Stability Indicating Chromatographic Methods for the determination of Trospium Chloride

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ABSTRACT

Two precise, accurate and sensitive high-performance liquid chromatographic and thin-layer chromatographic methods were developed and validated for the determination of Trospium chloride in presence of its degradation products. Forced degradation studies were performed using HCl and NaOH. Separation of the drug from its degradation products by HPLC was achieved using a X- Bridge C18 column and acetonitrile/methanol/0.05M potassium dihydrogen phosphate/triethylamine in a ratio of (25:25:50:0.2 by volume) as a mobile phase, pH was adjusted to 4 ± 0.1 with ortho-phosphoric acid. The flow rate was 1ml/min. Detection was performed at 215 nm. The linearity range was 0.5 to 18 µg/ml. The mean percentage recovery was 100.40 ± 0.45 %. The TLC method was used for separation of the drug from its degradation products using silica gel 60 F 254 plates; the optimized mobile phase was acetonitrile/glacial acetic acid (5:5 by volume). Quantitatively, the spots were scanned densitometrically at 215 nm. The linearity range was 2 to 16 µg/ spot. The mean percentage recovery was 99.96 ± 0.700 %. The degradation products were obtained in acid and alkaline stress conditions were the same, therefore the alkaline degradation products were prepared, identified and compared with the standard ones. Statistical comparison between the results obtained by these methods and those obtained by the official method was done, and no significance difference was obtained.

Keywords-Trospium chloride, stability studies, Degradation, HPLC, TLC, Benzilic acid.

1. INTRODUCTION

Trospium chloride (Fig.1) (spirop [8-azoniabicyclo [3.2.1] octane-8,1-pyrolidinium]-3 [(hydroxydiphenyl- acetyl)-oxy]chloride(1α, 3β, 5α) is an antimuscarinic agent indicated for the treatment of overactive bladder with symptoms of urge urinary incontinence, urgency, and urinary frequency [1].

Determination of Trospium chloride is described in European pharmacopoeia by titrimetric method [2]; using 0.1 M silver nitrate as titrant, then the end point was determined potentiometrically. Only two methods were reported for the determination of Trospium chloride, the first one was a fluorimetric method after derivatization with benoxaprofen chloride [3] while the second one was LC-MS method [4]. However, there is only one reported stability - indicating methods for the determination of the drug[5]. This paper presented the study of the acid and alkaline degradation of Trospium chloride, followed by the development of two chromatographic stability-indicating methods for the determination of the drug in its pure powder form and in pharmaceutical dosage form.

2. EXPERIMENTAL

2.1. Instruments

Agilent 1100 series, connected with an ultra violet detector set at 215 nm (Model G1316 A, Agilent 1100 series). The injector was a manual Rheodyne injector (Model 7725/7725L, Rohnert Park, CA., USA) equipped with 20 µl injector. The instrument was connected to an IBM compatible personal computer (PC), an HP disk jet 5652 printer and X- Bridge C18, (150x4.6mm.) column particle size 3.5µm (Waters). The mobile phase was filtered using a 0.45-mm Teflon membrane filter (Millipore, Milford, MA, USA) and degassed by ultrasonic vibrations J.P. Selecta, (Barcelona, Spain). For TLC method, the plates (Merck, Germany) used were coated with 0.25mm silica gel 60 F254 (Merck, Germany). The sample was applied to the plates using Hamilton micro syringe (10µl). A TLC scanner, dual wavelength flying spot (Shimadzu CS-9301, Tokyo, Japan) was used for scanning. The experimental conditions were scan mode = Absorbance mode, and wavelength = 215 nm. The spots were visualized using UV lamp at 254 nm for identification of the degradation product, IR Bruker Vector 22 8201 PC spectrometer (Bruker Instruments Ltd, Rheinstetten/ Karlsruhe, Germany) and Mass Spectrophotometer, Hewlett Packard Model 5988A GC/MS (Agilent Technologies, Wilmington, DE) were used.

2.2 Materials and Reagents

Trospium chloride-Pure sample was kindly supplied by Hekma Pharma, Egypt, B.N. 21787. Its purity was found to be 99.9% according to the official method. Methanol and acetonitrile for HPLC were purchased from s.d fine-chem limited (Mumbai, India). While sodium hydroxide, hydrochloric acid, potassium dihydrogen phosphate,
triethylamine, methanol, glacial acetic acid were obtained from ADWIC (Cairo, Egypt).

Trospian tablet was supplied by Hikma Pharma, (Cairo, Egypt), B.N.003. Each tablet is claimed to contain 20 mg of Trospium chloride. Benzoic acid was kindly supplied by Hekma Pharma (Cairo, Egypt), its purity was certified to be 99.9%

2.3 Chromatographic Conditions

2.3.1 HPLC method

The mobile phase was prepared by mixing acetonitrile/methanol/0.05M potassium dihydrogen phosphate/triethyamine in a ratio of (25:25:50:0.2 by volume) as a mobile phase, pH was adjusted to 4 ± 0.1 with o-phosphoric acid. The mobile phase was filtered using a 0.45-mm Teflon membrane filter (Millipore, Milford, MA, USA) and degassed by ultrasonic vibrations for 30 min prior to use. All determinations were performed at ambient temperature (25°C) under the following chromatographic conditions:

- Column: X-Bridge C18, (Waters; 150x4.6mm, 3.5 µm).
- Flow rate: 1ml/min
- Wavelength: 215 nm
- Injection volume: 20 µL

2.3.2 TLC method

The plates were first washed and developed with the mobile phase by mixing acetonitrile/glacial acetic acid (5:5 by volume), then activated for 15 minutes by placing in an oven at 100°C before use. Spots were applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase for one hour. The plates were developed in ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the following conditions:

Source of radiation: deuterium lamp.
Photomode: Reflection.
Scan mode: Absorbance.
Result output: Chromatogram and area under the peak.
Swing width: 10 mm.
Wavelength: 215 nm.

2.4 Preparation of the degradation products

Trospium chloride (100 mg) was refluxed with 50 ml 2M NaOH solution into a100-ml round-bottom flask for 2 hours and tested for complete degradation by TLC using acetonitrile/glacial acetic acid (5:5 by volume) as the mobile phase. Two spots were observed not corresponding to Trospium chloride. One spot was visualized under UV lamp at 254 nm, while the other spot was visualized after spraying with potassium iodobismuthate reagent. The degraded solution was then cooled at room temperature, neutralized with 2M HCl solution respectively till pH was approximately 7. The solution was nearly evaporated to dryness, cooled and transferred quantitatively with methanol to a volumetric flask 100-ml then the volume was completed to the mark to prepare solution of concentration (equivalent to 1mg/ml of intact Trospium chloride) in methanol and finally was filtered.

The degraded solution and the reference standard degradation product solution (benzilic acid) were spotted on TLC plate. The plate was developed with the previously mentioned mobile phase. The spots were visualized under UV lamp at 254 nm, the Rf value of the resultant degradation product 1 was compared with that standard degradation product, the results agreed with the published data.

2.5 Standard solutions

2.5.1 For HPLC method, Standard stock solutions of Trospium chloride was prepared in a concentration of (0.1mg/ml) by transferring 10 mg portion of Trospium chloride powder to a 100-ml volumetric flask and dissolved in 20 ml mobile phase, and then the volume was completed with mobile phase.

2.5.2 For TLC method

Standard stock solution of Trospium chloride was prepared in a concentration of (2mg/ml) by transferring 100 mg portion of Trospium chloride powder to a 50-ml volumetric flask and dissolved in 20 ml methanol, and then the volume was completed with methanol.

2.6 Laboratory prepared mixtures containing different ratios of Trospium chloride and its degradation product

2.6.1. HPLC method

Aliquots (1.62 – 0.18 ml) of Trospium chloride were accurately transferred from its stock standard solution (0.1mg/ml) equivalent to (162 – 18 µg) into a series of 10-ml volumetric flasks. Aliquots (0.18 – 1.62 ml) of degradation product solution (0.1 mg/ml) equivalent to (18 – 162 µg) were added, the volume was completed with mobile phase to prepare mixtures containing 10 – 90 % of the degradation product.

2.6.2 TLC method

Aliquots (7.2 – 1.6 ml) of Trospium chloride were accurately transferred from its stock standard solution (2mg/ml) equivalent to (14.4 – 3.2 mg) into a series of 10-ml volumetric flasks. Aliquots (0.8 – 6.4 ml) of degradation product solution (2 mg/ml) equivalent to (1.6 – 12.8 mg) were added, the volume was completed with the methanol to prepare mixtures containing 10 – 80 % of the degradation product.

2.7 Construction of Calibration Curves

2.7.1 For HPLC method

Aliquots equivalent to 5, 10, 20, 40, 80, 180 µg of the drug stock standard solution (0.1mg/ml) were transferred into a series of 10-ml volumetric flasks. The volume was completed to the mark with mobile phase. Aliquots equivalent to 20 µl of the previously prepared solutions were injected in triplicate into the liquid chromatograph at
ambient temperature (25°C) under the previously mentioned chromatographic conditions.

The chromatogram was obtained, the average peak area ratios obtained for each concentration of Trospium chloride to that of external standard 18µg/ml were plotted versus concentrations, and the regression equation was computed.

2.7.2 For TLC method

Aliquots equivalent to 20, 40, 60, 80,120, 160 µg of the stock standard solution (2mg/ml) were transferred into a series of 10-ml volumetric flasks. The volume was completed to the mark with methanol. Aliquots equivalent to ten µl of the prepared solutions, using 10 µl Hamilton syringe, were applied as separate compact bands 20 mm apart and 20 mm from the bottom of the plates. The chromatographic tank was saturated with the mobile phase for one hour in an ascending manner to a distance of 7 cm from the spotting line at room temperature, air-dried, and the plates were scanned under the previously mentioned chromatographic conditions. The scanning profile for Trospium chloride was obtained. The calibration curve relating the integrated peak area to the corresponding concentration was constructed and the regression equation was computed.

2.8 Application of the proposed methods for the analysis of laboratory prepared mixtures of Trospium chloride and its degradation products.

2.8.1 HPLC method

Aliquots equivalent to twenty µl from the prepared mixture were injected into the liquid chromatograph. Then the procedure was completed as described in subsection of 2.7.1. The concentration of Trospium chloride was calculated by substitution in the corresponding regression equation.

2.8.2 TLC Method

Aliquots equivalent to ten µl from the prepared mixture were spotted on TLC plates and the procedure was completed as described in subsection of 2.7.2. The concentration of Trospium chloride was calculated by substitution in the corresponding regression equation.

2.9 Application of the proposed methods for the analysis of Trospium chloride in pharmaceutical preparation.

2.9.1 HPLC method

Five tablets of Trospikan were weighed accurately and finely powdered in a small dish. An amount of powder equivalent to 10 mg Trospium chloride was accurately transferred into a 100 ml volumetric flask, 50 ml of the mobile phase was added. The flask was sonicated for 30 minutes, filtered into a 100-ml volumetric flask, then the volume was completed to the mark with mobile phase. Then the procedure was completed as described in subsection of 2.7.1. The concentrations of Trospium chloride were calculated by substitution in the corresponding regression equation.

2.9.2 TLC Method

Ten tablets of Trospikan were weighed accurately and finely powdered in a small dish. An amount of powder equivalent to 100 mg Trospium chloride was accurately transferred into a 50 ml volumetric flask, 25 ml of the methanol was added. The flask was sonicated for 30 minutes, filtered into a 50-ml volumetric flask, then the volume was completed to the mark with methanol. Then the procedure was completed as described in subsection of 2.7.2. The concentrations of Trospium chloride were calculated by substitution in the corresponding regression equation.

3. RESULT AND DISCUSSION

3.1 Separation and identification of degradation products

The stability of the drug was studied according to ICH guidelines Q2 (R1) [6] for:

(a) Stress Acid and Alkaline: 1M HCl/1M NaOH for 2 hours, 2M HCl/2M NaOH for 2 hours.

(b) Oxidative Condition: 3% H₂O₂ for 2, 4, 6 and for 10 hours.

(c) Thermal Degradation: at 100°C in an oven for 2, 4 and for 6 hours.

The degradation process under the previously mentioned conditions was followed using TLC and the compound was found to be liable to acid and alkaline degradations. There are two components which confirmed by TLC as indicated by the appearance of two spots with the same Rᵣ values, at the acid and alkaline degradation conditions after complete degradation. In this work, we concerned with the alkaline degradation of Trospium chloride as it is completely degraded under very mild conditions. Furthermore, standard solution of benzilic acid showed similar Rᵣ (0.71), UV, IR and MS spectra as the prepared degradation product 1. Trails were carried out to isolate and identify the degradation product 2 Rᵣ (0.25) which was invisible on TLC plates unless after successive spraying with potassium iodobismuthate reagent to give a very faint orange colour. Unsuccessful results were obtained; therefore we consider the solution of the degradation as one product.
In the present study, two stability-indicating methods for the simultaneous determination of Trospium chloride, in presence of its alkaline-induced degradation products were suggested.

3.2. Optimization of Chromatographic Conditions

3.2.1 HPLC method

HPLC has become the most versatile and wide spread technique used by the pharmaceutical industry for quality control. It has many applications in the field of pharmaceuticals including the quantitative determination of drugs present either alone or in presence of their degradation [7, 8]. The proposed method is based on the difference in the retention time between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product.

Different solvent systems with different ratios were tried; best separation was achieved upon using acetonitrile/methanol/0.05M potassium dihydrogen phosphate /triethylamine (25:25:50:0.2 by volume). The flow rate was 1 ml/min and the detector wavelength was 215 nm. Trospium chloride was completely resolved from its degradation product and its Rf value was 3.22 min, on the other hand the Rf value of the degradation product was 4.30. This would permit quantitative determination of Trospium chloride in presence of its acidic and alkaline-induced degradation product (Fig. 2).

System suitability test according to the United States Pharmacopoeia was used to verify that the resolution and reproducibility of the chromatographic system were adequate for the analysis to be done. Accordingly, system suitability was checked by calculating the column efficiency (N), resolution (R), selectivity (α) and tailing factor (T), where the system was found to be suitable, (Table 2).

3.2.2 TLC Method

Thin layer chromatography has become a well established technique for the assay of drugs either in binary or in multi-component mixtures [9].

The proposed method is based on the difference in the Rf between the intact drug and its degradation product. The suitable mobile phase has been selected to achieve the best separation the drug from its degradation product; other necessary conditions have been established. Different solvent systems with different ratios were tried; best separation was achieved upon using acetonitrile / glacial acetic acid (5:5 v/v). The instrumental conditions for densitometric measurement such as scan mode and wavelength detection were optimized. The scan mode chosen was zigzag mode, and the wavelength was 215 nm. Trospium chloride was completely resolved from its degradation product and its Rf value was 0.40. On the other hand the Rf value of the degradation product was 0.71. This would permit quantitative determination of the drug in presence of its degradation products (Fig. 3).

3.3. Method Validation

Validation of the proposed methods was made by measuring range, accuracy, precision, repeatabilities, interday precision, linearity and specificity. Results obtained are depicted in Table (1). This data render the applicability of the proposed methods for the quality control of the drug formulation.

3.4 Linearity

3.4.1 HPLC

The linear regression data for the calibration curves showed a good linear relationship over a concentration range of 0.5 – 18µg/ml and the regression equation was computed and found to be:

\[ A = 0.2713C + 0.0309 \]

Where A is the peak area ratio, C is the concentration of the drug in µg/ml and r is the correlation coefficient.

3.4.2 TLC

A linear relationship between the concentration of Trospium chloride and the integrated peak area was existing. The proposed method was found to be valid in the range of 2 – 16 µg/ spot and the regression equation was computed and found to be:

\[ A = 0.0460C + 0.0350 \]

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Where A is the integrated peak area, C is the concentration of the drug in µg/spot and r is the correlation coefficient.

### 3.4.3 Accuracy

The accuracy of two methods were assessed by the determination of pure Trosopium chloride samples within the linearity ranges, the mean accuracies are given in Table 1. The recovery percentages (recovery %) and relative standard deviations (RSD) revealed excellent accuracy.

### 3.4.4 Repeatability and intermediate precision

The repeatability and interday precision were evaluated by assaying three freshly prepared solutions of the drug in triplicate on the same day and on three successive days respectively at concentrations within the linearity range for the two methods. RSD% shows the precision of the methods (Table 1).

### 3.4.5 The specificity

The specificity of the methods was proved by the analysis of laboratory prepared mixtures containing different percentages of the degradation products. HPLC method was found to be specific for Trosopium chloride in presence of up to 90% of its degradation products, while the specificity of TLC method was achieved in presence of its degradation up to 80% (Table 3).

### 3.4.6 Assay of pharmaceutical formulation

The usefulness of the proposed methods for the analysis of Trosopium chloride was studied by assaying Trosikan tablet (Table 4). Standard addition technique was also applied to assess the validity of the proposed method (Table 4).

### 3.4.7 Comparison with the official method

Results obtained by the proposed method for the determination of pure samples of the drug were statistically [10] compared to those obtained by European pharmacopoeia's method and no significant differences were observed (Table 5).

### 4. CONCLUSION

The TLC and HPLC methods proposed are accurate, precise and reproducible. They are stability-indicating methods, so can be used for simple accelerated stability studies to predict the expiry dates of pharmaceuticals. Both methods complied with the validation guidelines of the International Conference on Harmonization and could be used for purity testing, stability studies, quality control, and routine analysis of Trosopium.

Table 1: Results of validation parameters of the responses and the regression equations obtained by the proposed methods

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HPLC Method</th>
<th>TLC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>0.5 – 18.0</td>
<td>2.0 – 16.0</td>
</tr>
<tr>
<td>µg/ml</td>
<td>µg/spot</td>
<td></td>
</tr>
<tr>
<td>Average accuracy (%)</td>
<td>100.40</td>
<td>99.96</td>
</tr>
<tr>
<td>Precision</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability a (%)</td>
<td>100.17 ±</td>
<td>100.30 ±</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.406</td>
<td>0.208</td>
</tr>
<tr>
<td>precision b (%)</td>
<td>100.32 ±</td>
<td>99.57 ±</td>
</tr>
<tr>
<td></td>
<td>0.247</td>
<td>0.574</td>
</tr>
<tr>
<td>Standard deviation (precision)</td>
<td>0.450</td>
<td>0.700</td>
</tr>
<tr>
<td>Relative Standard deviation (precision %)</td>
<td>0.448</td>
<td>0.700</td>
</tr>
<tr>
<td>Regression equation</td>
<td>0.2713</td>
<td>0.0460</td>
</tr>
<tr>
<td>Slope a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept a</td>
<td>0.0309</td>
<td>0.0350</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9995</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

\[ a n = 3\times3 \]
\[ b n = 3\times3 \]
\[ c Results of five determinations \]

Table 2: Parameters of system suitability test of HPLC Method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Obtained value</th>
<th>Reference value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trospium chloride</td>
<td>Degradation product</td>
</tr>
<tr>
<td>Selectivity factor (α)</td>
<td>1.33</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Resolution (R)</td>
<td>7.41</td>
<td>&gt;1.5</td>
</tr>
<tr>
<td>Capacity factor (K')</td>
<td>1.89</td>
<td>0.5-10</td>
</tr>
<tr>
<td></td>
<td>2.53</td>
<td>T=1 for atypical symmetric peak</td>
</tr>
<tr>
<td>Tailing factor (T)</td>
<td>0.86</td>
<td>Increase with the efficiency of the separation</td>
</tr>
<tr>
<td>Column efficiency (N)</td>
<td>9533</td>
<td>The smaller the value the higher the column efficiency</td>
</tr>
<tr>
<td></td>
<td>11736</td>
<td></td>
</tr>
<tr>
<td>Height equivalent to theoretical plates (HETP)</td>
<td>0.00157 cm/plate</td>
<td>0.00128 cm/plate</td>
</tr>
</tbody>
</table>
Table 3: Results of analysis of Trospium chloride in laboratory prepared mixtures containing different ratios of Trospium chloride and its degradation product in pure powder form by the proposed methods

<table>
<thead>
<tr>
<th>Degradation %</th>
<th>HPLC Method</th>
<th>TLC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (µg/ml)</td>
<td>Recovery %</td>
</tr>
<tr>
<td></td>
<td>Trospium Chloride</td>
<td>Degradation product</td>
</tr>
<tr>
<td>10</td>
<td>16.2</td>
<td>1.80</td>
</tr>
<tr>
<td>20</td>
<td>14.4</td>
<td>3.60</td>
</tr>
<tr>
<td>30</td>
<td>12.6</td>
<td>5.40</td>
</tr>
<tr>
<td>50</td>
<td>9.0</td>
<td>0.90</td>
</tr>
<tr>
<td>70</td>
<td>5.4</td>
<td>12.60</td>
</tr>
<tr>
<td>80</td>
<td>3.6</td>
<td>14.40</td>
</tr>
<tr>
<td>90</td>
<td>1.8</td>
<td>16.20</td>
</tr>
</tbody>
</table>

Mean ± RSD% 99.46 ± 0.63 Mean ± RSD% 100.01 ± 0.83

Table 4: Quantitative determination of Trospium chloride in pharmaceutical formulation by the proposed methods and results of application of standard addition technique

<table>
<thead>
<tr>
<th>Pharmaceutical formulation</th>
<th>HPLC Method</th>
<th>TLC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trospikan tablet 20 mg / tablet (Batch no. 003)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Found %&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.1 ± 1.34%</td>
<td>100.16 ± 0.77%</td>
</tr>
<tr>
<td>Recovery of standard added %&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.5 ± 0.99%</td>
<td>100.66 ± 1.00%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of six determinations  <sup>b</sup> Average of six determinations

Table 5: Statistical analysis between the results obtained for the determination of Trospium chloride in pure samples by the proposed methods and those obtained by the reported method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HPLC Method</th>
<th>TLC Method</th>
<th>European pharmacopoeia's method**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>100.40</td>
<td>99.96</td>
<td>99.95</td>
</tr>
<tr>
<td>S.D</td>
<td>0.450</td>
<td>0.700</td>
<td>0.644</td>
</tr>
<tr>
<td>R.S.D%</td>
<td>0.448</td>
<td>0.700</td>
<td>0.644</td>
</tr>
<tr>
<td>Variance</td>
<td>0.202</td>
<td>0.490</td>
<td>0.414</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Student's t (2.228)*</td>
<td>1.404</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>F test (5.05)*</td>
<td>2.049</td>
<td>1.183</td>
<td></td>
</tr>
</tbody>
</table>

*The values between parenthesis are the theoretical values of t and F at (p = 0.05).  
** Official method; Titrimetric method using 0.1 M silver nitrate as titrant, the end point was determined potentiometrically.
Fig. 1. Trospium chloride
Molecular formula = C_{25}H_{30}NO_{3}Cl; Molecular weight = 428

Fig. 2: HPLC chromatogram of a resolved mixture of Trospium chloride (10 µg/ml) and its degradation product (5 µg/ml) using the specified chromatographic conditions.

Fig. 3: Scanning profile of TLC chromatogram of Trospium chloride (2 – 16 µg/spot) at 215nm.
REFERENCES