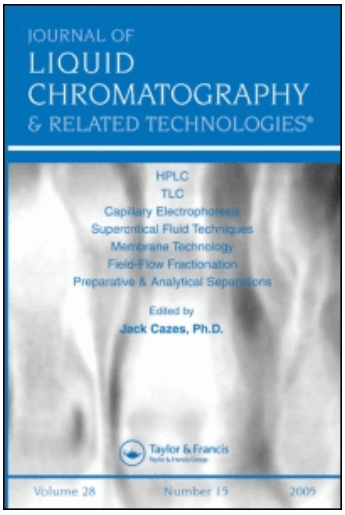

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Development of HPLC with UV-VIS Detection for the Determination of the Level of Oxytetracycline in the Biological Matrix

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Development of HPLC with UV-VIS Detection for the Determination of the Level of Oxytetracycline in the Biological Matrix

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Abstract: An isocratic reversed-phase high performance liquid chromatographic procedure was developed for the determination of oxytetracycline in the biological matrix. The procedure is based on isolation of the compound and the standard (oxytetracycline hydrochloride) from pig plasma using the Shimadzu C18 (500 mg) cartridge, with satisfactory recovery (92.50%) and specificity on a Varian ChromSep HPLC OmniSpher C18 (250 × 4.6 mm, 5 μm) column coupled with a UV-VIS detector set at 360 nm. The suggested technique was shown to be linear ($R^2 = 0.9999$) over the concentration range 25–500 ng/mL. These results clearly demonstrate that the HPLC method is a useful tool in many applications, particularly in veterinary medicine studies.

Keywords: Oxytetracycline, Biological matrix, HPLC determination, UV-VIS detection, Method validation

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INTRODUCTION

Oxytetracyclie (OTC) is a wide-spectrum antibiotic, belonging to the group of tetracyclines (TCs) with pronounced bacteriostatic activity. The spectrum of activity of the tetracyclines covers mainly Gram-positive and Gram-negative bacteria, Rickettsia, Chlamydia, and Mycoplasma.[1] TCs suppress the protein synthesis in the microbial cell by the tRNA and mRNA binding in the ribosomal complex. Antibiotics from this group are used mainly for therapy of systemic bacterial infections in various species of animals (including fish) and humans. OTC has been used for over 40 years in the medical field. OTC is well absorbed from the gastrointestinal tract. Food does not interfere considerably with absorption; although, the absorption is reduced significantly by dairy products or di- and trivalent ions, which takes part in the formation of chelate compounds. OTC is the most commonly used chemotherapeutic in animal husbandry.[1–8] It has generally been well tolerated in animals when administrated orally. Oral administration is contraindicated only in adult ruminant species. In humans, a variety of toxic and irritative effects have been reported from the use of OTC (gastrointestinal irritation, nausea, or vomiting). Intravascular administration may produce trombophlebitis. Long term therapy may cause changes in the peripheral blood (leucocytosis, thrombocytopenia). Oxytetracycline penetrates well in the body fluids and tissues, and attains high concentrations in the lungs, kidneys, and liver. Part of the antibiotic is reabsorbed from the intestines and further included in the enterohepatal circulation. OTC is excreted predominantly with urine.

Because of its antibacterial action, oxytetracycline is used widely not only in medicine but also in veterinary medicine, as well as in the meat production industry, where it is used as a feed additive or in drinking water to maintain optimal health for food-producing animals. Residues may remain in edible animal tissues and then affect the human health, and this is the reason why many researchers focus on the development of a rapid, accurate, and economical time and cost methods, for the determination of this antibiotic. Several papers have been published concerning the assay method for oxytetracycline.[9] Among them, bioassay and fluorometry, which are commonly used, lack sensitivity and specificity, while chromatographic methods are generally preferred for their greater selectivity and simplicity.[10,11] High performance liquid chromatography methods are effective in monitoring veterinary drugs, and these techniques have been reported for the determination of OTC concentrations in various biological matrices.[4,12–14] Common HPLC procedures is needed for sample pretreatment. Pretreatment procedures of samples based on protein precipitation,[15] liquid–liquid extraction,[16] and solid-phase extraction.[13] The use of the SPE column to isolate oxytetracycline from biological samples appears to be efficient and time saving.

The present paper describes a rapid and specific procedure for HPLC determination of the oxytetracycline content in pigs' plasma with the solid phase extraction as the clean up technique.

EXPERIMENTAL

Animals

All experiments were carried out on 8 healthy weaners of both sexes, 8–10 weeks of age, and with an initial weight of 17–23 kg (group I, $n = 8$). There was also one control group (group C, $n = 2$). The pigs were kept in individual pens. Before commencing the study, the pigs were marked with numbers. Feed and water were added ad libitum throughout the period of study. One day before the trials began, a venous catheter was positioned in the jugular vein.

Mepatar® 20% granulate containing 200 mg/mL OTC, was administrated orally at a dose of 20 mg/kg body weight. The oral doses were administrated individually through a stomach tube. Blood samples were collected from the jugular vein in heparin tubes 1, 1.5, 3, 4, 6, 8, and 10 hours after administration of the drug. Samples were centrifuged and the plasma was decanted and stored at -80°C until the day of analysis by HPLC.

Chemicals and Reagents

Mepatar® 20% (granulate 0.2 g OTC · HCl + 0.8 g saccharose combination) was purchased from a pharmaceutical company (TZF "Polfa" S.A., Warsaw, Poland). HPLC grade acetonitrile, methanol, and other chemicals ($C_2H_2O_4 \cdot 2H_2O$-oxalic acid dihydrate, $C_6H_8O_7 \cdot H_2O$-citric acid monohydrate, Na_2HPO_4-disodium hydrogen phosphate, $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$-ethylenediaminetetraacetic acid disodium salt dihydrate) were obtained from POCH chemical-company (Gliwice, Poland) at the highest purity available. An analytical standard (oxytetracycline hydrochloride EC No. 2058-46-0) was purchased from Sigma (St. Louis, MO, 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 chromatograph (Varian, Walnut Creek, CA, USA). It consisted of a solvent delivery pump (STAR 9002), a 10 μL volume manual injector, and a variable wavelength UV-VIS detector (all Varian Analytical Instruments, USA). Chromatographic separations were performed using a Varian ChromSep HPLC OmniSpher 5 C18 (250 × 4.6 mm) column.

A centrifuge (MPW 210), an analytical balance (Sartorius BP 61 S), 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.

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.

Extraction Procedure

Frozen plasma samples were thawed to room temperature prior to extraction. A sample of 1 mL of test plasma was transferred into a vial and mixed with 1 mL of methanol. After centrifugation for 15 min at 5500 rpm, 1.2 mL of the upper supernatant layer was transferred into a conical flask and diluted to 30 mL using the buffer 0.01 M EDTA-McIlvaine. The sample solution was applied to a cartridge (Shimadzu C18, 500 mg) activated with 5 mL of methanol and 5 mL of a deionized water and conditioned with 5 mL of a buffer (0.01 M EDTA-McIlvaine). The C18 cartridge containing the sample was washed with 1 mL of a buffer. OTC was eluted using ACN-0.1 M EDTA-McIlvaine buffer solution (50:50, v/v). The extract was evaporated to 2 mL under a stream of nitrogen. A 100-μL volume of elute was injected into the HPLC system. For the recovery study, 10 μL of a standard solution of OTC (10 μg/mL) was added to 1 mL of blank pig plasma sample.

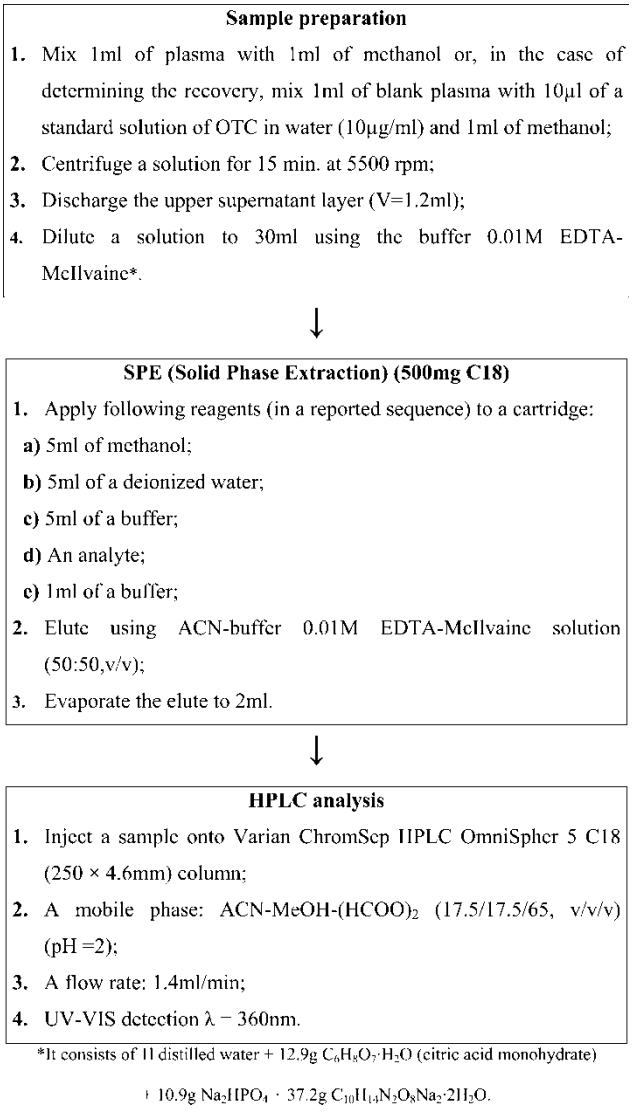
The extraction of OTC from the pigs' plasma was performed according to Scheme 1.

Preparation of Standard Solutions

A stock solution (1 mg/mL) of oxytetracycline hydrochloride was prepared by dissolving 100 mg of compound in 100 mL of distilled water. Working solutions (500, 200, 100, 50, 25 ng/mL) were prepared by appropriate serial dilution of the stock solution with distilled water. These solutions were then injected in order to obtain the calibration curve.

Validation of the Method

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



Scheme 1. Schematic diagram of an analytical process.

Linearity

Linearity was determined by constructing the calibration curve. Five standard concentrations of oxytetracycline in the range of 25–500 ng/mL were prepared and injected (10 µL). Before injection, the column was equilibrated for at least 60 min with the mobile phase flowing through the system.

The area of peak corresponding to OTC was plotted against the concentration of OTC in working solutions. The equation for the calibration curve is $y = (55.04 \pm 0.17)x + (29 \pm 42)$ and the correlation coefficient (R^2) equals 0.9999. The high value of the coefficient indicated good linearity of the calibration curve for the method in the considered concentration range. In addition, the acceptability ranges for the parameters of the curve (slope: $a = 55.04$; standard deviation of the slope: $s_a = 0.17$; intercept: $b = 29$; standard deviation of the intercept: $s_b = 42$ and standard error: $S_{xy} = 65.70$) have been calculated.

The plot is shown in Figure 1.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD-the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified and the LOQ-the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, were calculated.

The limit of detection ($LOD = 3 \cdot S_{xy}/a$, where S_{xy} is the standard deviation and a is the slope) was 3.58 ng/mL and the limit of quantification ($LOQ = 10 \cdot S_{xy}/a$) was 11.93 ng/mL.

Precision/Accuracy

The precision of the method was determined by calculating the relative standard deviation (RSD%) for the repeated measurements, and the accuracy as the standard deviation (SD) between nominal and measured concentrations. 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 the section Extraction Procedure. Each time a volume of supernatant was 1.2 mL. The area of a peak

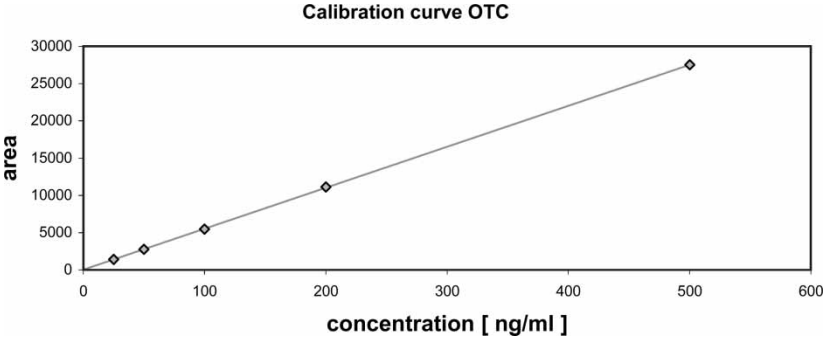


Figure 1. A calibration curve of oxytetracycline hydrochloride.

corresponding to an initial amount of OTC (S_p) in the spiked sample was calculated according to the equation

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

where S is a peak area of OTC obtained after SPE, 0.925 is the recovery, and 4 is a stoichiometric factor. S_p was then used in the determination of the OTC amount from the calibration curve. The equation for the curve was $y = 55.04x + 29$. The calculated standard deviation (SD) was 1.40 ng/mL and relative standard deviation (RSD) was 1.39%.

Recovery

The recovery of the solid phase extraction procedure was assessed by analyzing extracts of plasma samples containing oxytetracycline hydrochloride in pigs' control plasma spiked at 100 ng/mL. These spiked samples were prepared by adding 10 μL of standard solution of OTC (10 μg/mL) to separate 1.0 mL portions of the sample. The recovery from the tested plasma was calculated according to the equation

$$\text{Recovery}\,(\%) = \frac{4 \cdot S}{S_{std} \cdot V} \cdot 100\%$$

where S is a peak area of OTC obtained from the analysis of a spiked plasma (100 ng/mL), $S_{std} = 5479$ is a peak area for the standard (Figure 3), $V = 1.2$ mL is a volume of the supernatant, and 4 is a stoichiometric factor. The calculated OTC recovery was 92.50%.

RESULTS AND DISCUSSION

We were looking for a simple, effective, and precise method to determine the OTC amount in pigs' plasma. TCs (such as oxytetracycline, chlortetracycline, tetracycline) have similar chemical and physicochemical properties. They are amphoteric compounds soluble in polar and moderately polar organic solvents, and have the ability to form strong complexes with multivalent cations, organic molecules, and bind with proteins and silanol groups in the stationary phase. They are extractable with several organic solvents such as n-butanol, ethyl acetate, or methanol. For these reasons, the oxytetracycline antibiotic is difficult to isolate from biometrices.

Oka and Patterson extensively reviewed the literature to 1995 on the methodologies utilized for the analysis of tetracyclines. In addition to the review by Shaikh and Moats, both treatises indicated that there is an enormous variety of extraction procedures for TCs analysis. The one common factor in all of these procedures is that the majority use aquenos

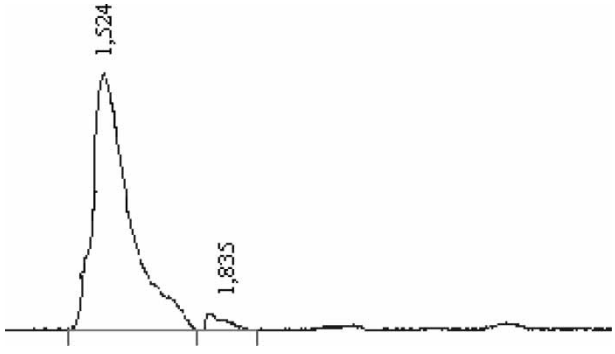


Figure 2. Chromatogram of a blank plasma.

solutions containing chelating agents to decrease the tendency for TCs to bind to metal ions in the matrix. EDTA, oxalic acid, and citric acid are the most commonly used chelating agents. McIlvaine's buffer, utilized in many extraction techniques contains citric acid.[17] The use of EDTA-McIlvaine's buffer, combined with SPE using alkyl-bonded silica cartridges for clean-up, was established by Oka et al. in 1985[18] and appears to be the current standard for the extraction of TCs from tissue matrices. The method used an oxalic acid methanol solution to elute the antibiotic from the cartridges. The addition of oxalic acid was found to be necessary to effect reproducible

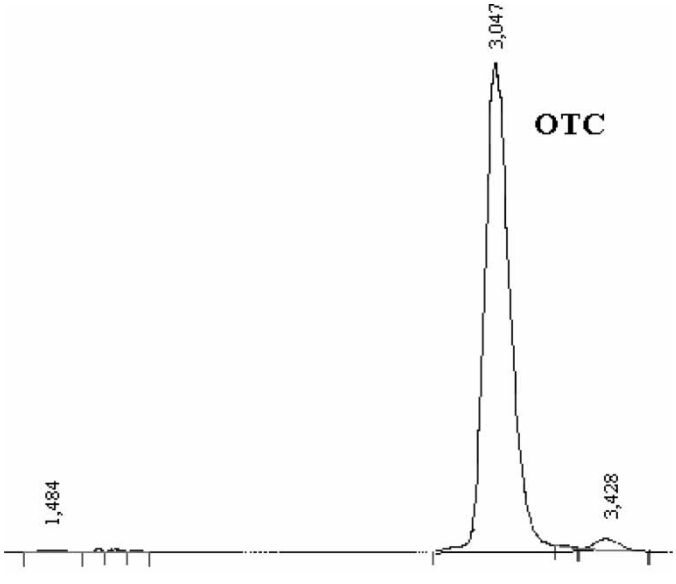


Figure 3. Chromatogram of OTC standard (water solution).

elutions; this was attributed to the presence of contaminations and free silanol groups on the cartridges. Oka et al. in 1997[13] utilized the same procedure but eliminated the use of oxalic acid, using instead an ethyl acetate-methanol mixture. The proposed method by Oka et al.[13] was used to determine the oxytetracycline content in pigs' plasma in our investigations. We utilized ACN-EDTA-McIlvaine buffer solution (50:50, v/v) to elute oxytetracycline from the cartridges. An isocratic HPLC for the determination of TCs has been reported using a mobile phase containing oxalic acid on a modified silica gel column.[19] The resolution and asymmetries of the TC peaks depend upon the pH of the aquenos oxalic acid solution in the mobile phase and the optimum pH is 2.0.[12] An oxalic acid was used in the mobile phase that was consisted of methanol-acetonitryle-0.01 M oxalic acid (17.5:17.5:65, v/v/v) (pH adjustment to 2) in our researches. The composition of the mobile phase (especially oxalic acid additive) caused sharp and no tailing peaks. Because TCs show strong UV absorption around 270 and 360 nm in neutral and acidic solutions,[12] we decided to use a UV detector, as the most conventional detection method for TCs.

Figures 2–4 show chromatograms of drug free plasma, plasma spiked with oxytetracycline standard (100 ng/mL), and pig plasma after oral administration of the drug. 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. The relationship between concentration (ng/mL) of OTC in plasma and time after administration of the drug is shown in Figure 5. Three hours after oral intake of

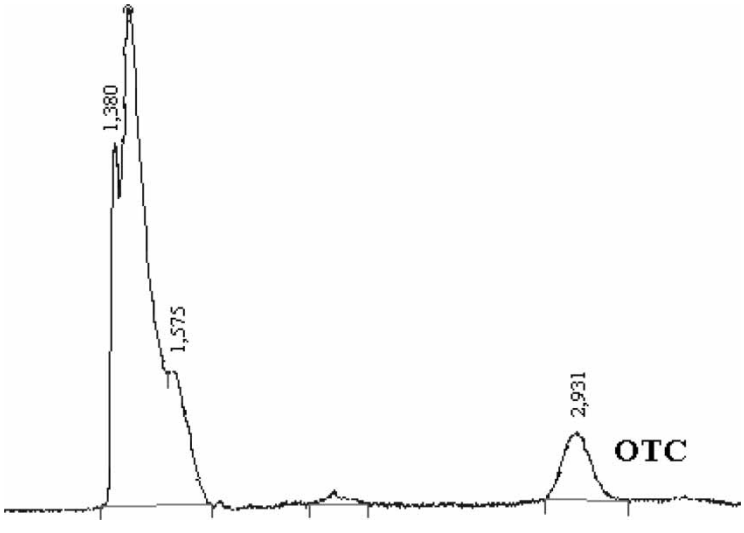


Figure 4. Chromatogram of a plasma sample taken from a pig (number IV) 1 h after administration of Mepatar®.

Table 1. Concentration of OTC (Mepatar® 20%) in plasma (ng/mL)

Number of animal	1 h	1.5 h	3 h	4 h	6 h	8 h	10 h
I	202	261	481	387	253	184	132
II	281	349	372	318	271	229	169
III	330	398	638	530	444	374	253
IV	127	258	346	453	412	282	156
V	270	438	542	423	292	239	139
VI	157	194	413	345	304	230	147
VII	138	183	366	286	233	157	98
VIII	269	351	371	297	241	157	93
Mean	221.8	304.0	441.1	378.8	306.3	231.5	148.4

Mepatar®, we observed that the mean maximum concentration (C_{max}) of OTC in the biological matrix reached a value of 441 ng/mL. From this moment, the concentration of OTC in plasma decreases and the smallest observed (during the investigated period of time) amount of the drug in blood (148.4 ng/mL) was 10 h after administration.

The HPLC method for the measurement of OTC in pigs 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,[4,13] milk,[14] and cheese[20] using the HPLC method. 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

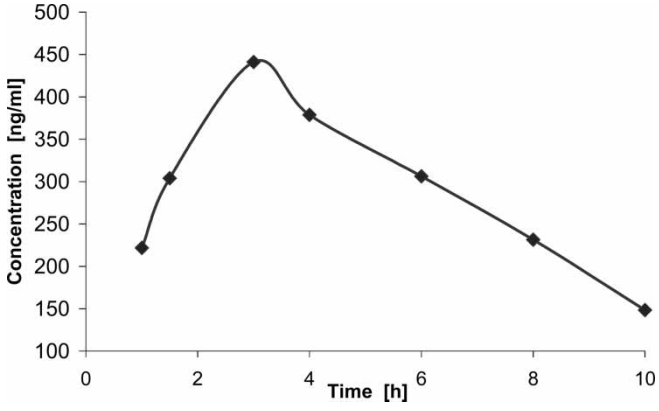


Figure 5. Plot concentrations of OTC in plasma vs. time after administration of the drug.

was already mentioned, the recovery was 92.50%. The reverse phase HPLC technique with UV-VIS detection was found to be a convenient and precise method for analysis of the residues of OTC in plasma samples.

CONCLUSION

We applied the solid phase extraction (SPE) and HPLC method as effective, reliable, and specific techniques to determine the oxytetracycline content in pigs' plasma. Results demonstrate that the suggested technique is characterized by good performance parameters: linearity $R^2 = 0.9999$, recovery = 92.50%, repeatability RSD $\leq$ 1.39%. 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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