IMPURITY PROFILING OF TOLVAPTAN TABLETS USING NEW STABILITY INDICATING UPLC METHOD

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ABSTRACT
The present research paper deals with development and subsequent validation of a new stability indicating UPLC method for the estimation of related compound and degradation impurities of Tolvaptan in tablet dosage form. Chromatographic separation was achieved on HSS C18, 100 mm x 2.1 mm, column with 1.7µm particles column, using 0.01M phosphate buffer and acetonitrile in gradient elution mode. The detector was set at 215 nm and the flow rate was kept at 0.6mL/min. The optimized method was found to produce symmetric and sharp peaks with good separation between process related impurities and degradation impurities. Formulation samples were subjected to forced degradation and stability indicating nature of the method was assessed by monitoring the sample using PDA detector. No interference was found due to any of the degradation impurities. The proposed method has been extensively validated in terms of specificity, precision, linearity, accuracy, limit of detection (LOD) and quantification (LOQ), and robustness. The precision was expressed with respect to the intra- and inter-day variation in the expected drug concentrations. The accuracy was expressed in terms of percent recovery of the known amount of impurities added to the sample preparation. The method was validated in terms of Specificity, Precision, Ruggedness, Accuracy, Robustness and Linearity as per ICH guidelines.

Key words: Tolvaptan, Method development, UPLC, Method validation, Forced degradation.

INTRODUCTION
Tolvaptan (Figure 1) chemically N-[(5R)-7-chloro-5-hydroxy-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl]carbonyl]-2-methylphenyl)-2-methylbenzamide, its molecular formula is C24H23N3O5 with a molecular weight of 448.941. Tolvaptan (Otsuka) is an orally administered nonpeptide 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.1,2

Very few methods have been published for the estimation of Tolvaptan using UV – visible Spectrophotometer,3 by HPLC4 in bulk and dosage forms, in biological matrices5. Till date there was no stability indicating method was available for Tolvaptan and its related compounds and degradation impurities in the literature. The present method is rapid, simple, sensitive and stability indicating reverse phase UPLC method in which all related compounds as well as degradation impurities were well separated. Present method has been validated as per ICH, current industrial trends and acceptable analytical practices6.

MATERIALS AND METHODS
Chemicals and Reagents
Samples of Tolvaptan and its four related compounds (Figure 2) were procured from Hetero Labs, Hyderabad, India. Gradient grade acetonitrile purchased from Baker, Germany. ACS grade potassium di hydrogen phosphate and ortho phosphoric acid were purchased from Sigma Aldrich, India. High pure water was used from Millipore Milli Q water purification system.

Equipment
The LC system, used for method development, forced degradation studies and method validation was Waters Acquity H-Class (manufactured by Waters corporation, USA) LC system with a diode array detector. The out put signal was monitored and processed using Empower 2 software (designed by Waters Corporation, USA) on Pentium computer (Digital Equipment Co).

Chromatographic conditions
The chromatographic column used was an Acquity HSS C18, 100 mm x 2.1 mm, column with 1.7µm particles. The mobile phase A consists of 10mM phosphate buffer with pH adjusted to 3.0 with dilute ortho phosphoric acid and acetonitrile in the ratio of 85:15(%v/v). The mobile phase B consists of acetonitrile and water in the ratio of 90:10(%v/v). The flow rate of the mobile phase was kept at 0.6 ml/min. The UHPLC gradient was set as: T/%B: 0/28, 1.5/40, 3.0/52, 4/76, 5.5/76, 5.8/28 and 8.0/28. The column temperature was maintained at 30°C and the wavelength was monitored at 215 nm. The injection volume is 2µl. Phosphate buffer and acetonitrile in the ratio of 1:9 used as diluent for samples preparation.

Preparation of solutions
Preparation of standard solution
A working solution containing of 2.5µg/ml of Tolvaptan was used for the determination of impurities (Figure 3-b).

Preparation of sample solution
A test solution containing of 500µg/ml of Tolvaptan was prepared by taking capsule powder equivalent to 50mg of Tolvaptan into a 100ml volumetric flask. Preparation of spiked sample solution
Another sample was prepared with spiked with related impurities (Related compound 1, Related compound 2, Related compound 3 and Related compound 4) at 0.5% of sample concentration i.e., 2.5µg/mL (Figure 3c).

Method validation
The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2 (R1)6. The described method has been extensively validated in terms of specificity, precision, linearity, accuracy, limit of detection (LOD) and quantification (LOQ), and robustness. The precision was expressed with respect to the intra- and inter-day variation in the expected drug concentrations. The accuracy was expressed in terms of percent recovery of the known amount of impurities added to the sample preparation.

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Specificity
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity was tested by injecting the sample by spiking with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix. Moreover, identification of each impurity was confirmed with relative retention times as compared with those of pure standards.

Forced degradation studies
Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions like thermal degradation (at 105°C), acid hydrolysis (using 5N HCl), base hydrolysis (using 5N NaOH), and oxidative degradation (using 5% H2O2) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well. To check and ensure the homogeneity (peak purity) of Tolvaptan peak in the stressed sample solutions, photo diode array detector was employed.

Precision
The precision of the related substance method was checked by injecting six individual sample preparations of (500µg/mL) Tolvaptan spiket with 0.5% of Related compound-1, Related compound, Related compound -3 and Related compound -4 with respect to analyte concentration 5% R.S.D. of area for Related compound -1, Related compound -2. Related compound -3 and Related compound -4 was calculated. The intermediate precision of the method was also evaluated using different analyst, different day and different make instrument in the same laboratory.

Limit of detection (LOD) and limit of quantification (LOQ)
The LOD and LOQ for Related compound -1, Related compound -2, Related compound -3 and Related compound -4 were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. Precision study was also carried at the LOQ level by injecting six individual preparations of Related compound -1, Related compound -2, Related compound -3 and Related compound -4 and calculating the % R.S.D. of the area.

Linearity
The linearity of the method was tested in order to demonstrate proportional relationship of response versus analyte concentration over the working range. It is usual practice to perform linearity experiments over a wide range of analyte. This gives confidence that the response and concentration are proportional and consequently ensures that calculations can be performed using a single reference standard/working standard, rather than the equation of a calibration line. The linearity of detector response to different concentrations of impurities was studied by preparing a series of solutions using Tolvaptan and its related impurities at five different concentration levels ranging from 0.05% to 0.75%w/w of test concentration (500µg/mL). The data were subjected to statistical analysis using a linear-regression model.

Accuracy
The accuracy of the related impurities method for the quantification of all four impurities in formulation samples. The study was carried out in triplicate at 0.05%, 0.1%, 0.2% 0.5% and 0.75% of the analyte concentration (500µg/ml). The % recoveries for Related compound -1, Related compound -2, Related compound -3 and Related compound -C were calculated from the slope and Y-intercept of the calibration curve.

Robustness
To determine the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between Tolvaptan and Related compound -1 was evaluated. To study the effect of flow rate on the resolution, the same was altered by 0.1 units, i.e. from 0.50 to 0.70 ml/min. The effect of pH on resolution of impurities was studied by varying ±0.2 pH units (at 2.8 and 3.2 buffer pH). The effect of column temperature on resolution was studied at 25 and 35°C instead of 30°C. All the other mobile phase components were held constant.

<table>
<thead>
<tr>
<th>Compound</th>
<th>USP Resolution</th>
<th>USP Failing factor (Rs)</th>
<th>Number of Theoretical plates (N)</th>
<th>Relative Retention time</th>
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<tbody>
<tr>
<td>Rel.compound-4</td>
<td>1.02</td>
<td>500896</td>
<td>0.34</td>
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<tr>
<td>Rel.compound-3</td>
<td>8.9</td>
<td>48010</td>
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<tr>
<td>Rel.compound-2</td>
<td>12.1</td>
<td>56289</td>
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<tr>
<td>Tolvaptan</td>
<td>17.6</td>
<td>70891</td>
<td>1.0</td>
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<tr>
<td>Rel.compound-1</td>
<td>9.2</td>
<td>94201</td>
<td>1.14</td>
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<tr>
<th>Stress conditions</th>
<th>% of Degradation observed</th>
</tr>
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<tbody>
<tr>
<td>Acid Hydrolysis (5N HCl)</td>
<td>40.9%</td>
</tr>
<tr>
<td>Base Hydrolysis (5N NaOH)</td>
<td>2.81%</td>
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<tr>
<td>Oxidation (5% H2O2)</td>
<td>1.36%</td>
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<table>
<thead>
<tr>
<th>Accuracy level</th>
<th>Rel. compound -1</th>
<th>Rel. compound -2</th>
<th>Rel. compound -3</th>
<th>Rel. compound -4</th>
</tr>
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<tr>
<td>Accuracy at 0.05%</td>
<td>98.3%</td>
<td>97.9%</td>
<td>99.3%</td>
<td>99.1%</td>
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<tr>
<td>Accuracy at 0.20%</td>
<td>98.1%</td>
<td>100.1%</td>
<td>97.9%</td>
<td>101.6%</td>
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<tr>
<td>Accuracy at 0.50%</td>
<td>100.2%</td>
<td>97.3%</td>
<td>98.5%</td>
<td>98.1%</td>
</tr>
<tr>
<td>Accuracy at 0.75%</td>
<td>100.6%</td>
<td>98.4%</td>
<td>100.8%</td>
<td>99.8%</td>
</tr>
<tr>
<td>Mean Recovery</td>
<td>99.3%</td>
<td>98.4%</td>
<td>99.1%</td>
<td>99.7%</td>
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n=3 number of determinations
Table 4: Precision and Ruggedness data

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<thead>
<tr>
<th>Parameter</th>
<th>Impurity-1</th>
<th>Impurity-2</th>
<th>Impurity-3</th>
<th>Impurity-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>%RSD (Precision)</td>
<td>1.82</td>
<td>2.03</td>
<td>2.80</td>
<td>1.98</td>
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<tr>
<td>%RSD (Ruggedness)</td>
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<td>3.16</td>
<td>1.38</td>
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</table>

n=6 number of determinations

Table 5: Robustness data

<table>
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<tr>
<th>Parameter</th>
<th>Deliberate change</th>
<th>Resolution</th>
<th>Minimum theoretical plates Tolvaptan</th>
<th>Maximum tailing factor</th>
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<td>Flow rate (0.6mL/min)</td>
<td>0.5mL/min</td>
<td>7.9</td>
<td>68110</td>
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<tr>
<td>Temperature (30°C)</td>
<td>0.7mL/min</td>
<td>7.8</td>
<td>70082</td>
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<tr>
<td>pH of buffer (3.0)</td>
<td>2.8</td>
<td>8.1</td>
<td>50360</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>7.6</td>
<td>48610</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Figure 1: Chemical Structure of Tolvaptan

Figure 2

(a) Typical chromatogram of Diluent as blank

(b) Typical chromatogram of diluted standard
RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The potential impurities present in bulk samples produced by Hetero Labs, Hyderabad, India. The main target of the chromatographic method is to get the separation of all impurities and degradants generated from analyte peak. Impurities were co-eluted by using different stationary phases like C18 and C8 and different mobile phases containing buffers like diluted ortho phosphoric acid, potassium phosphate and sodium per-chlorate with different pH and using organic modifiers like acetonitrile and methanol in the mobile phase. The elution order and peak shapes of related compounds are largely dependent on salt concentration and pH. The separation between tolvaptan & related compound 1 was achieved by optimizing gradient programme. Satisfactory chromatographic separation was achieved using a mobile phase mobile phase A consists of 10mM phosphate buffer with pH adjusted to 3.0 with dilute ortho phosphoric acid and acetonitrile in the ratio of (85:15%v/v). The mobile phase B consists of acetonitrile and water (9:1). The UHPLC gradient was set as: T/%B: 0/28, 1.5/40, 3.0/52, 4/76, 5.5/76, 5.8/28 and 8.0/28. In the optimized conditions the Tolvaptan, related compound-1, related compound -2, related compound -3 and related compound-4 were well separated with a...
resolution of greater than 7.9 and the typical retention times of related compound -1, related compound -2, related compound -3, related compound -4 and Tolvaptan were about 4.5 minutes, 2.7 minutes, 1.9 minutes, 1.3 minutes and 4 minutes (Figure 3) respectively. The system suitability results were given in Table 1 and the developed LC method was found to be specific for Tolvaptan and its three impurities, namely related compound -1, related compound -2, related compound -3 and related compound -4.

**Results of forced degradation studies**

Tolvaptan tablet dosage form samples were subjected to forced degradation. To identify interference from excipient, Placebo samples were also subjected to similar stress conditions and chromatograms were compared with that of formulation samples. The degradation of active ingredient was observed during oxidative stress, acid and base hydrolysis (Figure 4). Tolvaptan was found to be prone to degradation in acid stress condition when compared to base and oxidative stress conditions. All the degradation products were well separated, homogeneity and purity of all the peaks monitored using PDA detector. The mass balance of stressed samples was close to 98.7% (Table 2). This confirms the stability indicating power of the developed method.

**Results of method validation experiments**

**Precision**

The % R.S.D. of area of related compound -1, related compound -2, related compound -3 and related compound-4 in related substance method precision study was within 4% confirming good precision of the method.

**Limit of detection (LOD) and limit of quantification (LOQ)**

The limit of detection (LOD) of related compound -1, related compound -2, related compound -3 and related compound -4 were 0.0060%, 0.0033%, 0.042% and 0.0026% (of analyte concentration, i.e. 500 µg/ml) for 5 µl injection volume. The limit of quantification (LOQ) of related compound -1, related compound -2, related compound -3 and related compound-5 were 0.018%, 0.011%, 0.013 and 0.009% (of analyte concentration, i.e 0.5 mg/ml) for 2 µl injection volume. The method precision for related compound -1, related compound -2, related compound -3 and related compound -4 at LOQ level was below 5% R.S.D.

**Linearity**

Linear calibration plot for related substance method was obtained over the calibration ranges tested, i.e. 0.05% to 0.75% for related compound, related compound -2, related compound -3 and related compound -4. The correlation coefficient obtained was greater than 0.996. The results show that an excellent correlation existed between the peak area and concentration of related compound, related compound -2, related compound -3 and related compound -4.

**Accuracy**

The percentage recovery of related compound -1, related compound -2, related compound -3 and related compound -4 in formulation samples was ranged from 97.3 to 101.6 (Table-3 & 4) HPLC chromatograms of blank, pure sample and all four impurities spiked in Tolvaptan formulation sample were shown in Fig. 3 (Blank, sample, spiked).

**Robustness**

In all the deliberate varied chromatographic conditions (flow rate, pH and column temperature) the resolution between tolvaptan and related compound -1 was greater than 7, illustrating the robustness of the method.

**Stability of Analytical solutions**

The stability of the standard and sample solutions was tested at regular intervals. The stability of solutions was determined by comparing results with freshly prepared standard solutions. The differences in values were within 0.05% for identified and unidentified impurities and 0.2% for total impurities up to 48 hrs.

**CONCLUSION**

The validated stability-indicating UPLC method has proved to be rapid, simple, accurate, precise and reliable. The proposed method provides a good resolution between all the related compounds and potential degradants. The behavior of tolvaptan under various stress conditions were studied and presented for the first time. The information presented herein could be very useful for quality monitoring as well as impurity profiling of formulation samples during stability studies.

**ACKNOWLEDGEMENT**

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**REFERENCES**

6. Validation of Analytical Procedures: Methodology (Q2B), ICH Harmonized Tripartite Guideline

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