

Development and validation of an HPLC method for the determination of oxytetracycline and enrofloxacin in veterinary formulations

Dezvoltarea și validarea unei metode HPLC pentru determinarea oxitetraciclinei și enrofloxacinului din produse farmaceutice veterinare

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Cuvinte cheie: determinare simultană, oxitetraciclinei, enrofloxacin, metoda HPLC.

Abstract

An isocratic reversed phase high performance liquid chromatographic method with DAD detection was developed for the analysis of oxytetracycline and enrofloxacin. The mobile phase consisted in acetonitrile: methanol: 0.4% orthophosphoric acid adjusted to pH 2.7 with triethanolamine (20:10:70). The UV detection was carried out at 254 nm, and the flow rate was 1.0 mL/min. The separation was carried out on a Nucleosil C18 column, 5 μ m 250 mm x 4.6 mm, which was maintained at 20 °C. This method was validated by specificity parameters, linearity, limits of detection and quantification, precision and accuracy.

Rezumat

Determinarea oxitetraciclinei și enrofloxacinului s-a făcut printr-o metodă de cromatografie de lichid de înaltă performanță, prin eluție izocratică. Faza mobilă a fost formată din metanol: acetonitril: acid ortofosforic 0,4 % ajustat la pH 2,7 cu trietanolină (20:10:70). Detectia s-a făcut la lungimea de undă de 254 nm, folosind un debit de 1,00 mL/min. Separarea s-a realizat pe o coloană Nucleosil C18 column, 5 μ m 250 mmx 4,6 mm, menținută la temperatura de 20 °C. Metoda a fost validată urmărind parametrii: specificitate, liniaritate, limită de detecție și de cuantificare, precizie și acuratețe.

Introduction

Antibiotics are one of the common drugs of modern medicine, indicated for treatment of diseases by killing or destroying bacteria.

At present, there are more than 100 antibiotics which can cure minor or life-threatening infections.

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

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

Enrofloxacin is a broad-spectrum chemotherapeutic substance with strong

antibacterial effect against most pathogenic bacteria found in sick animals.

Enrofloxacin is used in veterinary medicine in the following species: cats, dogs, swine, poultry, for treatment of diseases caused by Gram-positive, Gram-negative bacteria and pathogenic agents such as:

- Mycoplasma,
- Chlamydia and
- Rickettsia.

Consequently, a simple, sensitive, safe and cost-effective HPLC method has been developed for the simultaneous determination of oxytetracycline and enrofloxacin in **Metrosept E – oily suspension**.

1. Materials and Methods

1.1. Reference materials and reagents

- The oxytetracycline hydrochloride standard and enrofloxacin standard were purchased from Fluka.
- The tested pharmaceutical product Metrosept E was provided by Romvac Company. Ultrapure water obtained in-house with Milli-Q system (Millipore, USA) was used for the preparation of all solutions.
- The HPLC-grade methanol and acetonitrile were provided by Merck. The orthophosphoric acid 85% was purchased from Merck.
- Triethanolamine necessary for pH adjustment was purchased from Fluka. The hydrochloric acid used for sample preparation was purchased from Fluka.

1.2. Chromatographic system and conditions

- The chromatographic system LC Hitachi Chromaster (Hitachi Group, Japan) consists of quaternary pump, auto sampler, 100 μ L loop, column thermostat, auto sampler thermostat and UV-VIS – diode array detector. The entire chromatographic system is controlled using EZChrom Elite/Open LAB Software.
- The chromatographic separation was carried out on a NUCLEOSIL C18, 5 μ m, 250 mm x 4,6 mm column. The mobile phase contains: phosphoric acid 0.4 %, pH 2.7 (TEA adjustment), methanol and acetonitrile (70:10:20).
- The parameters used for this method are 1 mL/min flow, 254 nm wavelength and 10 μ L injection volume.

1.3 Preparation of standard stock solutions

- The standard **oxytetracycline hydrochloride** solution was prepared by dissolving 10 mg standard in 10 ml HCl 0,01 M.
- The **standard enrofloxacin** solution was prepared by dissolving 10 mg standard in 10 ml methanol.

1.4 Samples preparation

- Weigh 1g (equal to 5 mg oxytetracycline, 25mg enrofloxacin) from Metrosept E suspension into a 50 ml flask.
- Add HCl 0,01M.
- Ultrasonate solution obtained for 10 min.
- After cooling, adjust the volume to 50 ml with HCl 0,01M and filter the solution through 1289-type quality filter paper.
- Dilute 1ml of solution to 10ml with water.
- After stirring, filter the solution through PVDF 0.45 μ m filter and inject it in the chromatographic system.

1.5. Chromatographic method validation

The validation of HPLC method for the simultaneous determination of oxytetracycline and enrofloxacin in the veterinary formulation was carried out in accordance with ICH guidelines.

The assessed parameters were:

- **specificity,**
- **linearity,**
- **limit of detection and quantification,**
- **precision and**
- **accuracy.**

Specificity was checked by calculating parameters such as: retention times, theoretical plates, asymmetry, resolution and area.

The **linearity** of the proposed method was determined based on calibration curves obtained by graphical representation of areas in the 10%-200% range vs. active substance concentrations.

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

Method precision was checked regarding both repeatability and intermediate reproducibility by calculating the standard deviation.

Method accuracy can be assessed by the recovery rates of the active substance.

The recovery study was carried out using sample solutions in the 80%-120% range.

2. Results and discussions

The development of HPLC method for the simultaneous determination of oxytetracycline and enrofloxacin involved the use of various chromatographic columns and mobile phase mixtures and it was eventually determined that the best separation had been obtained with mixture of phosphoric acid 0.4%, pH 2.7 (TEA adjustment), methanol and acetonitrile (70:10:20).

The active substances were separated with good asymmetry in 12 minutes.

The retention times were 4.9 and 6.9 for oxytetracycline and enrofloxacin.

A representative chromatogram of a sample of Metrosept E- oily solution is shown in figure 1. The identification of active substances was confirmed using the software which was also used to calculate other performance parameters such as resolution, asymmetry, retention times and theoretical plates. The results are shown in table 1.

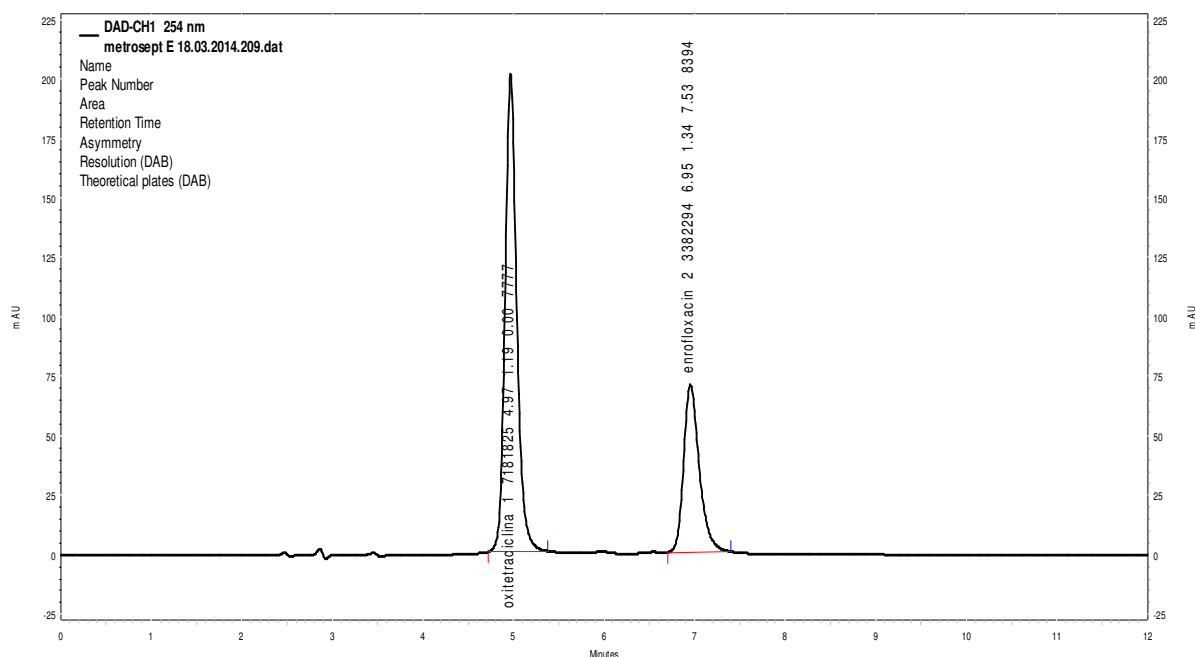


Figure 1. Representative chromatogram of Metrosept E- oily suspension

Table 1

Parameters calculated for the mixture in figure 1.

Parameter	Oxytetracycline hydrochloride	Enrofloxacin	Admissibility criterion
Retention time	4,97	6,95	%RSD <2
Area	7181825	3382294	%RSD <2
Theoretical plates	7777	8394	N>2000
Asymmetry	1,19	1,36	A _s (OXYTETRACYCLINE)<1,25 A _s (ENROFLOXACIN)<1,5
Resolution	0	8	R>2

The calibration curves obtained by graphical representation of the peak area vs. concentration and related statistical

calculations, the regression equation and correlation coefficient are shown in figures 3 and 4.

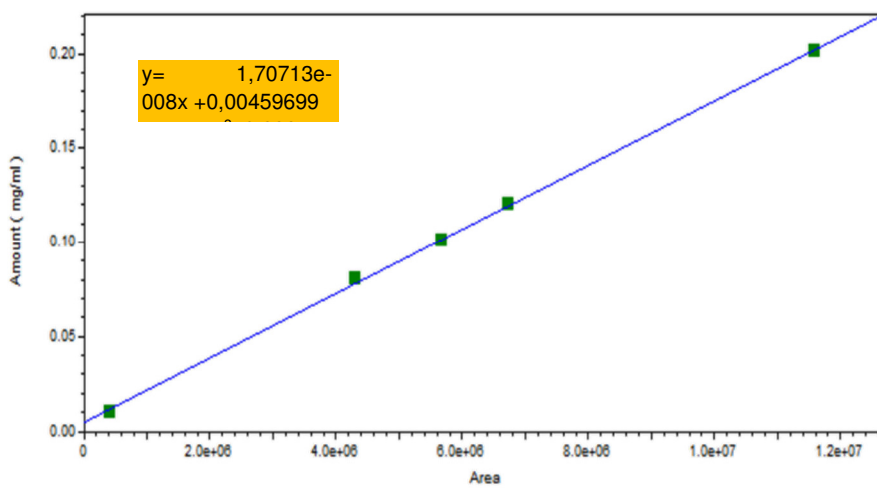


Figure 2. Calibration curve for oxytetracycline hydrochloride

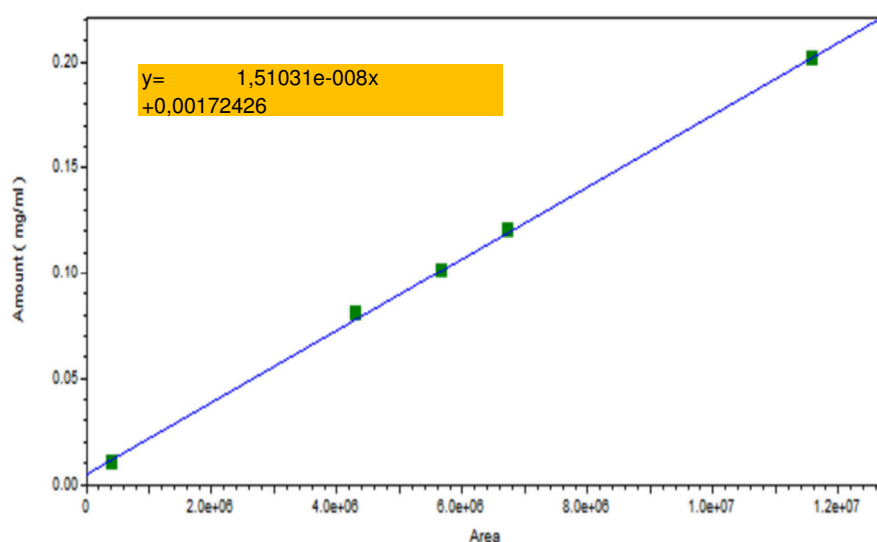


Figure 3. Calibration curve for enrofloxacin

Method sensitivity determined based on calibration curves, using the limit of detection and limit of quantification as parameters is shown in table 2. Results for precision are

shown in tables 3 and 4, allowing us to notice that relative standard deviation values are within specified limits (%RSD < 2).

Table 2

Parameters calculated for the mixture in figure 1.

Parameter	Oxytetracycline hydrochloride	Enrofloxacin
LOD µg/mL	0,33	0,402
LOQ µg/mL	1,109	1,341

Table 3

Results for precision

Parameter	Mean retention time	Mean area	%RSD	Admissibility criterion
Oxytetracycline hydrochloride				
Repeatability	4,989	7110678	0,256	%RSD<2,0
Reproducibility	4,982	7175381	0,286	%RSD<2,0

Table 4

Results for precision

Parameter	Mean retention time	Mean area	%RSD	Admissibility criterion
Enrofloxacin				
Repeatability	4,989	7110678	0,256	%RSD<2,0
Reproducibility	4,982	7175381	0,286	%RSD<2,0

Accuracy, revealed by the recovery rates of both active substances, is shown in tables 5 and 6. The recovery rates for oxytetracycline and enrofloxacin are between 100% and 126%.

Table 5

Results for accuracy

Concentration level %	Concentration of oxytetracycline hydrochloride * mg/mL	% recovery of oxytetracycline hydrochloride*	% recovery mean
80	0,0820	103,077	105,286
100	0,1039	109,273	
120	0,1234	103,510	

* mean of three determinations

Table 6

Results for accuracy

Concentration level %	Concentration of enrofloxacin* mg/mL	% recovery of enrofloxacin*	% recovery mean
80	0,0398	102,952	114,470
100	0,0525	126,853	
120	0,0625	113,606	

* mean of three determinations

3. Conclusions

- The proposed isocratic HPLC method proved to be selective, fast, precise and specific for simultaneous determination of both antibiotics in Metrosept E - oily suspension.
- Method selectivity is revealed by a resolution >2 between the active substances of the tested product.
- The proposed method is fast because it allows separation of oxytetracycline hydrochloride and enrofloxacin in 12 min.
- The short assay time and simplicity of the mobile phase indicate that the proposed method can be easily used for routine assays, quantitative dosage of oxytetracycline hydrochloride and enrofloxacin.
- In conclusion, this study demonstrates that the analytical method engaged is sensitive, selective and fast for the determination of oxytetracycline and enrofloxacin in Metrosept E- oily suspension.

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