8               | 104.0              |
|         | 96.0               | 99.2               | 98.9               | 104.0              | 97.6               | 104.0              |
|         | 96.5               | 100.0              | 100.0              | 104.8              | 98.1               | 105.1              |
| 100     | 96.8               | 99.2               | 96.1               | 100.7              | 98.5               | 101.7              |
|         | 97.5               | 99.9               | 96.9               | 101.7              | 99.2               | 102.4              |
|         | 94.5               | 97.6               | 93.7               | 98.7               | 96.9               | 99.5               |
| 150     | 100.0              | 101.8              | 102.3              | 103.6              | 101.3              | 104.2              |
|         | 97.8               | 99.9               | 99.4               | 100.9              | 99.0               | 101.9              |
|         | 99.3               | 100.7              | 100.3              | 102.0              | 99.7               | 102.7              |

### Table 3: Linearity of Tolvaptan and its related compounds

| Component | Slope | Intercept | Correlation coefficient (R) | R2     |
|-----------|-------|-----------|------------------------------|--------|
| TLPRC01   | 16165588 | -217.7    | 0.9999                       | 0.9998 |
| TLPRC02   | 22617017 | -172.1    | 0.9999                       | 0.9998 |
| TLPRC03   | 22381443 | -123.3    | 0.9999                       | 0.9998 |
| TLPRC04   | 26699897 | -96.8     | 0.9999                       | 0.9998 |
| TLPRC05   | 14039809 | -203.7    | 0.9999                       | 0.9998 |
| TLPRC06   | 30923614 | -158.0    | 0.9999                       | 0.9998 |
| TLP       | 25507228 | -37.0     | 0.9999                       | 0.9998 |
Tolvaptan and each impurity solutions were prepared individually at a concentration of 0.1 mg/mL and a solution of Tolvaptan spiked with all impurities at 0.1% level was also prepared and injected into the chromatographic system. Retention time and relative retention of Tolvaptan and their related substances were represented in Table 1. Typical chromatogram of Blank (diluent), Tolvaptan sample, Tolvaptan spiked sample and overlay chromatogram of blank, sample and impurities were represented in Figures 1, 2, 3 and 4 and specificity results represented in Table 1. The results of specificity study were proved that peaks of impurities are well resolved from each other and from the main drug substance.

**System suitability**

Reference solution was analysed as per the HPLC
### Table 7: Degradation Study

| Degradation parameter | Tolvaptan peak area | Purity Angle | Purity Threshold | Total area (Tolvaptan and its impurities) | Mass balance (%) |
|-----------------------|---------------------|--------------|------------------|----------------------------------------|-----------------|
| Control sample        | 12421909            | 0.07         | 1.02             | 12476272                               | 100.4           |
| Acid_RT               | 12432076            | 0.06         | 1.01             | 12404372                               | 99.8            |
| Acid_60 °C            | 12414217            | 0.07         | 1.01             | 12432076                               | 100.1           |
| Base_RT               | 12406129            | 0.06         | 1.02             | 12429058                               | 100.2           |
| Base_60 °C            | 12430087            | 0.08         | 1.02             | 12474479                               | 100.3           |
| Oxidation_RT          | 12440867            | 0.07         | 1.01             | 12499126                               | 100.6           |
| Oxidation_60 °C       | 12462285            | 0.07         | 1.02             | 12456129                               | 99.6            |
| Heat_105 °C           | 12393527            | 0.08         | 1.01             | 12439745                               | 100.4           |
| UV_200 wh/sq mt       | 12324845            | 0.06         | 1.01             | 12497566                               | 101.4           |

### Table 8: Robustness parameters

| Parameter | TLPR C01 | TLPR C02 | TLPR C03 | TLPR C04 | TLPR C05 | TLPR C06 |
|-----------|----------|----------|----------|----------|----------|----------|
| Normal    | 1.0      | 18.33    | 0.14     | 21.58    | 0.15     | 24.46    | 0.14     | 28.71    | 0.15     | 34.07    | 0.15     | 39.95    | 0.15     |
| Flow      | 0.9      | 19.26    | 0.14     | 22.57    | 0.15     | 25.27    | 0.14     | 29.56    | 0.15     | 34.90    | 0.15     | 40.84    | 0.15     |
| Flow      | 1.1      | 17.32    | 0.14     | 20.47    | 0.15     | 23.48    | 0.14     | 27.66    | 0.15     | 33.02    | 0.14     | 38.76    | 0.15     |
| Temperature | 28°C    | 18.23    | 0.15     | 21.62    | 0.15     | 24.35    | 0.14     | 28.63    | 0.15     | 33.95    | 0.15     | 39.90    | 0.15     |
| Temperature | 32°C    | 18.01    | 0.15     | 21.18    | 0.15     | 24.19    | 0.14     | 28.37    | 0.15     | 33.72    | 0.15     | 39.42    | 0.15     |
| pH        | 2.8      | 18.23    | 0.15     | 21.62    | 0.15     | 24.35    | 0.14     | 28.63    | 0.15     | 33.95    | 0.15     | 39.90    | 0.15     |
| pH        | 3.2      | 18.01    | 0.15     | 21.18    | 0.15     | 24.19    | 0.14     | 28.61    | 0.15     | 33.94    | 0.15     | 39.78    | 0.15     |

### Table 9: Solution stability study at 25°C and 2-8 °C

| Parameter | TLPR C01 | TLPR C02 | TLPR C03 | TLPR C04 | TLPR C05 | TLPR C06 |
|-----------|----------|----------|----------|----------|----------|----------|
| Initial   | 0.14     | 0.15     | 0.19     | 0.17     | 0.14     | 0.15     | 0.01     | 0.98     |
| 24 hrs    | 0.15     | 0.15     | 0.19     | 0.17     | 0.15     | 0.15     | 0.02     | 1.01     |
| 48 hrs    | 0.15     | 0.15     | 0.19     | 0.17     | 0.15     | 0.15     | 0.01     | 1.01     |
| Initial   | 0.14     | 0.15     | 0.19     | 0.17     | 0.14     | 0.15     | 0.01     | 0.98     |
| 24 hrs    | 0.15     | 0.15     | 0.18     | 0.17     | 0.14     | 0.15     | 0.02     | 0.99     |
| 48 hrs    | 0.15     | 0.15     | 0.19     | 0.17     | 0.15     | 0.15     | 0.01     | 1.01     |
method of analysis and observed that the resolution between all the impurities were more than 2.0. USP tailing factor for Tolvaptan was 1.12. Number of theoretical plate for Tolvaptan was 56354.

**Limit of detection and limit of quantification**

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculating the signal-to-noise ratio of 3:1 and 10:1, respectively for TLP, TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05 and TLPRC06 impurities with known concentration. The limit of detection found as 0.0015, 0.002, 0.0015, 0.0015, 0.002 and 0.0015 mg/mL and the limit of quantitation found as 0.007, 0.0045, 0.005, 0.005, 0.007 and 0.005 mg/mL for TLP, TLPRC01, TLPRC02, TLPRC03, TLPRC04, TLPRC05 and TLPRC06 impurities.

Accuracy

The study of accuracy of Tolvaptan spiked with known impurities for quantification was carried out in triplicate at LOQ, 25 %, 50%, 100% and 150% level (Table 2). The percentage recovery of all impurities has been calculated and found to be within the range of 80-120%. The results of accuracy of Tolvaptan and its related substances were represented in Table 2.

**Linearity**

Linearity was performed over a wide range of analyte which ensured that calculations could be performed using a single working standard rather than equation of a calibration line. Study of linearity of peak area versus concentration was demonstrated in the range of LOQ to 120% level of Tolvaptan and its impurities. Solutions were prepared from stock solution at seven concentration levels from LOQ to 120% level of analyte concentrations. The data was subjected to statistical analysis using linear-regression least-squares method. The calibration curve was found to be linear with correlation coefficient (R2) 0.9998 and the results are given in Table 3.

**System precision, method precision and intermediate precision**

The system precision was performed by analysing six replicate injections of Tolvaptan and its impurities. Results of percentage relative standard deviations are in the range of 0.2 - 0.7 for Tolvaptan and impurities. Precision at LOQ was also determined by injecting individual preparations of Tolvaptan spiked at LOQ level of its impurities and observed % of relative standard deviations in the range of 2.9 - 4.7. The precision of the method was determined by analysing samples of Tolvaptan spiked with impurities at 0.15% six replicate sample preparations. The % relative standard deviation values for precision and intermediate precision were found in the range of 0.0-3.6. Evaluating the variability of the results obtained for related substances with the analysis of Tolvaptan sample spiked with impurities, six times at the specification limit by different analysts, different instruments using different columns and different reagents on different days assessed the intermediate precision. The bias observed for individual impurities and total impurities were within the acceptance criteria. The percentage recovery of all impurities in drug substance has been calculated and the percentage recovery is found to be within the range as per ICH guidelines. System precision, method precision and intermediate precision results represented in Tables 4, 5 and 6.

**Degradation study**

Forced degradation has been performed for Tolvaptan and verified the stability indicating nature of method of HPLC. In order to assess the stability indicating nature, Tolvaptan sample was degraded forcefully under acid hydrolysis, base hydrolysis, oxidation with hydrogen peroxide, heat degradation at 105°C and UV degradation at 200 wh/sq mt. The degraded samples were analysed using a photodiode-array detector. No degradation was observed in acid, base, peroxide and heat, significant degradation was observed in UV light solution. The mass balance of Tolvaptan in all conditions observed in the range of 98 to 102 % and peak purity of Tolvaptan and its impurities were passed. The degradation results of Tolvaptan were summarized in Table 7.

**Robustness**

Robustness of method was carried out for all impurities by introducing miniscule changes in the chromatographic conditions which include mobile phase flow variation (± 0.1 mL/min), buffer pH variation (± 0.2) and column oven temperature (± 2 °C). The robustness results of retention time and % w/w of all impurities were summarized in Table 8.
Solution Stability

A solution of Tolvaptan spiked with the impurities at ICH specification level concentration and the impurities standard solutions were kept at room temperature (25°C) as well as in the refrigerator at 2-8°C. The solution stability was monitored at initial, 24 hours and 48 hours intervals and comparing the results indicates that the impurities standard solution and sample solutions were stable up to 48 hours at 2-8°C as well as at room temperature. The solution stability results of Tolvaptan and its impurities were summarized in Table 9.

CONCLUSIONS

In this current proposed study, impurities of Tolvaptan drug substance were estimated by using RP-HPLC method with photo diode array (PDA) detector. It was a simple, sensitive, accurate, linear, precise and robust method. The HPLC method was validated as per ICH guidelines, all impurities were well separated along with establishment of their limit of quantification values and was found to have excellent resolution for all the impurities indicating high sensitivity and selectivity of the method.

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Conflict of Interest

None.

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