Analysis of florfenicol in pig plasma using a validated PPT-HPLC-DAD method

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Abstract

High performance liquid chromatography (HPLC) has proven to be an effective tool for examining the disposition kinetics of florfenicol (FF), a synthetic broad-spectrum antibiotic used to treat infectious diseases in veterinary medicine. Modification and optimisation of the protein precipitation (PPT) sample preparation procedure and HPLC with diode array detector (DAD) instrumental method were carried out to ensure conditions suitable for FF analyses in pig plasma samples. Stable supernatants with good plasma mean recoveries of FF (99.8% ± 0.7%RSD) were achieved using 1% v/v phosphoric acid solution in methanol and 10% w/v sodium chloride in aqueous solution. The PPT-HPLC-DAD method's detection limit of 0.004 μg/mL and quantification limit of 0.013 μg/mL provides high sensitivity for analyses of FF in plasma samples. In addition, the optimisation of method conditions resulted in shorter extraction and analysis time and less solvent consumption, which stresses the sustainability of this method in analytical chemistry. The optimised and validated PPT-HPLC-DAD method was applied in a comparative study of FF in pig plasma after administration of veterinary medicinal products. The study was conducted on fattening pigs following repeated intramuscular administration of two similar solutions for injection at a dose of 20 mg FF/kg bodyweight (test groups 1 and 2). The solutions for injection contained the same FF concentration, *i.e.*, 300 mg/mL, but differed in excipients. The aim was to examine the influence of administrated solutions for injection on the extent of exposure to FF in pig plasma. The dynamics of kinetic profiles of FF in pig plasma from both treatments correspond to the FF kinetic profiles published in similar studies. However, differences were observed in the concentrations of FF, which were constant throughout the study, and statistical differences between the test groups were confirmed (P<0.05). Though these findings suggest the possible influence of excipients, a full comprehensive conclusion on the influence of administrated solutions for injection on FF exposure in pig plasma requires additional research.

Key words: *florfenicol; pig; plasma; PPT-HPLC-DAD; green method*

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Introduction

The amphenicols are a group of relatively small organic, lipid-soluble molecules with a phenylpropanoid structure. The first-in-class compound is chloramphenicol (CAP), a semisynthetic antibiotic derived from *Streptomyces venequelae*. CAP is a dichloro-substituted acetamide containing a nitrobenzene ring, amide bond and two hydroxyl groups. Even though CAP has been used therapeutically as an active pharmaceutical ingredient (API) for more than 70 years in human and veterinary medicine, it has been banned in food-producing animals for over 25 years due to the public health risk as a human carcinogen (Schwarz et al., 2004). A synthetic methyl-sulfonyl analogue of CAP is thiamphenicol (TIA), generally a safer substance though less active than CAP. Florfenicol (FF) is a TIA derivative with terminal fluorine instead of a primary hydroxyl group in amphenicol structure, which makes its antibacterial activity similar to CAP. It acts by binding to the 50S ribosome subunit of susceptible bacteria and inhibiting microbial protein synthesis. Due to its absorption/distribution, high bioavailability and good safety profile (Atef et al., 2001; Kim et al., 2008; Kawalek et al., 2016; De Ocenda et al., 2017; Balcomb et al., 2018; Chae et al., 2018; De Smet et al., 2018; Watteyn et al., 2018), FF has a practical application for infectious disease treatment in livestock and poultry production and aquaculture.

The wide application of the FF in food-producing animals has required the development of sensitive analytical methods. Liquid chromatography (Chae et al., 2018; Watteyn et al., 2018), gas chromatography (Shen et al., 2009; Kawalek et al., 2016), capillary electrophoresis (Kowalski et al., 2005) or enzyme-linked immunosorbent assay method (Wu et al., 2008) are analytical approaches that have been reported for FF determination in biomatrices. These methods were mainly developed for FF residue studies in edible tissues. However, from the operational and economic standpoints and regarding green strategies in analytical chemistry, they are generally less suitable for kinetics studies of FF assays in blood samples. The high performance liquid chromatography (HPLC) methods developed for pharmacokinetics/pharmacodynamics (PK/PD) studies of FF (Varma et al., 1986; Liu et al., 2003; Zhang et al., 2016) have proven to be very effective tools for establishing optimal dose and dosage intervals, and for examining the disposition kinetics and bioavailability of FF. Recently published PK/PD studies of FF in pig blood after intramuscular (IM) administration (Dorey et al., 2017; De Smet et al., 2018; Lei et al., 2018; Somogyi et al., 2022) have demonstrated similar FF distribution curves profiles in plasma samples, although the presented maximal concentrations of FF (C_{max}), times at maximal concentration (T_{max}) and areas under the plasma concentration-time curves (AUC) have some discrepancies attributed to differences in the dosing regimens and the manner of sample collection. According to the available literature, the studies of FF in pig plasma after repeated IM administration at a dose of 20 mg/kg bodyweight (BW) have not been fully documented.

Even though liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used techniques for the extraction of FF from blood samples, protein precipitation (PPT),

as a procedure characterised by a short process and low-solvent consumption, is recognised as one of the constitutive parts in the development process of greening the method for the analysis of FF. Accordingly, our research aimed to modify the PPT sample preparation procedure and HPLC with diode array detector (DAD) instrumental method conditions to have a sensitive, reliable and eco-friendly method applicable to determining the FF in pig plasma samples. Furthermore, the previously optimised and validated PPT-HPLC-DAD method was applied to compare the FF concentration in pig plasma after IM administration of the same FF dose of two similar solutions for injection containing the same FF concentration but differing in excipients.

Materials and methods

Animals

The present study included 18 clinically healthy, commercial crossbred fattening pigs with an average weight of 80 kg and housed on a commercial farm (Žito d.o.o., Lužani, Croatia). Throughout the study, animals had free access to water and feed. The study was performed in accordance with animal welfare principles (European Parliament and Council of the European Union, 2010) and approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Zagreb for a scientific research project (Ministry of Science, Education and Sports of the Republic of Croatia, no. 048-0481186-1184).

Solutions for injection

The study was conducted by using two commercial FF solutions for injection with the same FF concentration (300 mg/mL) but with different excipients. The solution for injection applied in the first treatment group (Test-1 group) contained *N*-methyl-2-pyrrolidone, propylene glycol and macrogol 300, while the solution for injection applied in the second treatment group (Test-2 group) contained dimethyl sulfoxide, propylene glycol and macrogol 400.

Animal experiment

The experimental design on pigs was conducted as previously described in the study of FF and florfenicol amine (FFA) in pig cerebrospinal fluid (Šandor et al., 2020). Briefly, animals were randomly assigned into three groups of six animals. Animals in each tested group received 2 IM injections of specified solution for injection at the FF dose of 20 mg/kg BW at a 48-hour interval. The third group served as the non-treated control.

Blood samples were collected by venipuncture prior to administration of the first dose and at 2, 4, 24, 48, 50, 52, 72, 96, 120, 144, 168, 192, 216 and 240 hours after administration of the first dose. Plasma samples were collected into plastic vacuum tubes containing K3-EDTA anticoagulant (Deltalab S.L.U., Barcelona, Spain). Serum samples, intended for use in the analytical method validation, were taken from pigs in the control group, collected in plastic vacuum tubes and kept at room temperature until complete clot formation. After collection, the samples were centrifuged at 3360 x g at room temperature for 15 minutes and stored at -20°C until HPLC analysis.

Chemicals

Glacial acetic acid and solvents (acetonitrile, ethanol, methanol and water) used in the analysis were HPLC grade and provided by Merck (Darmstadt, Germany). Analytical

grade ammonium hydroxide solution, tetrahydrofuran, phosphoric acid, hydrochloric acid and sodium chloride were obtained from Kemika (Zagreb, Croatia) while trifluoroacetic acid was from Merck (Darmstadt, Germany). Analytical standards of FF (Sigma-Aldrich, Steinheim, Germany) and CAP (Dr Ehrenstorfer, Augsburg, Germany) were supplied in the highest available purity.

Instrumentation

Chromatographic analyses were carried out using a TSP SpectraSystem HPLC (Thermo Separation Product, San Jose, USA) equipped with a binary pump, autosampler and DAD. An isocratic condition was achieved using a mobile phase consisting of water/acetonitrile (80:20 v/v, pH to 3.0 with phosphoric acid) at a flow rate of 1.3 mL/min. The reverse-phase LiChrospher RP C18 column (125 mm x 4.6 mm i.d., 5 μm; Merck, Darmstadt, Germany) was maintained at 30°C, and the injected volume was 30 μL. The eluent was monitored at 224 nm. ChromQuest Software, Version 5.0 (Thermo Fisher Scientific, San Jose, USA) was used for controlling the system operation, collecting and analysing the data.

HPLC optimization and validation

Quantitative analysis was performed using an internal standard (CAP) method. FF and CAP solutions were prepared in methanol at a concentration of 1.0 mg/mL. FF standard solutions were prepared freshly before calibration analysis in the range of 0.05–5.0 μg/mL. The concentration of CAP in all standard and sample solutions was 10.0 μg/mL. Prepared stock solutions were kept at 2–8°C for a maximum of one month. A system suitability test was performed by determination of the number of theoretical plates (N>2000), capacity factor (k'>1), precision (RSD_{area}≤2.0%), resolution (R_S≥2.0) and asymmetry factor (0.8≤A_f≥1.5). Plasma calibrators were also prepared by adding the appropriate volumes of FF and CAP standard stock solutions into blank plasma samples.

The validation of the PPT-HPLC-DAD method was performed in accordance with validation guideline (Eurachem, 2014). Linearity was assessed using an 8-point calibration curve with three replicates followed by a calculation of the FF analyte peak area to that of the CAP. The recoveries at 50%, 100% and 150% of nominal concentration (3.0 μg/mL) were analysed to express accuracy. Additionally, precision was determined at the same concentration levels and analysed on the same and different days and in different column batches. Sensitivity was determined by the limit of detection (LOD) and the limit of quantification (LOQ), and calculations were based on the standard deviation of the response and the slope. The matrix effect was evaluated in the range of 0.05–5.0 μg/mL by the ratio between the FF peak responses extracted from spiked plasma sample and the FF response from the standard aqueous solution. The stability studies of spiked/non-spiked standard solutions stored at -20°C were also conducted.

PPT procedure

The precipitants were chosen according to the results of the PPT efficiency test shown in Table 1. Deproteinization of pig plasma calibrators and samples was achieved by adding a mixture of phosphoric acid in methanol and an aqueous solution of sodium chloride. Briefly, frozen plasma samples were thawed at

room temperature. The 0.75 mL plasma was added to the test tube containing CAP stock solution, 10% w/v sodium chloride in aqueous solution and 1% v/v phosphoric acid solution in methanol. Each sample was mixed for 20 seconds and then centrifuged at 3360 x g for 10 minutes. The supernatants were filtered through 0.45 μm regenerated cellulose syringe filters before HPLC analysis.

PK statistical analysis

Statistical analyses were performed using Stata 13.1. (StataCorp. 2013., Stata Statistical Software: Release 13. College Station, TX: StataCorp LP, Texas, USA). For each pig, a non-compartmental analysis of PK parameters was performed for the obtained plasma concentrations (Chow and Liu, 2008). The following PK parameters were used for assessment: AUC_{0-48} – AUC from 0 to 48 h after the first injection; AUC_{48-96} – AUC from 48 to 96 h after the first injection, *i.e.*, AUC from 0 to 48 h after the second injection; AUC_{0-t} – AUC up to the last quantifiable concentration; C_h – the highest concentration of FF in plasma; $C_{h(0-48)}$ – C_h from 0 to 48 h after the first injection; $C_{h(48-96)}$ – C_h from 48 to 96 h after the first injection, *i.e.*, C_h from 0 to 48 h after the second injection; T_h – time at C_h in plasma; $T_{h(0-48)}$ – time at $C_{h(0-48)}$ in plasma; $T_{h(48-96)}$ – time at $C_{h(48-96)}$. The AUC was calculated using the trapezoidal rule. The Kolmogorov-Smirnov test was used to test for differences in the normality of the distribution of PK parameters between the test groups. Data were analysed by one-way analysis of variance (ANOVA) and Mann-Whitney U-test for non-parametric distributions. The results were presented as mean ± standard deviation (SD), considering the *P*-values of <0.05 as statistically significant.

Results and discussion

PPT-HPLC-DAD method

Blood sample preparation is a constant challenge because the protein composition can cause severe matrix interferences in chromatographic analyses. Plasma, as the main fraction, makes up to 56.6% of pig blood and contains 92% water and 6.51% w/v proteins with various molecular weights, consisting primarily of albumin, globulins and fibrinogen (Bah et al., 2013). These proteins, and others with smaller molecular weights, have to be extracted from plasma to make samples applicable for analytical analysis. LLE with acetonitrile or dichloromethane are sample preparation methods commonly used in kinetics studies of FF in plasma samples (Liu et al., 2003; Zhang et al., 2016; Chang and Tsai, 2018; Lei et al., 2018). However, in our study, the PPT procedure was chosen to achieve good extraction efficiency using less aggressive solvents and minimum waste production and to make an analytical method for determining the FF in plasma that is more environmentally friendly.

The simplicity of PPT based on the addition of organic solvents, acids and/or salts provides a quick sample clean-up of the high protein matrices. The organic solvent causes proteins to precipitate out of the solution by decreasing the dielectric constant in the blood sample, which leads to displacement of water from the hydrophobic layer of the protein surface and disruption of hydrophobic interactions between the proteins in the sample (French, 2017). Furthermore, added acid in reaction with the positively charged amino groups of the proteins creates an insoluble salt. The addition of high concentrations of salt affects the depletion of water from hydrophobic

protein surfaces even more by binding the metal ions through the hydrophobic layer of protein molecules. That changes the protein isoelectric point, which leads to protein solubility reduction (French, 2017).

The effects of precipitants used in this study are presented in Table 1 and in the chromatograms in Figure 1. Pig plasma deproteinization with ethanol yielded the highest recovery of FF, but a poor shape was observed in the chromatogram (Figure 1A). Tetrahydrofuran, methanol, 1% methanol solutions of hydrochloric acid and trifluoroacetic acid showed poor repeatability of FF or CAP. Precipitation in 1% methanol solutions of phosphoric and glacial acetic acid showed similar chromatograms (Figure 1B), solid FF recoveries and good repeatability (Table 1). However, CAP chromatograms demonstrated some interfering patterns.

The appearances of supernatants and their stability within 24 hours at room temperature were the criteria for the choice of precipitant, which is 1% v/v phosphoric acid solution in methanol. Sodium chloride was added to plasma in different concentrations (Figure 1C) to eliminate CAP interferences (Figure 1B). With an increase in the sodium chloride concentration, a decrease in the matrix pattern was observed in the chromatograms. Clear supernatants suitable for HPLC analysis (Figure 1D) with good FF recoveries and stability (Table 2) were obtained by the combination of 1% v/v phosphoric acid solution in methanol and 10% w/v sodium chloride in aqueous solution. These reagents/solvents are categorised as environmentally preferable, and their lower consumption follows sustainability in analytical chemistry (Alfonsi et al., 2008).

Table 1. Comparison of PPT efficiency of various precipitants in pig plasma (*n*=3)

Precipitants	Precipitation	Appearance of supernatant *		Recovery (mean±%RSD**)	
		initial	after 24 h	FF	CAP
Ethanol	good	clear	turbid	134.4±1.1	117.5±1.8
Acetonitrile	good	turbid	turbid	36.0±3.4	30.8±0.9
Tetrahydrofuran	slow	clear	turbid	129.4±7.4	119.0±2.6
Methanol	slow	turbid	turbid	115.8±7.5	98.2±4.6
Hydrochloric acid ***	good	turbid	turbid	116.1±1.6	97.7±6.3
Phosphoric acid ***	good	clear	clear	111.6±0.3	97.1±2.9
Glacial acetic acid ***	slow	clear	turbid	112.9±1.2	97.6±1.4
Trifluoroacetic acid ***	good	clear	turbid	112.5±1.4	98.5±10.5
1% w/v sodium chloride ****	good	turbid	turbid	136.0±3.4	97.1±1.4
5% w/v sodium chloride ****	good	clear	clear	125.2±1.5	102.0±4.4
10% w/v sodium chloride ****	good	clear	clear	99.8±0.7	81.9±1.2
15% w/v sodium chloride ****	slow	gelatinous	-	-	-

* At room temperature.
** Relative standard deviation, RSD.
*** 1% v/v acid in methanol.
**** Added to a mixture containing 1% v/v phosphoric acid in methanol.

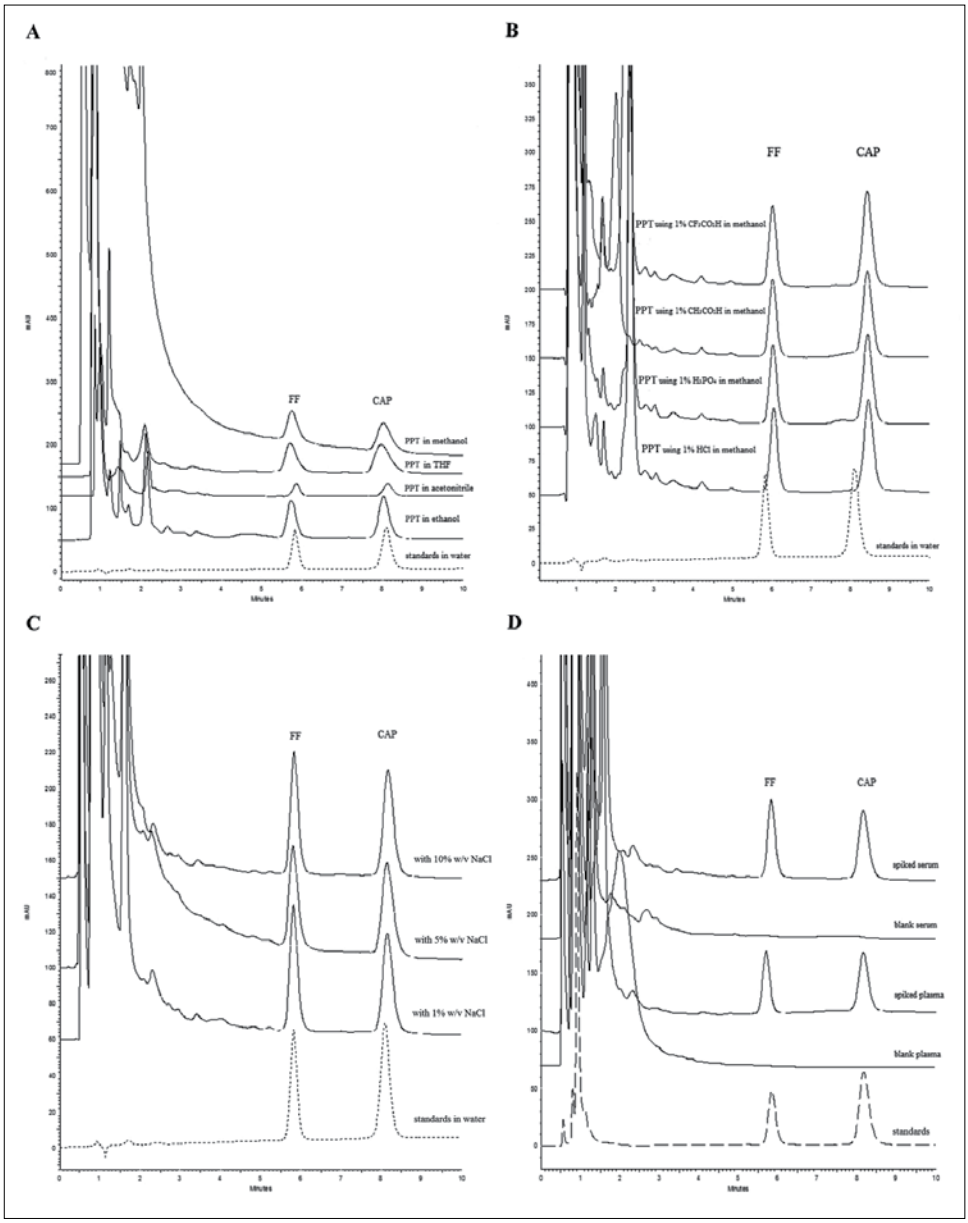

Figure 1. Typical FF and CAP chromatograms obtained using various precipitants in the PPT procedure: **A** plasma extracted with organic solvents; **B** plasma extracted with 1% v/v acid in methanol; **C** plasma extracted with 1% v/v phosphoric acid (H_3PO_4) in methanol and salt effect with different sodium chloride (NaCl) concentrations; **D** reference standards, blank plasma, spiked plasma, blank serum and spiked serum extracted with 1% v/v H_3PO_4 in methanol and 10% w/v NaCl in aqueous solution.

The selection of HPLC-DAD conditions for FF analysis in pig plasma was based on previous FF studies (Varma et al., 1986; Liu et al., 2003) and on optimisation and testing the method's robustness with various mobile phases at different pH values and column temperatures. Chromatographic separation using a mobile phase of 0.5 M ammonium acetate and acetonitrile (78:22) yielded a very low FF peak capacity (k'<5) and poor stabilisation of the solvent baseline compared to the mobile phase with a mixture of water and acetonitrile. Furthermore, the mobile phase with 10% acetonitrile showed a very low FF efficiency (n<800), while ratios up to 30% demonstrated poor FF selectivity (R_S<1.0). As for method robustness, a pH decrease of more than 10% in spiked plasma samples induced noisiness of the solvent baseline, while a 10% pH increase caused an interfering pattern of the FF or CAP peak. Column temperature changes made little difference regarding resolution, though temperatures higher than 35°C decreased the retention factor.

Validation results and typical chromatograms of the PPT-HPLC-DAD method are shown in Table 2 and Figure 1D. The linearity of the method was confirmed by Pearson's correlation coefficients (R^2 >0.99). Both the reference solution and spiked plasma in the matrix effect study showed a result of R^2 = 0.998. The obtained results are close to 1.0 with no statistical significance (P>0.05, ANOVA), indicating the absence of the matrix effect. The calculated LOD and LOQ were 0.004 μg/mL and 0.013 μg/mL, respectively. The repeatability and intermediate precision were below 5.0% RSD. Accuracy results of six replicates of fortified blank plasma samples were acceptable. The specificity was checked by an analysis of blank and spiked pig plasma samples obtained from the control group. Pig serum samples were also used to confirm the specificity of the PPT-HPLC-DAD method. The blank and spiked serum samples were extracted using the same PPT procedure (Figure 1D) and yielded a good mean FF recovery (95.4%, 2.3% RSD). The good ruggedness of the PPT-HPLC-DAD method has been proven through experiments performed during the optimisation of the PPT procedure (Table 1), chromatographic conditions (Figure 1) and stability study of the solutions (Table 2). Nevertheless, the comparison of PPT with other sample preparation approaches must also be considered. The use of SPE, dispersive liquid-liquid microextraction (DLLME) or dispersive solid-phase extraction (dSPE) could additionally confirm the robustness of the PPT-HPLC-DAD method for analyses of FF in pig plasma samples.

In the context of green sustainability strategy, the presented PPT-HPLC-DAD method showed several advantages over similar methods (Liu et al., 2003; Zhang et al., 2016; Chang and Tsai, 2018; Lei et al., 2018). The PPT sample preparation technique demands a smaller quantity of organic reagents and less toxic ones than acetonitrile (Chang and Tsai, 2018) or dichloromethane (Lei et al., 2018) used in the LLE procedure. In previous methods, the mobile phase composed of 33–30% acetonitrile or gradient program was used, while the PPT-HPLC-DAD method uses an isocratic mobile phase composed of 20% acetonitrile and a shorter C18-column. This reduces the analysis time and consequently generates less solvent consumption and smaller amounts of waste. Furthermore, green analysis is also characterised by minimal energy use of instrumental techniques (Galuszka et al., 2012). The methodology based on the HPLC technique coupled with ultraviolet

Table 2. Validation data of the PPT-HPLC-DAD method in pig plasma (*n*=6)

Validation criteria	Parameter	FF
Linearity	range (µg/mL)	0.05–5.0
	slope	2.773
	intercept	-0.022
	R^2	0.9942
Precision	intraday	0.56%RSD
	intermediate (*n*=12)	2.16%RSD
Accuracy	50% recovery (%)	94.43±5.01
	100% recovery (%)	91.53±1.40
	150% recovery (%)	87.97±7.97
Sensitivity	LOD (µg/mL)	0.0040
	LOQ (µg/mL)	0.0130
Stability	Δ FF conc. at -10 to -20°C	0.79%/72 h

detection uses less energy (<0.1 kWh/sample) than LC coupled with a tandem mass spectrometry system (1.5 kWh/sample), making the PPT-HPLC-DAD method more energy efficient.

FF in the plasma of treated pigs

The optimised and validated PPT-HPLC-DAD method was used to determine the FF in pig plasma after repeated IM administrations of two similar solutions for injection. The evaluation was based on the observation of API in plasma samples, *i.e.*, FF as the parent compound, because its absorption rate and peak concentration proved to be more sensitive than its metabolite, *i.e.*, FFA (Committee for Veterinary Medicinal Products, 1999). The disposition of FF in samples is illustrated by arithmetic

Table 3. Overview of plasma PK parameters of FF for the test groups (Test-1 and Test-2) after repeated IM administration to pigs (*n*=6) at a dose of 20 mg FF/kg BW

Parameters	Unit	Test-1	Test-2
$C_{h(0-48)}$	µg/mL	2.06±0.0002	2.24±0.001*
$C_{h(48-96)}$		1.80±0.02	1.83±0.0004
C_h		2.06±0.0002	2.24±0.001
$T_{h(0-48)}$	h	4.0±0.0	3.0±0.01
$T_{h(48-96)}$		52.0±0.0	52.0±0.0
T_h		4.0±0.0	3.0±0.01
AUC_{0-48}	µg·h/mL	37.58±0.02	48.85±0.02*
AUC_{48-96}		43.84±0.01	52.42±0.01*
AUC_{0-t}		89.10±0.01	123.39±0.03*

* $P<0.05$, compared with Test-1 group

plots of FF plasma concentration *vs.* time (Figure 2), and the results obtained by PK statistical analysis are given in Table 3.

The presented results show similar kinetic profiles of the FF concentrations in pig plasma of both tested groups (Figure 2). Following the administration of both doses, the FF concentration rapidly increased after 2 hours and decreased within the next 44 hours. Between 48 and 192 hours after administration of the first dose, the FF concentration decreased more slowly with a plateau between 168 and 216 hours. This dynamic of the FF kinetics in both groups is due to the repeated IM dose of FF given at 48-hour intervals and its further distribution in the organism. Despite similar kinetic FF concentrations in pig plasma of both tested groups, some differences were observed. The highest mean plasma concentration of FF in the Test-2 group was achieved within 48 hours after the first injection ($C_{h(0-48)}$) and was statistically significantly higher (P<0.05) compared to the Test-1 group (Table 3). Furthermore, statistical analysis showed that AUC values for the Test-2 group were statistically significantly higher (P<0.05) compared to Test-1 group for all tested periods, *i.e.*, AUC_{0-48}, AUC_{48-96} and AUC_{0-t}. These findings suggest differences in the absorption rate of FF from the injection site after administration of each solution for injection which could be explained by differences in the composition of excipients. Both solutions for injection used in our research have an identical pharmaceutical form and contain the same FF strength, but the distinction between them is manifested in the qualitative composition

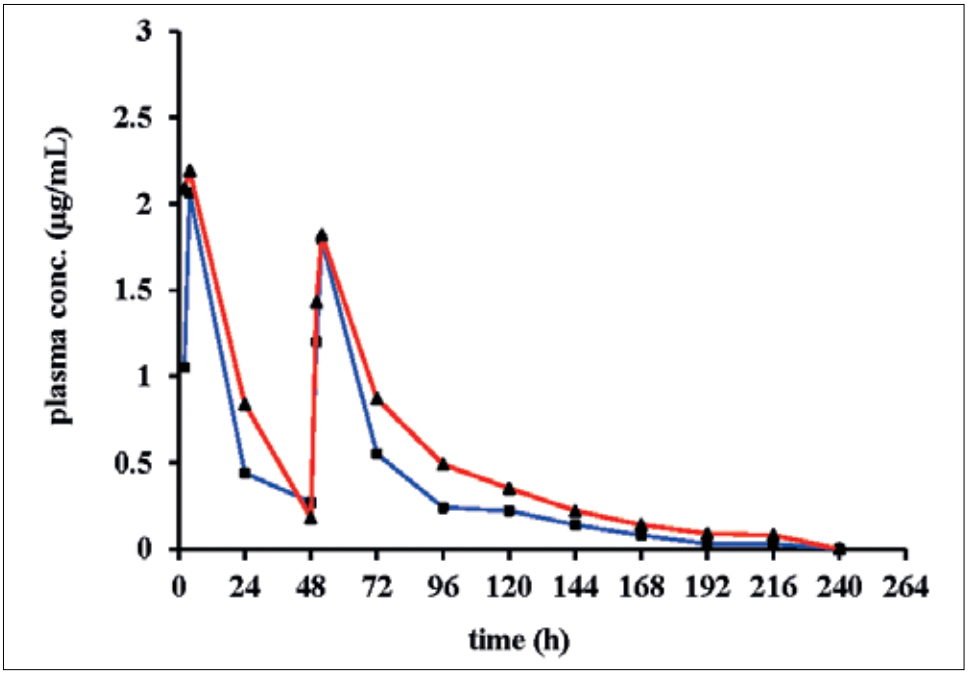

Figure 2. The arithmetic plot of mean plasma concentration of FF *vs* time after repeated IM administration at a dose of 20 mg/kg BW on pigs (n=6) in Test-1 (—) and Test-2 (—) group

of the excipients, *i.e.*, *N*-methyl-2-pyrrolidone and dimethyl sulfoxide. Due to the very low water solubility of FF (approximately 1.3 mg/mL), these organic solvents are commonly used to afford FF solubility in commercial formulations. They are dipolar aprotic solvents with low toxicity and often simultaneously act as co-solvents and complexing agents in veterinary drug formulations (Strickley, 2004; Jouyban et al., 2010; Patil and Shivaprakash, 2013). However, their difference in the mechanism of action and molecular structures manifested in the larger planar nonpolar region of *N*-methyl-2-pyrrolidone may have some impact on the API delivery system (Williams and Barry, 2012).

Kinetic profiles of the FF in pig plasma obtained in this study correspond to the exposure of FF in profiles of similar studies related to the dose of 20 mg FF/kg BW (Liu et al., 2003) or dosing regimen, *i.e.*, repeated IM administration (De Smet et al., 2018). However, distinctions were observed in the C_h and T_h values. In the present study, the $C_{h(0-48)}$ determined in pig plasma samples from the Test-1 group was 2.06 μg/mL at 4 hours and from Test-2 group was 2.24 μg/mL at 3 hours (Table 3). Liu et al. (2003) reported PK values of 3.2 μg/mL in plasma at 0.91 hour after a single dose of 20 mg FF/kg BW. Comparing these results with ours obtained within 48 hours after administration of the first dose, lower peak concentrations of FF were achieved a few hours later in both test groups. This was expected since the blood sampling schedule in this study did not include frequent sampling around the predicted T_{max}, *i.e.*, within the first two hours (Committee for Veterinary Medicinal Products, 1999). Nevertheless, the results of the presented study can be useful for future detailed research.

Conclusions

The proposed PPT-HPLC-DAD method proved to be suitable for the qualitative and quantitative determination of FF in pig plasma samples. The combination of the simple sample preparation procedure and optimal chromatographic conditions for FF analyses makes the PPT-HPLC-DAD a more environmentally friendly method.

The profiles of FF concentration in pig plasma investigated in this study are comparable to the literature. The differences observed in FF concentrations were constant throughout the study and were statistically confirmed between the treated groups. These findings suggest the possible influence of excipients, though additional detailed research is required to reach a full and comprehensive conclusion on the influence of administrated solutions for injection on FF exposure in pig plasma.

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Analiza florfenikola u plazmi svinja primjenom validirane PPT-HPLC-DAD metode

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Tekućinska se kromatografija visoke djelotvornosti (HPLC, engl. *High Performance Liquid Chromatography*) pokazala učinkovitim alatom za ispitivanje kinetike florfenikola (FF), sintetitčkog antibiotika širokog spektra koji se u veterinarskoj medicini koristi za liječenje zaraznih bolesti. Modifikacija i optimizacija postupka pročišćavanja uzoraka krvi tehnikom precipitacije proteina (PPT) i instrumentalne metode sustava HPLC-a uz detekciju UV-detektorom s nizom fotodioda (DAD, engl. *Diode Array Detector*) provedene su sa svrhom postizanja pouzdane analitičke metode za određivanje FF-a u plazmi svinja. PPT postupkom priprave uzoraka dodavanjem 1 % otopine fosforne kiseline u metanolu (V/V) i 10 % vodene otopine natrijevog klorida (m/V) dobiveni su stabilni nadtalozi s dobrim srednjim analitičkim povratima FF-a iz plazme (99,8%±0,7%RSD). Postignuta granica detekcije PPT-HPLC-DAD metode od 0,004 µg/mL i granica određivanja od 0,013 µg/mL omogućuju dobru osjetljivost određivanja FF-a u plazmi; optimizacijom uvjeta metode skraćeno je vrijeme ekstrakcije i analize te je smanjena količina potrošnje otapala čime je postignuta održivost metode u analitičkoj kemiji. Primjenom optimirane i validirane PPT-HPLC-DAD metode provedena je usporedna analiza FF-a u plazmi svinja nakon primjene veterinarsko-medicinskih proizvoda. Tovnim su svinjama u istim eksperimentalnim uvjetima dvokratno intramuskularno davane otopine za injekcije u dozi od 20 mg FF/kg tjelesne mase (Test-1 i Test-2 skupina). Otopine za injekcije sadržavale su istu koncentraciju FF-a, tj. 300 mg/mL, ali su se u pomoćnim tvarima razlikovale. Istraživanje je provedeno u svrhu ispitivanja utjecaja primijenjenih otopina za injekciju na opseg izloženosti FF-a u plazmi svinja. Dinamike kinetičkih profila FF-a u plazmi svinja utvrđene našim istraživanjem oba tretmana usporedive su s kinetičkim profilima objavljenim u sličnim studijama. Međutim, uočene su razlike u koncentraciji FF-a koje su tijekom cijelog istraživanja bile konstantne, a potvrđene su i statističke razlike između Test-1 i Test-2 skupine (P<0,05). Iako rezultati upućuju na mogući utjecaj pomoćnih tvari, potrebno je provesti dodatna istraživanja za sveobuhvatni zaključak o utjecaju primijenjenih otopina za injekcije na izloženost FF-a u plazmi svinja.

Ključne riječi: *florfenikol, svinja, plazma, PPT-HPLC-DAD, zelena metoda*