RAPID AND VALIDATED HPLC ASSAY FOR THE DETERMINATION OF OXYTETRACYCLINE IN BIOLOGICAL MATERIAL

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Abstract

A rapid, accurate, simple, and reproducible high performance liquid chromatographic (HPLC) method for the determination of oxytetracycline hydrochloride (OTC) in porcine plasma has been developed and validated. The drug and the standard were eluted from 5 µm Omni Spher (Varian) C18 column (250 mm × 4.6 mm I.D.) at room temperature. The mobile phase was composed of ACN-MeOH-(HCOO)2 (17.5:17.5:65 v/v/v) (pH adjusted to 2). A flow rate was 1.4 mL/min. The effluent was monitored using a UV-VIS detector set at 360 nm. The retention time of OTC was about 3 min. The suggested technique is characterised by superior performance parameters: linearity R² = 0.9999, recovery = 92.50%, repeatability RSD ≤ 1.39%. These results demonstrate the validity of the HPLC method for the analysis of OTC HCL.

Key words: piglets, biological material, oxytetracycline, HPLC.

Oxytetracycline (OTC) is one of the most commonly used antibiotics in animal production (3, 5, 9). It belongs to the tetracycline group of antibiotics. OTC is a compound produced in broth by fermentation from Streptomyces rimosus. It has broad-spectrum of antimicrobial activity. In particular, it is active against Gram-positive and Gram-negative bacteria, Rickettsia, Coxiella, Chlamydia, Mycoplasma, and some protozoa. This antibiotic, as a bacteriostatic agent (2, 3, 5), is widely used in the treatment of respiratory (pharyngitis, pneumonia, bronchitis), urinary, and gastrointestinal infections, because of its activity and good penetration into the tissues. Oxytetracycline is generally well tolerated when administered orally or intramuscularly as an aqueous solution (2, 3).

Several HPLC methods for the determination of oxytetracycline in plasma have been described (1, 6, 8). This paper presents a simple, highly sensitive, rapid, and economical HPLC method for the determination of OTC HCL in plasma.

Material and Methods

Chemicals and reagents. Acetonitrile and methanol, HPLC grade, were obtained from Labscan (Dublin, Ireland). Dihydrate of oxalic acid (C2H2O4·2H2O), monohydrate of citric acid (C6H8O7·H2O), di-sodium hydrogen phosphate (Na2HPO4) and di-sodium ethylenediaminetetraacetate dihydrate (Na2EDTA·2H2O), analytical grade, were obtained from POCH (Gliwice, Poland). Chlorotetracycline analytical standard (EC No. 200-591-7) was purchased from Sigma (St. Louis, USA). Water was purified by the reverse osmosis method with Milli-Q-Plus 185 system (Millipore, Molsheim, France).

Apparatus. The chromatographic system used was a Varian liquid chromatography (Varian, Palo Alto, USA). It consisted of a solvent delivery pump (STAR 9002), a 10 µl manual injector, and a variable wavelength UV-Vis detector. Chromatographic separations were performed using a Varian ChromSep HPLC OmniSpher 5 C18 column (250 mm × 4.6 mm I.D.) (Palo Alto, USA). The system was controlled under Varian Star Chromatography Workstation Version 4.51 (Varian, Palo Alto, USA) software installed on an IBM-PC Pentium computer. Sample preparation was performed using MPW 210 centrifuge (Mechanika Precyzjna, Warsaw, Poland.), BP 61S (Sartorius, Paris, France) analytical balance, cartridges C18, 500 mg (Shimadzu, Tokyo, Japan), AGA Labour vacuum pump (Prague, Czech Republic), and SPE extraction chamber 16 x 75 mm 51 (Varian, Palo Alto, USA).

Animals. The study was carried out on 10 healthy male and female weaned piglets, 8-10 weeks of age and with an initial weight of 17–23 kg. Eight of the piglets were assigned to the experimental group (group I) and two to the control group (group C). One day before the trials began; a venous catheter was positioned in the jugular vein. The animals were housed in individual pens. Before commencing the study, the pigs were marked with numbers. Feed (antibiotic-free diet) and water were given ad libitum throughout the period.
of study. OTC was administered orally at the dose 20 mg/kg body weight. The oral doses were administered individually through a stomach tube. Blood samples (5 ml) were collected from the jugular vein into heparinised tubes at 1, 1.5, 3, 4, 6, 8, and 10 h after the administration of the drug. The samples were centrifuged and plasma was decanted and stored at -80°C until the day of analysis by HPLC.

**Chromatographic procedure.** A mobile phase consisted of MeCN-MeOH-(water solution of HCOO)2 (17.5:17.5:65% v/v/v, pH 2). The mobile phase was pumped isocratically at a flow rate of 1.4 mL/min and the column effluent was monitored at a wavelength of 360 nm. All analyses were performed at ambient temperature.

### Results

Optimised chromatographic conditions were set and the method was validated by the determination of the following parameters: linearity, precision, and accuracy, limit of detection (LOD), and limit of quantification (LOQ).

**Linearity.** Linearity was determined by constructing the calibration curve. For the construction of the curve, five standard concentrations of oxytetracycline in the range of 25-500 ng/mL were prepared. Before the injection of the solution, the column was equilibrated for at least 60 min with the mobile phase flowing through the system.

The peak area of the chromatogram was plotted against the concentration of OTC in the plasma to obtain the calibration curve.

The equation for the calibration curve was \( y = (55 \pm 1.7) x + (29 \pm 42) \) and the correlation coefficient \( R^2 \) equalled 0.9999. The high value of the coefficient indicated good linearity of the calibration curve for the method. The plot is shown on Fig. 1.

**Limit of detection and limit of quantification.** The LOQ, taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, and the LOD, taken as the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified, were calculated. The limit of detection (LOD = 3\( S_{xy}/a \), where \( S_{xy} \) is the standard deviation and \( a \) is the slope) was 3.58 ng/mL and the limit of quantification (LOQ = 10\( S_{xy}/a \)) was 11.93 ng/mL.

**Precision/Accuracy.** The precision and the accuracy of the method were determined by repeatability. The repeatability was examined by four of OTC samples at the same concentration, on the same day, under the same experimental conditions. The standard deviation (SD) was 1.40 ng/mL and relative standard deviation was 1.39%.

**Recovery.** The determination of the OTC recovery from control plasma was performed according to the scheme (Fig. 2).

### Discussion

The HPLC method for the measurement of OTC in porcine plasma was fully validated. The proposed method is selective and sensitive enough for the determination of OTC in sample matrix in veterinary diagnostic laboratories. This makes it valuable and adequate in many applications, particularly in studies performed on animals. Other authors determined residues of tetracyclines (including oxytetracycline) in animal tissues (7), milk and cheese (4) using HPLC method. According to our best knowledge, the recovery has never reached the level of 90%.

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**Fig. 1.** A calibration curve of oxytetracycline hydrochloride. Chromatographic conditions: OmniSpher C18 column - 5 µm (250 mm × 4.6 mm I.D.); mobile phase – ACN-MeOH-(HCOO)2 (17.5:17.5:65% v/v/v), pH 2; UV-Vis detector - 360 nm; temperature - ambient, flow rate – 1.4 mL/min., injection volume – 10 µL.
Preparation of the supernatant
1. Prepare the solution: 1ml plasma + 100 ng/mL standard solution of OTC + 1 ml methanol;
2. Centrifuge a solution for 15 min, 5, 500 rpm;
3. Discharge the upper supernatant layer;
4. Dilute a solution to 30 ml by means the buffer 0.01M EDTA-McIlvaine*.

SPE (Solid Phase Extraction) (500 mg C18)
1. 5 ml of methanol;
2. 5 ml of deionised water;
3. 5 ml of buffer;
4. Analyte;
5. 1 ml of buffer;
6. Elute using ACN-buffer 0.01M EDTA-McIlvaine solution;
7. Evaporate the elute to 2 ml.

HPLC analysis
1. OmniSpher C18 column (250 mm × 4.6 mm);
2. Mobile phase: ACN-MeOH-(HCOO)₂ (17.5:17.5:65, v/v/v), pH 2;
3. Flow rate: 1.4 mL/min;
4. UV-VIS detection \( \lambda = 360 \text{ nm} \).

*It consists of 1 l of distilled water + 12.9 g of C₆H₈O₇·H₂O (citric acid monohydrate) + 10.9 g of Na₂HPO₄ + 37.2 g of Na₂EDTA·2H₂O.

The recovery from the tested plasma was calculated according to the equation:

\[
\text{recovery \ [%] } = \frac{4 \cdot S}{S_{wz} \cdot V} \cdot 100\% 
\]

where:
- \( S \) - peak area (OTC) from the analysis of plasma;
- \( S_{wz} \) - peak area for the standard and equals 5479 (Fig. 2)
- \( V \) - volume of the supernatant.

The calculated of OTC HCL recovery was 92.50%.

In this study the recovery of OTC was determined from blank plasma samples spiked at 100 ng/mL. As was already mentioned, the recovery was 92.50%. The reverse-phase HPLC technique with UV-VIS detection was found to be convenient and precise for analysing the residues of OTC HCL in plasma samples.

This work shows the improved HPLC assay for the determination of OTC in plasma. The results show that the suggested technique is characterised by superior performance parameters: linearity \( R^2 = 0.9999 \), recovery = 92.50%, repeatability RSD ≤ 1.39%, and samples preparation procedure does not influence the analysis result quality. It can be concluded that the developed HPLC method can be successfully applied for analysis of OTC HCL in plasma.

**References**


