Development and validation of a HPLC method for the determination of metronidazole, oxytetracycline and furazolidone in veterinary formulations

Dezvoltarea si validarea unei metode HPLC pentru determinarea metronidazolului, oxitetraciclinei si furazolidonei din produse farmaceutice veterinare

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Cuvinte cheie: determinare simultană, metronidazol, furazolidonă, oxitetraciclinei, metoda HPLC.

Abstract
An isocratic reversed phase high performance liquid chromatographic method with DAD detection was developed for the analysis of metronidazole, oxytetracycline and furazolidone. The mobile phase consisted of pH 2.5 phosphate buffer solution, methanol and acetonitrile (70:18:12). The UV detection was carried out at 264 nm, and the flow rate was 1.100 mL/min. The separation was carried out on a Nucleosil C18 column, 5 µm 250 mmx4.6 mm, which was maintained at 25 °C. This method was validated by system suitability parameters, linearity, limits of detection and quantification, precision and accuracy.

Rezumat
Determinarea metronidazolului, oxitetraciclinei si furazolidonei s-a facut printr-o metoda de cromatografie de lichid de inalta performanta, prin elutie izocratica. Faza mobila a fost formata din fosfat de potasiu monobazic 50 mM cu pH 2.5, metanol si acetonitril (70:18:12). Detectia s-a facut la lungimea de unda de 264 nm, folosind un debit de 1.100 mL/min. Separarea s-a realizat pe o coloana Nucleosil C18 column, 5 µm 250 mmx4.6 mm, mentinuta la temperatura de 25 °C. Metoda a fost validata urmarind parametrii: system suitability, liniaritate, limita de detectie si de cuantificare, precizie si acuratete.

Introduction
Oxytetracycline is a broad spectrum antibiotic cu used in the veterinary medicine to inhibit gram-positive and gram-negative bacteria synthesis.

The European Committee has approved the use of oxytetracycline in a wide range of species: cats, dogs, sheep, goats and swine.

Both oxytetracycline and oxytetracycline hydrochloride contain impurities that in the starting material should not exceed the European Pharmacopeia-required limits.

Furazolidone is a nitrofuran antibacterial with a nitro group in its molecular structure.

This group has a broad antibacterial and antiparasitic action and this is why nitrofurans are widely used in the treatment of gastrointestinal infections in cage birds, dogs and cats.

Metronidazole is effective against protozoa and anaerobic bacteria, being used for prevention and treatment as well.

The proposed method is simple, precise, specific and, above all, appropriate for the determination of metronidazole, oxytetracycline and furazolidone in Enterogurd M- powder.

1. Materials and method
1.1. Reference materials and reagents
- The metronidazole standard was purchased from Sigma.
• The oxytetracycline standard was purchased from the European Pharmacopeia.
• The furazolidone standard was purchased from the USP.
• The studied pharmaceutical product, Enteroguard M – tablets was supplied by Romvac Company.
• Ultrapure water was used for the preparation of all solutions, obtained in-house with a Milli-Q system (MILLIPORE, USA).
• The HPLC methanol and acetonitrile were supplied by Merck. The HPLC monobasic potassium phosphate and hydrochloric acid were supplied by Fluka. The 85% orthophosphoric acid, used for pH adjustment, was purchased from Merck.
• Dimethylformamide (DMF) used for sample preparation was purchased from Sigma.

1.2. Chromatographic system and conditions

The chromatographic system used, LC Surveyor (Thermo Electron Corporation, USA) is provided with quaternary pump, autosampler, 25 µL loop, column thermostat, autosampler thermostat and UV-VIS – diode array detector. The entire chromatographic system is controlled with ChromQuest soft.

The chromatographic separation was done on a Nucleosil C18, 5 µm, 250 mm x 4.6 mm column. The mobile phase contains: 50 mM monobasic potassium phosphate with pH 2.5, methanol and acetonitrile (70:18:12). The 1.1 mL/min flow rate, 254 nm wavelength and 10 µL injection volume are parameters set for this method.

1.3. Preparation of standard stock solutions

The standard metronidazole solution was prepared dissolving 10 mg of standard in 10 mL methanol.

The standard furazolidone solution was prepared dissolving 10 mg of standard in 10 mL DMF.

The standard stock oxytetracycline hydrochloride solution was prepared dissolving 10 mg of standard in 10 mL HCl 0.01 M.

1.4 Sample preparation

In a volumetric flask (de 50 mL) was weighed 0.254 g of Enteroguard M powder, (equal to 25 mg metronidazole, 15 mg oxytetracycline and 5 mg furazolidone). 5 mL HCl 0.1M, 8 mL DMF and 15 mL water were poured over this amount and the solution obtained was sonicated for 10 minutes.

1.5. Validation of the chromatographic method

This method was validated studying various parameters: specificity, linearity, limit of detection and quantification, precision and accuracy.

Specificity was checked calculating parameters such as: retention time, theoretical plates, asymmetry, resolution and capacity factor.

To determine calibration curves linearity, five concentrations within 10% - 200% were prepared from stock solutions of metronidazole, oxytetracycline and furazolidone.

The calibration curves were used for the determination of limits of detection and quantification.

Method precision was determined by injecting six individual samples of Enteroguard M powder.

Method accuracy can be assessed by the recovery percents of metronidazole, oxytetracycline and furazolidone.

The recovery study was conducted using sample solutions with concentrations within 80%-120%.
2. Results and discussions

The objective of this study was to develop a HPLC method allowing separation of metronidazole, oxytetracycline and furazolidone.

A common chromatogram of a sample of Enterogurd M-powder is described in Figure 1. The active substances were prepared with good asymmetry in eight minutes. The retention times were 2.5, 4.1 and respectively 5.3 for metronidazole, oxytetracycline and furazolidone.

The identification of active substances was confirmed using a soft calculating other performance parameters as well, such as resolution, asymmetry, retention time and theoretical plates, table 2.

The statistical assessment of results for active substances in Enteroguard M – powder is shown in table 3.

![Common chromatogram of Enteroguard M - powder](image.png)

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Retention Time</th>
<th>Area</th>
<th>Resolution</th>
<th>Asymmetry</th>
<th>Capacity factor</th>
<th>Theoretical plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td>2.593</td>
<td>2519440</td>
<td>0.00000</td>
<td>1.01999</td>
<td>2</td>
<td>6726</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>4.155</td>
<td>2692618</td>
<td>0.00006</td>
<td>0.97944</td>
<td>3</td>
<td>5021</td>
</tr>
<tr>
<td>Hydrochloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furazolidone</td>
<td>5.300</td>
<td>1422186</td>
<td>0.000035</td>
<td>0.90892</td>
<td>4</td>
<td>8673</td>
</tr>
</tbody>
</table>

Fig.1. Common chromatogram of Enteroguard M - powder
### Table 2

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Metronidazole</th>
<th>Oxytetracycline</th>
<th>Furazolidone</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD mg/mL</td>
<td>0.000358</td>
<td>0.000130</td>
<td>0.001109</td>
</tr>
<tr>
<td>LOQ mg/mL</td>
<td>0.001194</td>
<td>0.000432</td>
<td>0.003696</td>
</tr>
<tr>
<td>Regression equation</td>
<td>(y=2.26968e-008x–0.00134127)</td>
<td>(y=1.21776e-008x–4.94260e-006)</td>
<td>(y=7.58344e-008x–4.94260e-006)</td>
</tr>
<tr>
<td>Correlation coefficient (r²)</td>
<td>0.998711</td>
<td>0.999608</td>
<td>0.999608</td>
</tr>
<tr>
<td>Linearity domain mg/mL</td>
<td>0.005-0.1</td>
<td>0.003-0.06</td>
<td>0.001-0.02</td>
</tr>
<tr>
<td>Repeatability %RSD n=6</td>
<td>0.356</td>
<td>0.301</td>
<td>0.333</td>
</tr>
<tr>
<td>Reproducibility %RSD</td>
<td>0.525</td>
<td>0.383</td>
<td>0.500</td>
</tr>
<tr>
<td>% recovery</td>
<td>100.7</td>
<td>97.49</td>
<td>89.72</td>
</tr>
</tbody>
</table>

### Conclusions

In this study, the analytical parameters for the three active substances were optimized using high performance liquid chromatography.

Separation was isocratic with a mobile phase consisting of 50 mM monobasic potassium phosphate with pH 2.5, methanol and acetonitrile (70:18: 12) and a flow rate of 1.100 mL/min.

The chromatographic analysis time was short (8 min) and peak resolution was good for all active substances.

In conclusion, this study demonstrates that the analytical method applies is sensitive, selective and fast for the determination of metronidazole, oxytetracycline and furazolidone in Enteroguard M–powder.

### References

5. European Pharmacopoeia 7.0 (2011), 2651-2653.