Development and Validation of a Spectrofluorimetric Method for the Assay of Tetracycline in Capsules

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Abstract

The purpose of this study is to develop and validate a method for the analysis of tetracycline capsules by spectrofluorimetry. A pH 9 borate buffer was used as diluent of tetracycline after reaction with magnesium salt at the excitation wavelength of 372 nm and 516 nm of emission. A linear response was observed between 0.25 μg/mL and 1.5 μg/mL with a correlation coefficient (R) of 0.9998. The detection and quantification limits found are 0.0125 μg/mL and 0.0412 μg/mL respectively. The proposed method proved trueness with a recovery between 99.88% and 101.10%. The relative standard deviations of repeatability and intermediate precision found ≤ 2.88% reflected a good precision of the method. The proposed method is therefore valid within the limits of 90% to 110%. The proposed method was applied to the quality control of 9 tetracycline samples from market and gave results in accordance with the pharmacopoeia standards.

Keywords

Validation, Spectrofluorimetry, Tetracycline

1. Introduction

Tetracycline hydrochloride (THC) is a broad-spectrum polyketide antibiotic produced by the genus Streptomyces. It exerts a bacteriostatic effect on bacteria by reversibly binding the 30S ribosomal subunit of bacteria and blocking the binding of tRNA to the ribosome acceptor site. It also binds to some extent to
the bacterial 50S ribosomal subunit and can alter the cytoplasmic membrane causing leakage of intracellular components of bacterial cells [1].

A large number of analytical techniques for the determination of tetracycline and its degradation products have been reported, particularly in biological fluids and pharmaceuticals such as spectrophotometry [2] [3] which is of limited utility due to its non-specificity. More efficient separative methods, such as capillary electrophoresis [4] and high performance liquid chromatography [5] [6] using UV and fluorescence detectors require a lot of time, proven expertise and a very high operating cost. This limits their use in routine quality control and especially in developing countries with limited resources. The aim of this study is to validate a spectrofluorometric method for tetracycline assay in capsules by using the accuracy profile approach [7]. This method has proved to be simple, sensitive, fast, efficient and cheap.

2. Material and Method

2.1. Materials

2.1.1. Apparatus

A Perkin Elmer luminescence spectrometer model LS 45® (Perkin Elmer instruments, Massachusetts, USA) connected to a Fujitsu Siemens computer loaded with the FLwinlab® application software was used.

2.1.2. Reagent

The following reagents and chemicals of analytical grade (except specific indication) were used:

Potassium dihydrogenphosphate (KH$_2$PO$_4$), hydrogenphosphatedisodium (Na$_2$HPO$_4$), boric acid, Sodium hydroxide both from sigma-aldrich, Tetracycline hydrochloride (Figure 1) was provided by the European Pharmacopeia.

Purified water was produced in situ with a Milli-Q Ultrapure Water System (Millipore, Molsheim, France).

2.2. Method

2.2.1. Analytical Parameters

Optimum reaction conditions have been studied such as: The solvent, Effect of magnesium sulphate, Stability of tetracycline in water and Influence of pH.

![Figure 1. Structure of tetracycline hydrochloride.](image-url)
2.2.2. Preparation of Solutions for the Determination of the Calibration Curve

10.00 mg of THC are dissolved in 100 mL of distilled water to obtain the standard solution (SS). 0.5-1.5-2-2.5 and 3 mL of the SS are transferred respectively into the 200 mL volumetric flasks and diluted to the mark. 5 mL of the pH9 buffer solution are added to each 5mL of dilution followed by 2.5 mL of the MgSO4 solution (0.75 M).

2.2.3. Accuracy Profile

Accuracy is the total error after the sum of systematic error (trueness) and random errors (precision). It is represented by an accuracy profile for the area of measures (75% - 125%). Accuracy of the method is established through the accuracy profile described by Feinberg.

The accuracy profile approach allows to determine simultaneously the recovery and precision of the method using standard and validation solutions as previously described [8].

1) Preparation of Standard Solution

Three solutions of concentration of 0.75-1-1.25 μg/mL are prepared. 5 mL of each dilution, 5 mL of the pH 9 buffer solution and 2.5 mL of the MgSO4 solution (0.75 M) are mixed in a conical tube. The reading of the fluorescence intensity is carried out after 25 min at 372 nm of excitation and 516 nm of emission.

2) Preparation of Test Solution

The reference sample for validation is reconstituted from the active ingredient (tetracycline) and the matrix (Lactose, Mannitol, magnesium stearate and starch), thus the stock solution is made of 10 mg of tetracycline and 10 mg of the matrix dissolved in 100 mL of water and filtered. Solutions of concentrations of 0.75-1-1.25 μg/mL are prepared. 5 mL of the buffer solution at pH 9 and 2.5 mL of the MgSO4 solution (0.75 M) are added to 5 mL of each solution. The reading of the fluorescence intensity is carried out after 25 min at 372 nm of excitation and 516 nm of emission.

2.2.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection is estimated by diluting a solution of known concentration until the lowest near detectable signal is different from that of blank, while the limit of quantification is 3.3 times the limit of detection [9]. They were determined as specified in the ICH (International Conference on Harmonisation) protocol [10].

2.2.5. Application of the Method

A test sample of 10 mg of tetracycline is carried out on the powder emptied from 5 capsules (taking into account the average weight). After dissolution in 100 mL of distilled water, homogenization with ultrasound for 10 minutes and filtration, 2 mL of the filtrate are diluted to 200 mL to obtain a solution of 1 μg/mL of THC. To 5 mL of the 1 μg/mL solution are added 5 mL of Borate buffer at pH 9 and 2.5 ml of the 0.75 M MgSO4 solution. The reading of the fluorescence inten-
sity is carried out after 25 min at 372 nm of excitation and 516 nm of emission.

3. Results

3.1. Study of the Optimal Reaction Conditions

3.1.1. Effect of Solvent on Fluorescence Intensity
A solution with a cloudy appearance is obtained when the magnesium sulfate is put into the THC solution in basic medium. This solution is unusable due to the formation of insoluble compounds. On the other hand, in the water a weak signal is observed as it appears in Table 1.

3.1.2. Influence of pH
Signals are stronger with basic buffers than acidic buffers (Figure 2). An excellent and stable signal is observed at pH 9. The use of MgSO₄ at pH 5.6 helped to boost the fluorescence of tetracycline dissolved in water (non fluorescent). The complex formed between THC and Mg gave a good stability in function of time.

3.2. Study of Selectivity
The comparison of data found during the analysis of THC pure product and when it is with the other substances which accompany it in galenic presentation (the excipients) gave two spectra (Figure 3 and Figure 4).

3.3. Graph Calibration
Employing the conditions described in the procedure, a calibration curve was drawn in Figure 5.

![Figure 2](image.png)

Effect of pH on the fluorescence intensity.

<table>
<thead>
<tr>
<th>Settings</th>
<th>NaOH 0.5 μg/mL</th>
<th>H₂O 5 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>MgSO₄ à pH 5.6</td>
<td></td>
</tr>
<tr>
<td>Λex</td>
<td>380 nm</td>
<td></td>
</tr>
<tr>
<td>Λem</td>
<td>520 nm</td>
<td></td>
</tr>
<tr>
<td>FI⁺</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>Observation</td>
<td>Non exploitable</td>
<td>stable</td>
</tr>
</tbody>
</table>

Table 1. Effect of magnesium sulfate on the bypass.
Figure 3. Spectrum of pure TCH.

Figure 4. Spectrum of TCH in the matrix.

Figure 5. TCH Calibration Curve.

\[ y = 288.7x + 10.86 \]
\[ R^2 = 0.999 \]
The equation of regression way \( y = 289.11 + 10.448 \) with a correlation coefficient \((R)\) of 0.9998.

### 3.4. Accuracy Profile

The recovery data, precision and confidence interval are shown in Table 2 and Figure 6. For trueness, recoveries were between 99.88% and 101.10%. For intra-day and inter-day precision the RSD (relative standard deviation) between 1.57% and 2.88% were lower than 5%.

### 3.5. Application of the Validated Method for the Determination of TCH in Pharmaceutical Preparations

To make the application of the method effective, it has been used for the direct determination of TCH in pharmaceutical preparations. Nine lots of THC capsules were separately selected for quantitative determination of THC. The THC content in samples collected from the market is determined qualitatively by the validated method and results are presented in Table 3.

### 4. Discussion

Although Tetracycline is soluble in aqueous solutions, water and methanol, it is far from stable in these solvents. Most often, it is transformed into Epitetracycline hydrochloride (ETC). This epimerization reaction is reversible. In the presence of divalent cations the reaction is not made [11].

Dissolved in water, no signal was found for TCH; on the other hand dissolved in water in the presence of Mg at a pH of 5.6, a signal was found at 380 nm of

![Figure 6. Diagram of the accuracy profile of the proposed method.](image-url)

<table>
<thead>
<tr>
<th>concentration</th>
<th>Trueness</th>
<th>Repeatability RSD</th>
<th>Intermediate precision RSD</th>
<th>Tolerance</th>
<th>Lower tolerance limit</th>
<th>Higher tolerance limit</th>
<th>Lower limit</th>
<th>Upper tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>101.10%</td>
<td>2.88%</td>
<td>2.88%</td>
<td>7.04%</td>
<td>94.06%</td>
<td>108.13%</td>
<td>90%</td>
<td>110%</td>
</tr>
<tr>
<td>100%</td>
<td>99.88%</td>
<td>1.57%</td>
<td>1.57%</td>
<td>3.84%</td>
<td>96.04%</td>
<td>103.72%</td>
<td>90%</td>
<td>110%</td>
</tr>
<tr>
<td>125%</td>
<td>101.07%</td>
<td>1.80%</td>
<td>2.07%</td>
<td>5.06%</td>
<td>96.01%</td>
<td>106.13%</td>
<td>90%</td>
<td>110%</td>
</tr>
</tbody>
</table>

\( T = 2.45; \text{ddl} = 6; \alpha \text{ risk} = 0.05. \)
Table 3. Results of samples assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>99.20 ± 0.91</td>
<td>101.90 ± 0.54</td>
<td>99.00 ± 2.02</td>
<td>103.10 ± 2.83</td>
<td>98.50 ± 0.96</td>
<td>108.20 ± 1.26</td>
<td>105.30 ± 0.63</td>
<td>104.00 ± 0.46</td>
<td>94.50 ± 1.02</td>
</tr>
</tbody>
</table>

Mean: mean concentration; SD: standard deviation.

excitation and 520 nm of emission (Table 1). This coincided with the results found by Rodríguez et al. [12] using methanol as a diluent and other divalent cations selective for tetracycline [13] [14].

It turns out that pH 9 is the one that gave the highest signal (Figure 2). THC is more fluorescent under these conditions, i.e. stable at pH > 8 [12].

Thus, for the analysis of THC, distilled water (used as diluent), Borate at pH 9 (buffer) and MgSO₄ 0.75 M (not only to boost fluorescence but also to keep the stability of the THC) are retained in this proposed method.

Linearity was established by the least squares regression method of the calibration curve. This standard curve was obtained in the range of 0.25 to 1.5 μg/mL. The correlation coefficient is greater than 0.9998. The results show an excellent correlation between the fluorescent intensity and the concentration of the analyte. These values are in accordance with ICH specification [10].

The trueness recoveries were between 99.88% and 101.10%. For intra-day and inter-day precision the relative standard deviation (RSD) between 1.57% and 2.88% were lower than 5%.

These values below 5% indicate a good precision confirmed by the accuracy profile (Figure 6) which clearly shows the good quantitative method performance throughout the validation field [15].

The student test was used at 5% risk and at a degree of freedom of 6, the found t is 2.45, thus, the confidence interval found per level varies from 3.84 to 7.04%. These values are in accordance with ICH specifiable accepted limit for tetracycline content varies from 90 to 125% for the American Pharmacopoeia [5] for our method the limit or interval of validity fixed is between 90% - 110%.

With regard to the profile drawn (Figure 6), we note that the lower and upper bounds found vary from 94.06% to 108.13%. This method is therefore valid in this concentration range.

Limits of detection and quantification are determined based on signal-to-noise ratios, comparing signals measured from samples of known concentrations of THC with the signal from the blank. Experimentally, the detection limit (LD) found is 0.0125 μg/mL and the limit of quantification (LOQ) is 0.0412 μg/mL. This LOQ obtained is approximately 35 times lower than that (1.739 μg/mL) reported for the spectrophotometric method [3]. It is close (0.026 μg/mL) to that reported for HPLC [6].

The content of our samples is quantitatively determined. The results found vary from 94.5% to 108.5%. Margins of (100 ± 10)% have been set as the acceptable limit for the method used. The results of the samples met the official acceptance criteria.
5. Conclusions

The present work demonstrates that the method is fair, faithful, selective and sensitive. The linear response function was found for a range of concentrations ranging from 0.25 to 1.5 μg/mL with a correlation coefficient of 0.9998.

Considering its low limit of detection and quantification, its sensitivity and selectivity, the method seems competitive with respect to titration and spectrophotometry which are reported as other analytical techniques. Although high performance liquid chromatography has a similar sensitivity, the method developed gives the advantage of being easy, fast, less expensive and avoids the tedious extraction procedure, the use of expensive and sometimes toxic organic solvents.

The validated spectrofluorimetry method was successfully applied in the direct determination of THC in 9 batches of pharmaceutical preparations.

It is therefore usable as a technique for routine analysis of tetracycline in pharmaceutical preparations.

References


