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DEVELOPMENT OF SOME ANALYTICAL METHODS FOR QUALITY CONTROL OF VETERINARY MEDICINES

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(As in Ph. D. Thesis)

INTRODUCTION

Pharmaceutical forms used in veterinary field, in opposition with those for human use, contain more active substances than drugs used in humans. This may seem unusual for those involved in the human medicine industry, but it should be taken into account that the animals are stubborn and therefore it is necessary to administer, through a single injection, several active principles. Therefore, most pharmaceutical forms for animals contain more than two active substances. Some excipients which are also added may interfere with active substances in the determination of the above-mentioned compounds.

At present, in most technical documentation of veterinary medicinal products, manufacturers propose one analytical method for each active species and only sometimes for excipients, as well. In this way, the time needed to analyse a finished product is quite long. For this reason, the cost of the finished product will be increased according to the number of active substances added.

In view of these considerations of saving human resources, materials and time and bringing innovation from a scientific point of view, this work aims at:

MAIN GOAL

- Development of analytical methods for quality control of veterinary medicines.

OBJECTIVES

- Development of chromatographic methods for the determination of three or more active substances in veterinary pharmaceuticals;
- Development of spectrometric methods for the determination of three or more active substances in veterinary pharmaceuticals.

I. THEORETICAL PART

The theoretical part consists of two chapters: the first is related to the active substances and the existing methods in the scientific literature for determining their quality, and the second one presents the methodologies for the development of the chromatographic and spectrometric methods.

In the first chapter are presented the main characteristics of the active substances and excipients covered by this thesis as well as the summary description of the analytical methods of interest which are included in the monographs of the European Pharmacopoeia's 8th edition of the active substances / excipients. To these, methods published in the literature are added. Here are some methods to assay the following:

- chloramphenicol, tylosin tartrate, prednisolone, vitamin B12 and benzyl alcohol, which are compounds found in the medicine CTP12 solution;

- vitamins B1, B6 and B12 as well as carprofen, compounds that are to be found in the composition of Artro-Vet tablets;

- fenicols: chloramphenicol, florfenicol and thiamphenicol, found in veterinary products;

- metronidazole, oxytetracycline and furazolidone, substances given in the composition of Enteroguard tablets.

Considering the literature study pointing out the active substances in the pharmaceutical veterinary medicinal product which are the subject of this PhD thesis the following conclusions can be drawn:

- these substances have monographs in the European Pharmacopoeia, with the exception of furazolidone and florfenicol;
- there are analytical methods for their quantitative determination in finished and biological products;
- most developed methods are based more on liquid chromatography and less on gas chromatography;
- there are spectrometric, potentiometric methods and very few volumetric methods;
- all these methods allow the determination of active substances of interest by themselves or in combination of no more than three, such as the determination of metronidazole, furazolidone and oxytetracycline by HPLC [1]
- there is no analytical method in the literature to allow, for example, the determination of chloramphenicol, tylosin tartrate, prednisolone and vitamin B12 (as in product CTP12) or vitamins B1, B6, B12 and carprofen (as in product Artro-Vet).

The second chapter presents the strategy for the development of some chromatographic methods based first on the structural characteristics of the substances and second on the chemical

equilibria. To this, choosing the chromatographic columns, the mobile phases and the appropriate wavelength for the best detection of the substances are also discussed.

The chapter also deals with different aspects related to the determination of substances found in a multicomponent mixture by means of UV-VIS absorption spectrometry. These determinations are based on the Bouguer-Lambert-Beer law and on the property of absorbance to be additive at the same wavelength. The fundamental principle of derivative spectrometry, namely zero-crossing, is applied to multicomponent mixtures that cannot be analysed by zero-order spectrometry.

II. GENUINE EXPERIMENTAL PART

The genuine experimental part discusses the results obtained in the development of some chromatographic (in Chapter 3) and spectrometric methods (in Chapter 4) used for the control of the quality of some veterinary medicines. All developed methods have been tested according to international rules [2, 3]; parameters such as selectivity, linearity, accuracy, precision and robustness have been evaluated.

3.1. Development of a chromatographic method for the determination of active substances in CTP 12

One of the most important Romanian manufacturers on the veterinary medicine market has developed a finished product containing four active substances, namely chloramphenicol (200 mg / mL), tylosin tartrate (55 mg / mL), prednisolone (5 mg / mL) and vitamin B12 (0.1 mg / mL). CTP 12 is a synergetic combination that associates a bacteriostatic (chloramphenicol) with a bactericide (tylosin) and an anti-inflammatory (prednisolone) to which vitamin B12 is added as a general tonic. Any under-dosing or overdosing of these 4 active substances makes treatment ineffective and also can lead to side effects. Therefore, a rigorous control of their content in the finished product is required.

As presented in the theoretical part of this thesis, the four active substances mentioned above were never quantitatively determined by a single analytical method. That is why we developed and published [4] a new HPLC method, which would allow the determination of the four above mentioned active substances together with the one excipient existing in the composition of CTP 12.

The chromatographic method was developed using two chromatographic columns, namely Hypercarb and HyPurity Advance, purchased from ThermoElectron Corporation USA. The Hypercarb column has the following characteristics: 100×4.6 mm internal diameter and 5 μm particle size, whereas HyPurity Advance has the following features: 250 mm length, 4.6 mm internal diameter and 5 μm particle size. Throughout the separation, the column was kept at 25 ° C. The separation was performed isocratic, using a mobile phase of water with 40% (v / v) methanol as an organic modifier. Injection volume was 10 μL. The flow rate of the mobile phase was 0.8 mL / min. Detection was performed at 260 nm.

The representative chromatogram for a synthetic sample containing chloramphenicol 2 mg / mL, tylosin tartrate 0.55 mg / mL, prednisolone 0.05 mg / mL, vitamin B 12.00 mg / mL and benzyl alcohol 0.1 mg / mL is shown in Figure 1.

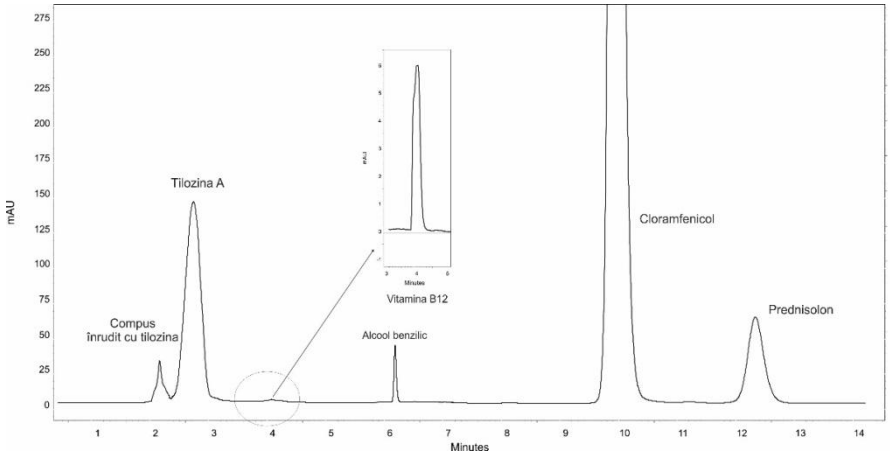

Figure 1. Representative chromatogram for a synthetic sample containing 2 mg / mL chloramphenicol, 0.55 mg / mL tylosin tartrate, 0.05 mg / mL prednisolone, 0.001 mg / mL vitamin B12 and 0.1 mg / mL benzyl alcohol

The method was applied to CTP12 commercial products. A very good agreement between the amounts of active substances and excipients claimed by the manufacturer and those calculated using the new HPLC method was obtained.

3.2. Development of a chromatographic method for the determination of carprofen and vitamins B1, B6 and B12 in Artro-Vet

A pharmaceutical form containing several active substances, namely carprofen (100 mg), vitamin B6 (50 mg), vitamin B1 (50 mg) and vitamin B12 (0.1 mg) per tablet is marketed in

Romania under the name Artro-Vet. It is recommended to be administered both during growth to very active puppies in order to ensure and improve their mobility and also to dogs with a symptomatic osteoarthritis. The monograph in the European Pharmacopoeia [5] presents a potentiometric titration method for carprofen analysis.

Until the publication of the paper [6] resulting from this thesis, no analytical method was available in the literature to determine the four active substances.

Two chromatographic columns LiChrosorb RP-18 (150 × 4.6 mm, 5 μm) and Hypersil GOLD aQ (250 × 4.6 mm, 5 μm) were used to develop and optimize the HPLC method. The best choice was the Hypersil GOLD aQ column, which allows the use of a fully mobile aqueous phase. Thus, the following chromatographic conditions were tested: gradient elution using a mobile phase composed of phosphate buffer (pH = 2.65) and methanol as organic modifier. The gradient, expressed as a variation of the phosphate buffer percentage in the mobile phase, was as follows: from 0 to 1 minute - 98% phosphate buffer; between 1 and 7.50 minutes - 50%; then kept constant until 11 minutes - 50%; then up to 15 minutes decresed to 0% and finally increased to 98% between minutes 15 to 22 after which it was maintained at this composition for a further 2 more minutes. Mobile phase flow rate was 1 mL / min, and detection was at 292 nm. The injection volume was 10 μL.

Under these chromatographic conditions the following chromatogram shown in Figure 2 was obtained.

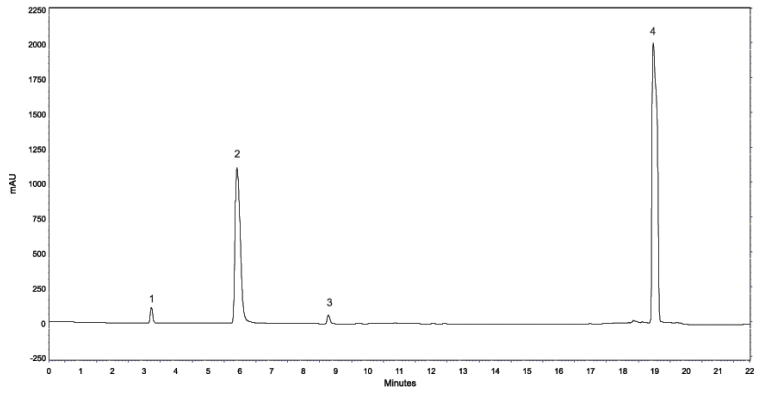

Figure 2. Representative chromatogram for carprofen mixture (0.5 mg / mL), vitamin B1 (0.25 mg / mL), B6 (0.25 mg / mL) and B12 (0.5 μg / mL) 1-vitamin B1, 2-vitamin B6, 3-vitamin B12, 4-carprofen

The method was applied to Artro-Vet samples and the results obtained were consistent with those reported by the manufacturer.

3.3. Development of a chromatographic method for the determination of fenicols

While chloramphenicol, CAP and thiamphenicol, TAP have monographs [7, 8], the quality control of florfenicol is not described in any pharmacopoeial monograph or other official document. Therefore, there is a need for a chromatographic method for the determination of florfenicol in different veterinary pharmaceuticals. Consequently, we developed a new chromatographic method [9] for the determination of fenicols in pharmaceutical products and milk samples. This method uses a Hypercarb column (with porous graphitic carbon stationary phase) with the following characteristics: 100 × 4.6 mm and particle size of 5 μm) purchased from ThermoElectron Corporation, USA. Separation was performed isocratically at 25 ° C using a mobile phase of water and acetonitrile as organic modifier (40:60 v / v), having a flow rate of 0.8 mL / min. Detection was performed at 260 nm. The injection volume was 10 μL. The chromatogram recorded for the separation of the three fenicols is shown in Figure 3.

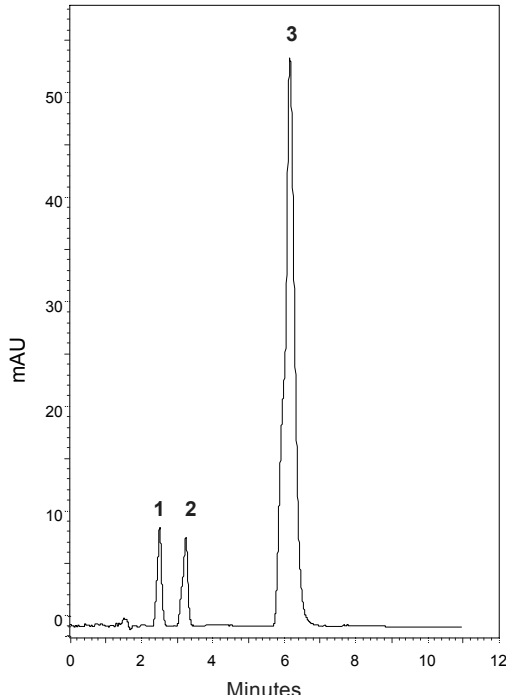

Figure 3. Representative chromatogram for the mixture of fenicols (0.1 mg / mL): 1-thiamphenicol, 2-florfenicol, 3-chloramphenicol.

As can be seen from above, the separation is performed in 7 minutes with a good resolution between all three fenicols.

For this method, the decision limit (CCα) and the detection capability (CCβ) were calculated, as it was applied for the determination of the fenicols from different milk samples. In addition, this method can be used to determine the florfenicol content from various veterinary medicines, especially since there are currently no official monographs for this compound.

4.1. Development of a spectrometric method for determination of active substances from CTP 12

Concentrations of active substances are determined by derived-spectrometry using equations based on Bouguer-Lambert-Beer-law. For example, for multi-component mixtures, /X plus Y plus Z/ the Lambert-Beer law can be written for the wavelength at which the first-derived spectrum of compound Z crosses the abscissa, in the form of:

$$I^{am} = \frac{d\varepsilon^X}{d\lambda} \times C^X + \frac{d\varepsilon^Y}{d\lambda} \times C^Y \qquad (1)$$

- C^X și C^Y are the concentrations of X and Y, expressed in mol / L;
- I^{am}- is the intensity of the first order derivative spectrum, $\frac{dA}{d\lambda}$ is the intensity of the I-derived spectrum, $\frac{dA}{d\lambda}$ measured for the mixed solution, at the wavelength at which the first order derivative spectrum of compound Z crosses the abscissa;
- $\frac{d\varepsilon^X}{d\lambda}$ și $\frac{d\varepsilon^Y}{d\lambda}$ are the molar absorptions, first order derivative spectrum, expressed in L × mol-1 × cm-1 for compounds X and Y, determined experimentally at the wavelength at which the first-derivative spectrum of compound Z crosses the abscissa.

The CTP 12 product is in the form of a homogeneous brownish-green solution that makes it virtually impossible to develop a method by UV-VIS absorption spectrometry. Therefore, the following attempts were aimed to obtain the I, II or III derivative spectra that best meet the *zero-crossing* condition. As in the chromatographic method, the greatest difficulty in establishing the best conditions for the spectrometric method was determined by the large variety of concentrations and the diversity of structures in the CTP 12 composition.

The experimental conditions in which the *zero-crossing* method can be applied have been investigated. Thus, derivative spectra were obtained by using the Spectra Manager program and

the only ones that allow the application of the *zero-crossing* methodology were second-order derivative spectra. The image obtained after their overlapping is shown in Figure 4.

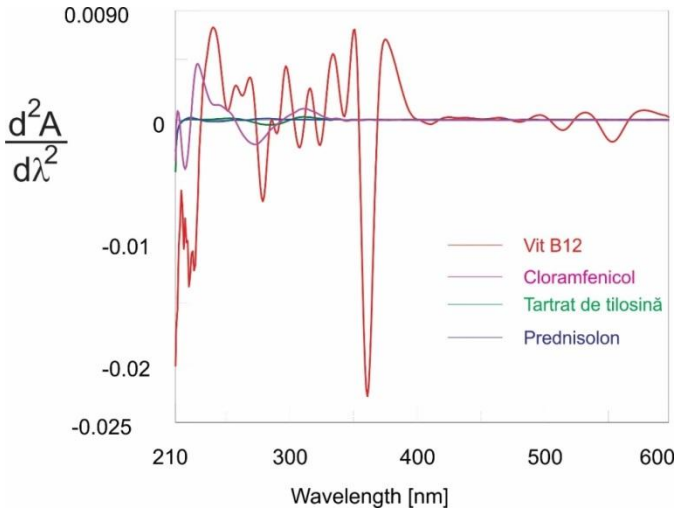

Figure 4. Second-derivative spectra for active substances in CTP12 recorded for wavelength range 210-600 nm

What is remarkable as concerning these substances is that the *zero-crossing* condition for the series of second-derivative spectra at different wavelengths is met. This is quite rare for mixtures of four compounds. The image obtained for this situation is shown in Figure 5.

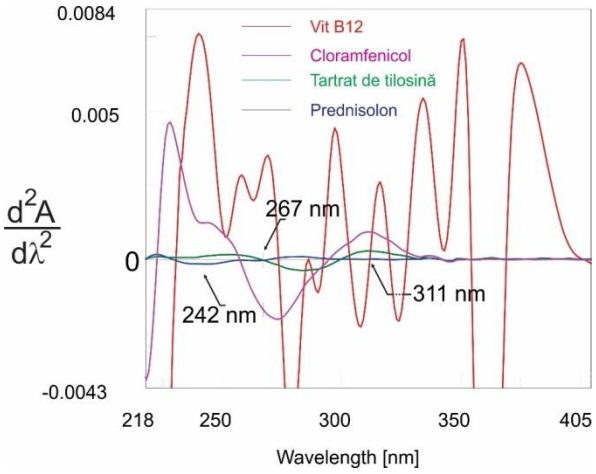

Figure 5. Second-order derivative spectra for active substances in CTP12 recorded for the wavelength range 218-405 nm.

Based on the spectra shown in Figures 4-5, the conditions for the determination of the four active substances in the CTP 12 composition have been established. Thus:

- at the wavelength of 552 nm vitamin B12 can be determined in the presence of all other active substances in the formula CTP 12;
- at the wavelength of 267 nm, only the vitamin B12 and chloramphenicol contribute to the intensity of the spectrum of the second-derivative spectrum of the active substance mixture of the formula CTP 12; the tylosin tartrate and prednisolone compounds have the magnitude of the second-order derivative spectrum zero and do not influence this value. Knowing the concentration of vitamin B12 in the finished product, determined at 552 nm, the chloramphenicol concentration in the sample can be calculated based on the additivity of the intensity of the derivative spectra and the molar absorbtivity second order derivative for both vitamin B12 and chloramphenicol;
- the tylosin tartrate concentration can be determined at the wavelength of 311 nm, since no other compounds other than tylosin and chloramphenicol contribute to the intensity of the derivative spectrum of the mixture; the concentration of chloramphenicol was calculated using the *zero-crossing* methodology applied at the wavelength of 267 nm;
- at the wavelength of 242 nm, prednisolone can be determined because the concentration of all other species in the CTP formula is known and well as the molar absorbtivities.

The method was applied for the determination of the four active substances in the CTP 12 composition and the results compared to those obtained by the original HPLC method presented in Chapter 3.1.

4.2. Development of a spectrometric method for determination of substances from Enteroguard

As presented in subchapter 2.2.2. such mixtures of compounds can be analysed by using the *zero-crossing* method for one of the variants of the I, II or III derivative spectra. Therefore, using the Jasco V530 spectrometer software, the derivative spectra were obtained and those that best met the *zero-crossing* condition are the first-derivative spectra. The image obtained for them is shown in Figure 6.

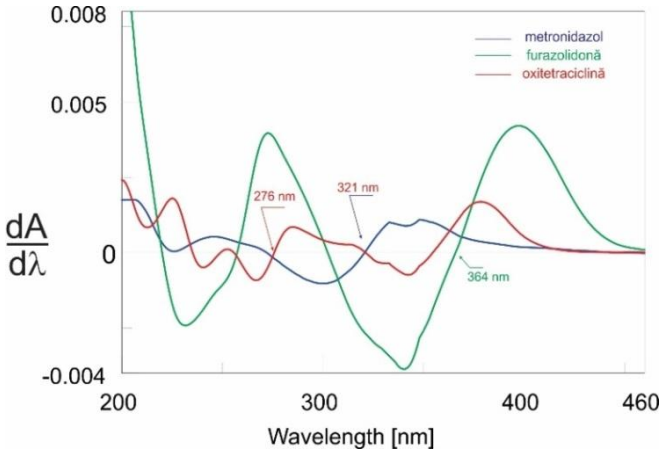

Figure 6. First-order derivative spectra for the three active substances

It can be seen from Figure 6 that the first-order derivative spectrum provides the best spectral conditions for the determination of the three compounds in the mixture, namely:

- at the wavelength of 321 nm, the first-order derivative spectra of metronidazole and oxitetracycline cross the abscissa, while that of furazolidone has a maximum, different from zero; at this wavelength the concentration of furazolidone in the sample can be determined;
- at the wavelength of 276 nm, the intensity of the first-order derivative spectra of oxytetracycline is zero; knowing the previously determined concentration of furazolidone, the concentration of metronidazole in the sample can be calculated;
- at the wavelength of 364 nm, the intensity of the furazolidone firdt-derivative spectrum is zero, knowing that the concentration of metronidazole can determine the concentration of oxytetracycline.

The new spectrometric method developed allowed quantification of the active substances in Enteroguard M - tablets.

GENERAL CONCLUSIONS

Both the aim of this paper and its objectives were achieved in studies that are the subject of the doctoral thesis, by elaborating and publishing for the first time in the literature three chromatographic methods that can be used for the control of the quality of some veterinary medicine. To all these two other new methods are added, based on the principle of *zero-crossing* of derivative spectrometry, that can be used both in the in-process as also in the finished product control.

BIBLIOGRAPHY (selection)

1. Tauber, V., Chiurciu, V., Development and validation of a HPLC method for the determination of metronidazole, oxytetracycline and furazolidone in veterinary formulations, *Veterinary Drug,* 8 (1): 73-79, 2014

2. ICH Topic Q2A. Validation of Analytical Methods: Definitions and Terminology, step 5 (CPMP/ICH/381/95)

3. ICH Topic Q2B. Validation of Analytical Procedures: Methodology, step 4 (CPMP/ICH/281/95)

4. Stan, G.-S., Badea, I. A., Aboul-Enein, H. Y., HPLC method for quantification of five compounds in a parenteral form used in treatment of companion animals, *J. Chromatogr. Sci.*, 54(9): 1567-1572, 2016

5. European Pharmacopoeia, 8.0 version General Monographs, Carprofen for veterinary use, 07/2008:2201, 1778-1779, Council of Europe, Strasbourg, France

6. Stan, G.-S., Mitoi, B.E., Mihăilă, A., Badea, I. A., Aboul-Enein, H. Y., Determination of Carprofen in the Presence of some Hydrosoluble Vitamins of Group B by HPLC Method*, Curr. Anal. Chem.*, 11(4): 273-278, 2015

7. European Pharmacopoeia, 8.0 version General Monographs, Chloramphenicol 01/2008:0071, 1844 – 1845, Council of Europe, Strasbourg, France

8. European Pharmacopoeia, 8.0 version General Monographs, Thiamphenicol, 01/2015, 1844, Council of Europe, Strasbourg, France

9. Stan, G.-S., Moldovanu, F., Badea, I. A., Determination of phenicols using a fast and reliable HPLC method developed on Hypercarb stationary phase, *Rev. Chim.,* 68(3): 545-548, 2017.