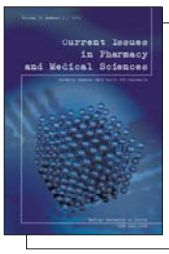

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Validation of the doxycycline hydrochloride determination by high-performance liquid chromatography technique

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ABSTRACT

A sensitive and reproducible chromatographic method was developed and validated for the quantification of doxycycline hydrochloride in veterinary product – Doxycyclinum 50%. The analysis was performed on HPLC (Shimadzu) apparatus with diode array detector. The separation was provided with C_{18} column (4.6mm×100 mm, 3 μm particle size) at mobile phase flow rate 1ml/min and detection at 254 nm. The method was tested in the range 50-150% nominal DOXY concentration resulting good linearity with correlation coefficient r=0.9999. The estimated LOD and LOQ elaborated method amounted to 0.46 and 1.55 mg/L. The recovery values were from 99.70 to 100.9% with RSD 0.78%. It was confirmed that the reagents used for testing of samples and solvents do not interfere with the active substance. Moreover, the significant influence of temperature and flow rate on the retention time was ascertained. The carried out research demonstrates that the method is validated and the obtained results are accurate and reproducible. The elaborated method can be suitable to routine analysis in quality control laboratory.

Keywords: doxycycline hydrochloride, validation, HPLC

INTRODUCTION

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to assess the quality, reliability, and consistency of analytical results. Validation is an integral part of any good analytical practice. Validation of the methods and processes in the pharmaceutical industry is also used to ensure the health safety of the product [1, 3, 5, 7].

In this paper, we described the validation of doxycycline hydrochloride (DOXY) determination by high performance liquid chromatographic method.

Doxycycline (DOXY) is an antibiotic belonging to the tetracycline group, which was discovered about 60 years ago by Duggar. It represents the most widely used group of antibiotics. Tetracycline compounds can be divided into short, moderate, and prolonged biological half-life period. Doxycycline was discovered in 1966 and put into use in humans in the early 1970s. It has the form of a yellow crystalline, hygroscopic powder. Easily soluble in water and methanol, but quite difficult in ethanol [2, 4, 6].

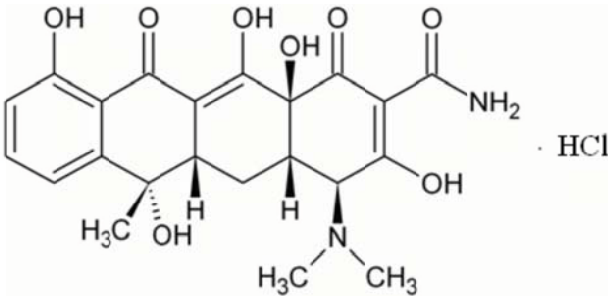

Fig. 1. The doxycycline structure

According to the current guidelines for manufacturing and registration of drugs, if the material is in a pharmacopoeial monograph, it is preferred during the registration process. Additionally, analytical methods described in pharmacopoeial monographs do not need to be submitted for a registration dossier validation. On the other hand, this situation results in the fact that pharmaceutical companies are developing analytical methods for raw material. It is thus a "negative" effect especially if the substance is more and more practically applied and is used in many forms of pharmaceutical drugs or passes from hu-

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man to veterinary medicines [2, 4, 6, 8]. In this respect, it is worth noting that the Doxycycline is now very widely used in treatment of humans and animals. Use of Doxycycline in production animals requires the development of new analytical methods for analysis of the drug in the animal tissues.

[2, 8]. The fact of doxycycline being pharmacopoeial substance does not exempt researchers from the need to develop analytical methods for the determination of doxycycline in proprietary medicinal products. Each drug has its own specifications and related analytical methods that must be validated. The main purpose of this work was to prove the assumptions: the selectivity, the linearity, the scope of method, accuracy, precision, limits of detection and quantification, stability of the prepared samples, insensitivity to small changes in the method and chromatographic system suitability.

MATERIAL AND METHODS

All used reagents and solvents were of analytical grade. Doxycycline hydrochloride was purchased from Sigma, Poland. Pharmaceutical preparation Doxycycline 50% (Biofaktor sp. z o. o., Gorzów Wlkp.) was obtained from local pharmacy.

HPLC SHIMADZU (Kyoto, Japan) system with auto sampler SIL-20AC HT and photodiode multi-wavelength detector (SPD-M20A diode array detector) was applied. The chromatographic data were recorded and processed by the *LCsolution version 1.23 SP*.

Analyses were carried out with a C_{18} column (4.6mm×100 mm), 3 µm particle size, Supelco, Poland). The mobile phase consisted of tert-butanol, 0.2M tetrabutyloammonium hydrogen sulphate, 0.1 M EDTA (60:5:1) up to volume with distilled water. The mobile phase flow rate was 1 mL/min, and the detector was monitored at 254 nm. All the chromatographic operations were carried out at 60°C.

Selectivity. The influence of the main component of veterinary medicinal product Doxycycline 50% on the determination of biological active substance was studied. For this purpose, a sample of the mobile phase, the solvent sample (0.01 M HCl), the placebo with all excipients, certified doxycycline hydrochloride standard solution and the sample Doxycyclinum 50%, were prepared. The effect peaks originating from the reagents and the placebo ingredients for the main peak of doxycycline hydrochloride in the standard as well as a sample in the drug were observed. No influence of the matrix on the analyzed substance should be confirmed. Acceptance criterion should be adopted if the peaks interfering with the signal of the main peak cannot be greater than 1.0%.

The linearity of the method. To determine the linearity of the method the sample of the veterinary medicinal product Doxycyclinum 50% was prepared according to the drug formulation. For the test, the solutions containing 50%, 80%, 100%, 120% and 150% of DOXY standard were prepared and analyzed. Each sample was measured five times. The required linearity must be at least 0.999.

Accuracy of the method. In order to determine the accuracy of the method there were prepared three samples of 80-120% of the nominal concentration. It is assumed that the recovery needs to be between 97-103% of nominal DOXY concentration for the medicinal product Doxycyclinum for each level.

Intra and inter-day precision. Intra-day precision was determined by analysis of solutions of Doxycycline 50% at three doxycycline hydrochloride concentration levels (19.16; 38.31 and57.47 mg/g) five times on the same day. Inter-day precision was determined by analysis of the same concentration on five different days over a period of one week. The acceptance was that the RSD value was lower than 2.0% for the active ingredient determination.

Precision under repeatability. In order to determine the precise method for the determination of doxycycline hydrochloride in the veterinary medicinal product analysis was performed in five series of Doxycyclinum 50%. The acceptance was that the RSD value was lower than 2.0% for the active ingredient determination.

The limit of quantification and detection. In order to determine the limit of quantification and detection in the DOXY assay the noise of blank was analyzed. The solution was injected six times. The limit of detection (LOD) calculated as the concentration, which generated a peak about 3 times as high as the noises height, and the limit of quantitation (LOQ) calculated as the concentration, which generated peak about 10 times as high as the noises height, was found as 3.

Insensitivity (robustness). Insensitivity determines the behavior of the analytical method by small but significant changes in the parameters. For this purpose, we analyzed the effect of factors such as the flow of the mobile phase and the temperature of the column at a retention time of the component denoted. For this purpose, the sample was prepared in accordance with the standard method and subjected to analysis by changing the temperature and flow rate. It should be assumed that the changes cannot be greater than 1.0%.

Stability. In order to check the stability of the method of determination of doxycycline hydrochloride content by HPLC, the reaction of the standard solution and sample drug Doxycyclinum 50% was analysed for a period of seven days.

Between analyses, the standard sample solution was stored in a refrigerator at a controlled temperature of about 5.0°C. The calculating relative standard deviation for the surface area of doxycycline hydrochloride in Doxycyclinum 50% should be lower than 2.0%.

DISCUSSION OF RESULTS

Selectivity. The obtained results indicate that the method is selective because of the excipients, solvents and mobile phase do not affect the main peak originating from doxycycline hydrochloride. The acceptance criterion was met. The chromatogram of 50% Doxycyclinum is presented in Fig. 2.

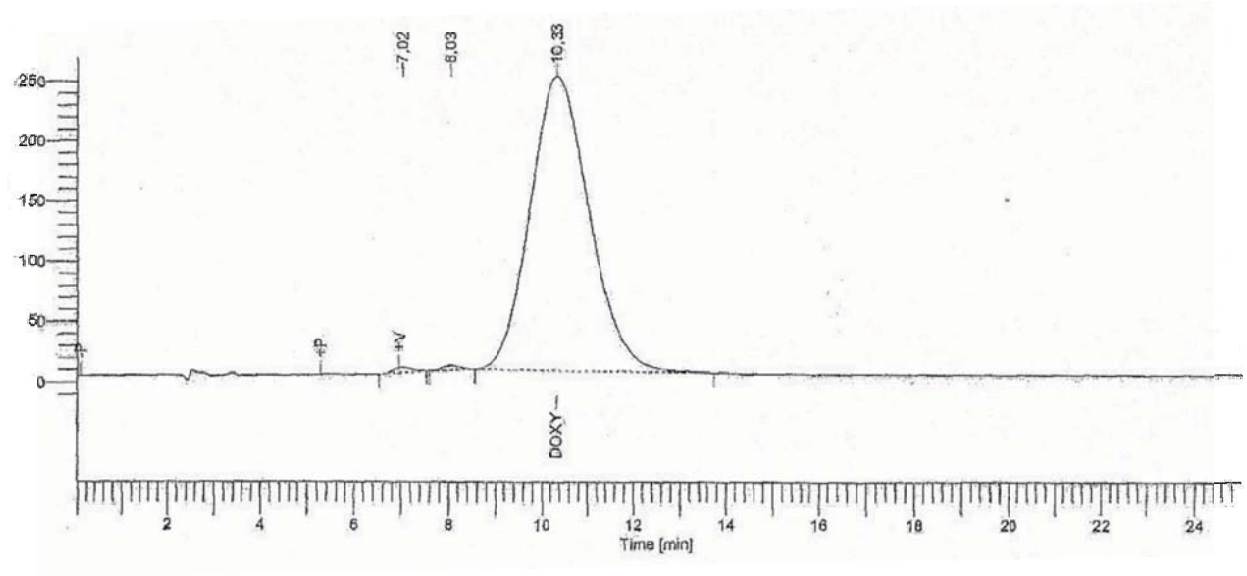

Fig. 2. The chromatogram of Doxycyclinum 50% (condition of analysis are described in the text)

The linearity of the method. The results obtained during linearity test are listed in Table 1.

Table 1. The results of the linearity test (n=5)

Concentration of DOXY [%]	Theoretical amount [mg/g]	Calculated amount [mg/g]	Recovery [%]
50	19.16	19.34±0.32	100.9
80	30.65	30.56±0.08	99.70
100	38.31	38.21±0.04	99.74
120	45.97	46.03±0.17	100.1
150	57.47	57.57±0.26	100.2

In the studied range (19.16-57.47 mg/L) we obtained the correlation coefficient r = 0.9999 which indicates a linear relationship between the concentrations of analyte and the response of detector. The calibration curves equation was as follows: y = 0.3829x+0.0506.

Accuracy of the method. In assessing the mean recovery at each level, it can be stated that the method is accurate and meets the established criteria for acceptance. The recovery of the sample with a nominal content of active substance of 80% was 99.70%, while with the content of 100% and 120% it was 99.74% and 100.1% respectively.

The recovery results are presented in Table 1. The set up criteria were fulfilled, thus, the method is accurate.

Intra and inter-day precision. The intra-day and inter-day relative standard deviations for doxycycline hydrochloride determination in the studied veterinary product were in the ranges 1.06-1.22% and 1.26-1.53%, respectively. These small values indicate that the method is precise and meets the established criteria for acceptance.

Precision under repeatability. The results of these analyses are listed in Table 2.

Table 2. The results of precision under repeatability

Sample number	Mass of sample [g]	Batch	Concentration [mg/g]
1a	0.0529	1	50.11
1b			49.61
2a	0.0597		51.43
2b			51.48
1a	0.0497	2	50.19
1b			50.54
2a	0.0555		51.06
2b			51.76
1a	0.0511	3	51.03
1b			51.53
2a	0.0519		51.11
2b			51.14
1a	0.0449	4	50.41
1b			50.93
2a	0.0421		51.58
2b			51.64
1a	0.0409	5	51.52
1b			51.68
2a	0.0411		51.20
2b			51.30

The above results imply that the precision of the method is good, because the relative standard deviation (RSD) is 1.14%. The acceptance criterion was met.

The limit of quantification and detection. Based on the analysis of the blank solution there are calculated minimum amounts of the substance that can be quantified with appropriate precision and accuracy. The value of the limit of quantification (LOQ) is 1.55 mg /L. This means that it is the minimum concentration of the sample for

which we can calculate the content with appropriate precision and accuracy. When it comes to the limit of detection (LOD), it is smaller and is 0.46 mg /L. This means that the concentration of the active substance, which is the hydrochloride salt of doxycycline, is detectable.

Insensitivity (robustness). The test results on the effect of temperature and flow rate on the doxycycline hydrochloride retention time are listed in Table 3 and 4.

Table 3. The influence of flow rate on the retention time

Flow rate, min/ml	Retention time, min
1 (according to the method)	10.33
0.6	16.21
1.6	8.03

Table 4. The influence of temperature on the retention time

Temperature, °C	Retention time, min
60 (according to the method)	10.33
30	12.02
50	10.55

The above results indicate that the flow rate significantly affects the retention time. The method is also more sensitive to temperature changes. Lowering the temperature of the column temperature control to 30şC significantly affects the retention time. To obtain reproducible results, conditions such as flow rate and temperature should be carefully controlled.

Stability. On the basis of the obtained results it can be concluded that both the standard solution and the proprietary medicinal product sample are stable for a period of seven days. The relative standard deviation is below 2.0% and amounts to 1.42% for the standard solution and 0.78% for the formulation – Doxycycline 50%.

In our study, a simple, fast and reliable HPLC method was developed and validated for the determination of doxycycline hydrochloride in veterinary preparation – Doxycycline 50%. From the obtained results, we concluded that the suggested method characterized high sensitivity, accuracy, reproducibility, and specificity. Moreover, this method is simple and inexpensive and can be employed for the routine quality control of doxycycline hydrochloride.

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