
SHORT COMMUNICATION

**DETERMINATION OF OXYTETRACYCLINE
IN BIOLOGICAL MATRIX*****CEZARY KOWALSKI, MAŁGORZATA POMORSKA,
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Oxytetracycline (OTC) has been used for years in medical field. It is a wide-spectrum antibiotic, belonging to the group of tetracyclines with bacteriostatic activity (1-4). OTC chemical name is 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-tetracene-2-carboxamide. OTC possesses broad antimicrobial spectrum, similar to tetracycline. The antibiotic displays high activity against aerobic Gram-positive and Gram-negative bacteria, Rickettsia, Mycoplasma and Chlamydia. OTC is used for the treatment of respiratory, urinary and alimentary tract infections, because of its activity, low toxicity and good penetration into tissues (1-3, 5). OTC is rapidly absorbed from the gastrointestinal tract. The absorption is reduced significantly by dairy products due to the calcium content, which takes part in the formation of chelate compounds. OTC may cause photosensibilisation (1-4).

Several methods have been published describing the determination of OTC in premixes and animals tissues (7,9-12). These method are, however, time-consuming and aren't as accurate as the method described in this paper. The present report deals with a simple, sensitive, rapid and economical HPLC method for determination of OTC in plasma.

The aim of the present study was to develop a time saving, cost-effective and sensitive method for determination of OTC in biological matrix.

EXPERIMENTAL**Chemicals and reagents**

Oxytetracycline standard (oxytetracycline hydrochloride EC No. 2058-46-0) was purchased from Sigma (St. Louis, MO, USA). Methanol, ace-

tonitrile and other chemicals (oxalic acid dihydrate, citric acid monohydrate, disodium hydrogen phosphate, ethylenediaminetetraacetic acid disodium salt dihydrate (POCH Gliwice) were of analytical and HPLC grade. HPLC grade water was produced by means of a Milli-Q-Plus 185 system (Millipore, Molsheim, France). Mepatar® 5% powder (0.05 g OTC-hydrochloride + 0.95 g saccharose combination) was purchased from a pharmaceutical company (TZF „Polfa” S.A., Warsaw, Poland).

Apparatus

Analyses were performed with a Varian liquid chromatograph (Varian, Walnut Creek, CA, USA) equipped with a solvent delivery pump (STAR 9002), a 10 µL volume manual injector, a variable wavelength UV-VIS detector (all Varian Analytical Instruments, USA). Compounds were separated on a Varian ChromSep HPLC OmniSpher 5 C18 (250 × 4.6 mm) column. A centrifuge (MPW 210), an analytical balance (Sartorius BP 61S), cartridges (Shimadzu C18, 500 mg), a vacuum pump (AGA Labor, Warsaw, Poland), Vortex (WL-1, Bio-mix, Warsaw, Poland), and extraction chamber SPE (Varian, USA, 16 × 75 mm) were also used. Detection was performed at 360 nm.

Animals

The studies were performed on 8 healthy weaned piglets of both sexes, 8 weeks of age and weighing 17-23 kg (group I, n = 8). There was also one control group (group C, n = 2). Animals were housed in individual pens. Before starting the study, the piglets were marked with numbers. Feed and water (antibiotic-free diet) were added *ad libitum* throughout the period of study. One day before the

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Table 1. Concentration of OTC after oral administration in piglets'.

Number of piglet	1 h	1,5 h	3 h	4 h	6 h	8 h	10 h
I	349,0645	402,7315	550,1582	825,1231	560,5759	471,1571	352,9316
II	193,1063	208,2937	381,2481	274,5628	193,1142	174,5685	97,6236
III	97,6244	186,3134	368,2647	265,1443	211,8334	139,2752	83,5594
IV	118,4538	137,7412	188,5608	252,1124	242,4669	190,0362	146,0141
V	417,1980	725,6022	496,8068	394,0445	320,8892	241,1778	167,8591
VI	266,3121	343,1397	145,9726	123,3544	106,6905	78,6914	56,2835
VII	184,1236	357,2024	511,3645	467,2899	354,0467	195,2358	124,6367
VIII	211,68134	241,5271	392,4747	294,1156	219,5487	182,7351	115,2862
Mean	229,6955	325,3189	379,3563	361,9684	276,1457	209,1096	143,0243

trials began, a venous catheter was positioned in the jugular vein. All procedures involved in the study were approved by Local Ethical Commission of the Agriculture University.

Experimental design

Mepatar® 5% powder containing 0.05 g OTC/1 g of saccharose, was administered orally at a single dose of 20 mg/kg body weight. The oral doses were administered individually through a stomach tube. Blood samples were collected from the jugular vein into heparinized tubes at time 1, 1.5, 3, 4, 6, 8, and 10 h after administration of the drug. Samples were centrifuged, plasma was decanted and stored at -80°C until the day of analysis by HPLC.

Sample preparation

1 mL of plasma was mixed with 1 mL of methanol. After centrifugation (15 min. at 5500 rpm) the upper supernatant layer was discharged. The solution was diluted to 30 mL using the buffer 0,01 M EDTA-McIlvaine. Next, the following reagents were applied to a cartridge (in reported

sequence): methanol, deionized water, buffer, an analyte and buffer. After this, analyte was eluted using ACN – buffer 0.01 M EDTA-McIlvaine solution. Sample was evaporated and redissolved in mobile phase, and the analysis was performed by HPLC.

Chromatographic conditions

A mobile phase consisted of ACN-MeOH-(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 AND DISCUSSION

The method was validated by the determination of the following parameters: linearity, precision, accuracy, limit of detection and limit of quantification and recovery.

Linearity was determined by generating the calibration curve. Working standard solutions, rang-

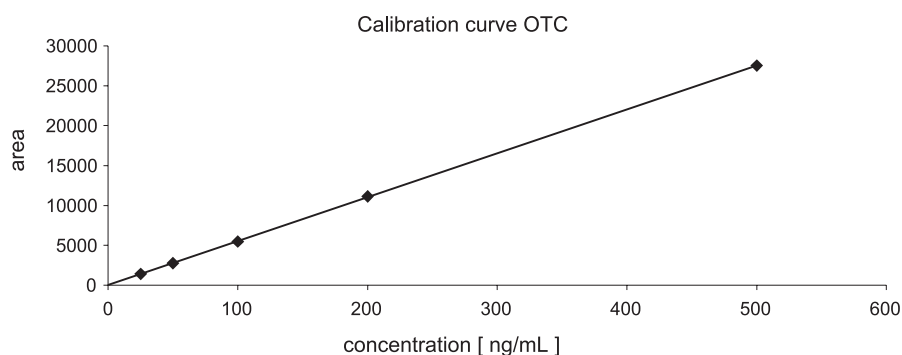


Figure 1. A calibration curve of oxytetracycline hydrochloride.

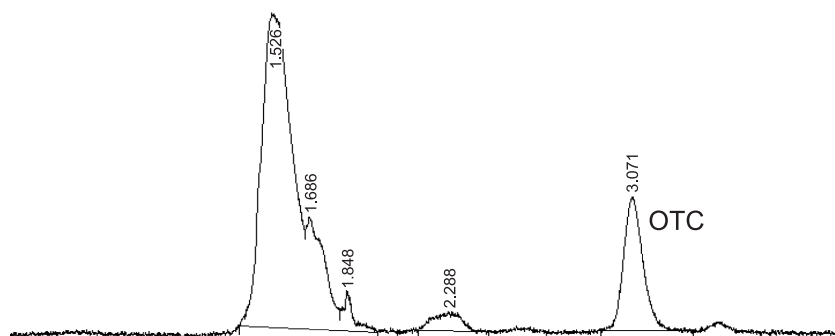


Figure 2. Chromatogram of a plasma sample taken from a piglet (no. I) 3 h after administration of Mepatar® 5% powder.

ing from 25 ng/mL to 500 ng/mL were prepared separately by appropriate dilution of the stock solution (1 mg/mL) with HPLC-grade water and analyzed under the optimized conditions. Before injection, the column was equilibrated for at least 60 min with the mobile phase flowing through the system. The linearity of the method was evaluated by plotting calibration curves of peak area against the concentration of each analyte. The equation for the calibration curve was $y = 55.035x + 28.875$ and the correlation coefficient (R^2) equals to 0.9999 (Tab. 1.) The high value of the coefficient indicated good linearity of the method in the considered concentration range. The plot is shown in Figure 1.

The precision and the accuracy of the method were determined by repeatability. Four drug-free plasma samples (1 mL) were spiked with oxytetracycline standard to 100 ng/mL. All samples were processed according to the procedure described in section: Sample preparation. Each time a volume of supernatant was 1.2 mL. The area of a peak corresponding to an initial amount of OTC (S_p) in the spiked sample was calculated according to the equation:

$$S_p = \frac{4 \times S}{1.2 \times 0.925}$$

where S is a peak area of OTC obtained after SPE.

S_p was used in the determination of OTC amount from the calibration curve. The calculated standard deviation (SD) was 1.40 ng/mL and relative standard deviation (RSD) was 1.39 %.

Limit of detection (LOD) and limit of quantification (LOQ). The calculated values of LOD and LOQ were 3.58 ng/mL and 11.93 ng/mL, respectively.

Recovery. Known amount of OTC-hydrochloride was added to blank piglets' plasma, extracted

and analyzed according to the procedure described in section: Sample preparation. The recovery from the tested plasma was calculated according to the equation:

$$\text{Recovery [\%]} = \frac{4 \times S}{S_{std} \times V} \times 100\%$$

where S is a peak area of OTC obtained from the analysis of spiked plasma (100 ng/mL), $S_{std} = 5479$ is a peak area for the standard and $V = 1.2$ mL is a volume of the supernatant. The calculated OTC recovery was 92.50%.

Figure 2 shows the chromatogram of pig plasma after oral administration of the OTC formulation. There was no peak at the retention time corresponding to OTC on the blank plasma chromatogram. The results of the determination of OTC in pigs' plasma are reported in Table 1. After the determination of concentrations of tiamulin in plasma, the „PK Solution” computer program was used to calculate the pharmacokinetic parameters. The maximum average concentration (C_{max}) of OTC in plasma was found to occur 2.9 h after oral administration of the drug and was 474.9 ng/mL. From this moment concentration of OTC in plasma was decreased and the smallest was observed at 10 h after administration. The AUC, $t_{1/2}$ and MRT were also calculated. They were 2628.3 [ng \times h/L], 3.65 h, and 7.0 h, respectively.

CONCLUSIONS

The HPLC method for the measurement of OTC in piglets' plasma was fully validated and showed good sensitivity, reproducibility, linearity and selectivity. This makes it valuable and adequate in many applications, particularly in veterinary medicine studies. Other authors determined residues of

tetracyclines (including oxytetracycline) in animal tissues, milk and cheese using HPLC method [7,10,12]. According to our best knowledge the recovery has never reached the level of 90%. 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 method for analysis of the residues of OTC in plasma samples. It can be concluded that the developed method in the present study can be successfully applied for analysis of OTC in plasma.

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