Development of an SPE-HPLC-DAD method for the experimental study of florfenicol and florfenicol amine in pig cerebrospinal fluid

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Abstract

A study of florfenicol (FF) and its metabolite florfenicol amine (FFA) in pig cerebrospinal fluid was conducted following repeated intramuscular administration of the original (reference) and a generic veterinary medicinal product (VMP) under the same experimental conditions (20 mg FF/kg body weight, 48-hour interval). Both VMPs are solutions for injection containing FF as an active substance in the concentration of 300 mg/mL and have been authorized in Croatia for use in cattle and pigs. In this study, clinically healthy pigs were randomly divided into three groups. The first group was treated with the reference VMP, the second with the generic VMP, while the third served as the control group. Animals were sacrificed at 216, 288 and 384 hours after the first drug administration. Cerebrospinal fluid samples were analysed by the optimized and validated high-performance liquid chromatography-diode array detector method (HPLC-DAD). The solid-phase extraction (SPE) technique was chosen for sample preparation. The HPLC-DAD method provides good linearity over the concentration range of 0.05 to 5.00 μg/mL for FF and FFA. Limits of detection were 0.0023 μg/mL for FF and 0.0100 μg/mL for FFA. Extraction recoveries of FF were from 86.6% to 111.8%, and of FFA from 91.7% to 98.8%. The SPE-HPLC-DAD method has been demonstrated to be a selective, sensitive and suitable analytical method for the determination of FF and FFA in cerebrospinal fluid. The present study was based on a preliminary study that quantified FF in pig plasma at 216 hours after the first application of reference or generic VMP. However, FF and FFA were not detected in any of the cerebrospinal fluid samples during the experimental period. According to the nature of biological fluids, the SPE-HPLC-DAD method can be suitable for further pharmacokinetic studies of FF in pig plasma and serum after intramuscular administration of VMPs.

Key words: *florfenicol; florfenicol amine; pigs; cerebrospinal fluid; veterinary medicinal products; HPLC-DAD*

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Introduction

Florfenicol (FF) is a synthetic broad-spectrum antibiotic developed for veterinary use. It belongs to the amphenicol group of antibiotics (Figure 1), like its structural analogues thiamphenicol and chloramphenicol (CAP). Original (reference) and generic veterinary medicinal products (VMPs) containing FF as an active substance are available for parenteral or oral administration in cattle, pigs, fish, chickens and sheep. FF is used for metaphylaxis and treatment of respiratory infections etiologically associated with *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica* and *Mannheimia haemolytica* (Dowling, 2000). The mechanism of FF action includes protein synthesis inhibition at the ribosomal level of susceptible bacteria.
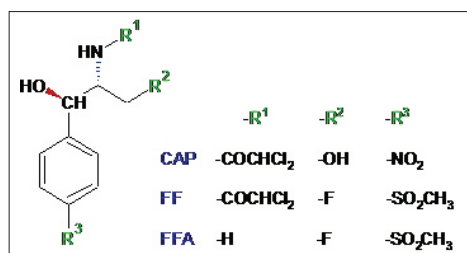

Figure 1. Chemical structures of amphenicols (CAP - chloramphenicol; FF - florfenicol; FFA - florfenicol amine; R[1-3] – functional groups)

FF is distributed mainly into the lungs, muscle, bile, kidney and urine. Radiometric studies of ^{14}C-FF conducted in pigs following various routes of administration (EMA, 1999) showed that FF has a major fraction of radioactivity (45 to 60%) in urine, followed by florfenicol amine (FFA, 11.2 to 17%), its marker residue. In pharmacokinetic (PK) and pharmacodynamic (PD) studies of FF in pigs, peak plasma concentrations in the range from 3 to 12 μg/mL were reported within 5 hours of intramuscular administration (Voorspoels et al., 1999; Liu et al., 2003; Geng et al., 2015; Qian et al., 2017; de Smet et al., 2018; Lei et al., 2018). The dispositions of FF in cerebrospinal fluid (CSF) were studied mainly in calves. After intravenous administration of FF to calves at a dose of 20 mg/kg body weight (bw), FF penetrated into CSF, reaching a peak concentration of 4.7 μg/mL (de Craene et al., 1997). The outcomes of FF exposure in pig CSF after repeated intramuscular administration of 20 mg FF/kg bw have not been fully documented.

Plasma, serum and CSF are very similar biological fluids. CSF is nearly protein-free compared with plasma, for CSF formation begins as plasma is filtered across the permeable choroidal capillaries (Johanson et al., 2008). Due to the similar composition, CSF is a matrix in which experimental data can provide preliminary information about PK/PD changes in plasma or serum. Furthermore, the penetration of antibiotics into CSF following various routes of drug administration and their concentration levels are relevant data for neurological toxicity studies. Neurological toxicity associated with antibiotic use (Grill and Maganti, 2011) or combined administration of antibiotic and antiepileptic/anti-inflammatory drugs (Esposito et al., 2017) have shown that the toxic effects are directly related to brain tissue and CSF concentrations of administrated antibiotics.

A considerable number of analytical approaches for FF determination in biological fluids, edible tissues or feedstuffs have been reported. Those include gas chromatography (Shen et al., 2009; Liu et al. 2010), high(ultra)-performance liquid chromatography (HPLC or UPLC) with ultraviolet detector (Yang et al., 2017; Patyra and Kwiatek, 2019), mass spectrometer (Li et al., 2020)

or fluorescence detector (Xie et al., 2011; Wang et al., 2019), liquid chromatography-tandem mass spectrometry (Imran et al., 2017; Xie et al., 2018; Kverme et al., 2019), capillary electrochromatography (Valette et al., 2004) or immunochemical techniques (Fodey et al., 2013). However, no reports have described the method for FF or FFA quantification in pig CSF samples after repeated administration of the recommended dose of generic or reference VMPs.

The aim of this study was to develop a selective, sensitive and rapid HPLC method for FF and FFA determination in CSF. Due to possible antibiotic penetration into the CSF, we investigated the FF and FFA exposure in pig CSF after repeated intramuscular administration of reference or generic VMP.

Materials and methods

Animals and experimental design

The study was conducted using reference and generic VMPs from the Croatian market. Both VMPs are solutions for injection containing FF as an active substance in the concentration of 300 mg/mL and are indicated for use in cattle and pigs.

In this study, 18 clinically healthy pigs, commercial crossbred hybrids weighing 80-90 kg were used. Animals were housed on a commercial farm (Žito d.o.o., Lužani, Croatia) under conditions compliant with good veterinary practices. Throughout the study, animals had *ad libitum* access to feed and water. Pigs were randomly divided into three groups of six, where one group was treated with the reference VMP, one with the generic VMP, and one served as the control group. Both VMPs were administrated in the recommended FF dose of 20 mg/kg bw by deep intramuscular injection using a 16-gauge needle into the neck muscles, twice at 48-hour intervals. CSF samples were taken from the cranial cavity of slaughtered

animals and collected in sterile tubes at 216, 288 and 384 hours after the first drug administration. Samples were stored at -20 °C until analysis.

The experiment was performed in abidance with the animal welfare principles, and was categorized as non-aggressive. Animals were slaughtered at the end of the fattening period. The study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Zagreb. This study was supported by the Croatian Veterinary Institute (Zagreb, Croatia) under research project no. 048-0481186-1184 "The effects and transformation of veterinary drugs and vaccines in animals" granted by the Ministry of Science, Education and Sports of the Republic of Croatia.

Standards and sample preparation

FF (99.0% assay purity) was obtained from Sigma-Aldrich (Steinheim, Germany), CAP (99.8% assay purity) was provided by Dr Ehrenstorfer (Augsburg, Germany), while FFA (98.1% assay purity) came from Schering-Plough Essex Chemie AG (Luzern, Switzerland). HPLC-grade acetonitrile and water were purchased from Merck (Darmstadt, Germany). Analytical grade phosphoric acid (85 wt% in water) and ammonium hydroxide were obtained from Kemika (Zagreb, Croatia). FF and CAP standard stock solutions were prepared in methanol at the concentration of 1.0 mg/mL and stored at 4 °C for one month. CAP was used as an internal standard. Standard solutions of FF and FFA (0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg/mL), and CAP (10.0 μg/mL), were prepared in a solvent mixture of water and acetonitrile (80:20 v/v, pH 3.5) freshly before calibration analysis. CSF calibrators were prepared by adding the appropriate volumes of FF, FFA and CAP standard stock solutions into blank CSF to contain the same concentrations as standard solutions. Before HPLC-analyses, CSF calibrators

and CSF samples were purified using a solid-phase extraction (SPE) procedure, which was selected based on the data by Shen et al. (2009) and modified. CSF samples (100 μL) were spiked with 10.0 μg CAP and diluted to 1 mL with solvent. AccuBOND ODS C18 cartridges (100 mg/mL, Agilent Technology, Palo Alto, CA, USA) were preconditioned with 2 mL methanol and 2 mL water. The CSF solution (1 mL) was applied to the cartridge and washed with 2 mL water. Analytes were eluted with 2 mL of a mixture of methanol and 5% ammonium hydroxide in water (70:30 v/v). The extract was evaporated to dryness at 50 °C under a gentle stream of nitrogen gas. The residue was reconstituted in 1 mL solvent for HPLC analyses.

HPLC instrumentation and method validation

The HPLC TSP SpectraSystem (Thermo Separation Products, San Jose, CA, USA) with diode array detector (DAD) was used. Data were analysed using the ChromQuest Software Version 5.0 (Thermo Fisher Scientific, San Jose, CA, USA). The separations of FF, FFA and CAP were carried out on a Zorbax C8 SB column (250 mm x 4.6 mm i.d., 5 μm; Agilent Technology, Santa Clara, CA, USA). The column temperature was maintained at 30°C, and the flow rate of the mobile phase (Table 1) was kept at 1.0 mL/min. The injection volume was 30 μL. Column eluates were detected at 224 nm using a full spectral scanning covering the wavelength range spanning from 192 to 360 nm. Quantification was performed using an internal standard method.

The analytical method was validated concerning its specificity, linearity, accuracy, precision (repeatability and intermediate precision), limits of detection (LOD), limits of quantification (LOQ), and robustness. The specificity of the method was assessed by testing the level of any interference at FF, FFA

and CAP retention times in solvent, FF, FFA or CAP reference solution, blank and spiked CSF samples. Two calibration curves per matrix (solvent, CSF) were constructed at six (spiked) levels using the ratio of FF (FFA) and CAP peak area versus FF (FFA) concentration. At each level, samples were analysed in triplicate. The calculation of the LODs and LOQs was based on the standard deviation of the response and the slope. Precision was assessed in terms of repeatability and intermediate precision by analysing spiked matrix samples having the FF and FFA nominal concentration of 1.0 μg/mL. Recoveries were determined in spiked samples at 50%, 100% and 150% of the nominal concentrations. Samples were stored at -20 °C and tested during 7 days to determine the stability of spiked and non-spiked standard solutions. The matrix effects were evaluated by FF, *i.e.* FFA analysis of non-spiked and spiked samples at each concentration level. Data were analysed using the one-way analysis of variance (ANOVA; $P<0.05$). The uncertainty of the SPE-HPLC-DAD method was evaluated according to the Eurachem/Citac Guide (Eurachem, 2012). The estimation of uncertainty was based on method validation studies (overall bias, precision, and recovery) and data obtained from the calibration certificates of standards, balance, volumetric flasks and pipettes.

Table 1. HPLC-DAD mobile phase elution conditions

Time (min)	% A (water/ acetonitrile, 80:20 v/v)	% B (acetonitrile)
0	100	0
18.0	80	20
18.5	100	0
20.0	100	0

Results and discussion

The development of the HPLC-DAD method for FF and FFA determination in CSF was a challenge due to the limited number of collected pig CSF samples from the control group. Therefore, the selection of a reversed-phase C8 column was based on published data (Wrzesinski and Crouch, 2003; Boison, 2004; Holmes et al., 2012). Optimization and robustness testing of the HPLC-DAD method included combining various mixtures of A and B component of mobile phase (70:30, 80:20 and 90:10 v/v) at the time of gradient elution (Table 1) and isocratic elution of the 100% mobile phase (water/acetonitrile, 80:20 v/v). The mobile phase elution conditions present in Table 1 demonstrated the best FF, FFA and CAP optimization results for method resolution (R_S>2.0), capacity (k'>1), asymmetry (1.0) and efficiency (N>2000).

The SPE technique was selected to achieve easier and faster FF and FFA extraction from CSF samples. Furthermore, the criterion was also to select a solvent that would provide efficient extraction and high recoveries for FF, FFA and CAP. FFA is a polar amphenicol, a weak base that mainly exists in cationic form in acid solution, meaning that FFA has a better solubility at high pH (Zhang et al., 2008). Only the non-polar amphenicols FF and CAP are efficiently extracted from C18 cartridges when using acetonitrile, ethyl acetate or methanol as eluents. To increase recovery of the FFA, a mixture of methanol and a 5% aqueous ammonium hydroxide solution was used. This extraction yielded good recoveries for FF and FFA (Table 2).

The validation results for the FF and FFA determination in CSF samples with the SPE-HPLC-DAD method are summarised in Table 2. Calibration curves were obtained using linear regression analysis and the ratio of the standard area to internal standard area over the analyte concentration. The coefficients of determination (R^2) were higher than 0.99, indicating acceptable linearity in the tested range from 0.05 to 5.0 μg/mL for standard and spiked samples. The sensitivity of the method was established from the LOD and LOQ. In the calculation of LODs and LOQs, the residual standard deviation of a regression line was used as the standard deviation. The SPE-HPLC-DAD method used for the FF and FFA determination in CSF ensured LOD at 0.0023 μg/mL for FF and 0.0100 μg/mL for FFA. Recently reported LOQs for FF in pig plasma are in the range from 0.04 to 0.10 μg/mL (Geng et al., 2015; Qian et al., 2017; de Smet et al., 2018). Our method achieved a lower LOQ for FF in CSF (0.007 μg/mL). However, it should be taken into account that the CSF in relation to plasma is a clear, colourless, nearly acellular, low protein fluid, meaning that CSF purification is easier. The specificity of the SPE-HPLC-DAD method was checked by the preparation and analysis of blank and spiked pig CSF samples obtained from the control group. Typical chromatograms of standard solution, blank pig CSF and fortified CSF sample are shown in Figure 2. The retention times of either analytes or internal standard in spiked CSF samples are identical to the peaks in the standard solution. No interfering peaks were observed in the blank CSF at the retention time of FF, FFA or CAP. Repeatability and intermediate precision were below 1.0%. Accuracy was studied using fortified blank CSF samples having FF and FFA concentrations of 0.5, 1.0 and 1.5 μg/mL. Six replicates per concentration were analysed. Mean recovery was 99.8% ± 1.0% RSD for FF, and 94.1% ± 9.5% RSD for FFA. All these results showed acceptable precisions and satisfactory recoveries.

The matrix effect was characterized by the matrix factor, *i.e.* the ratio between the FF (FFA) peak response extracted from the spiked CSF sample over the

Table 2. Validation parameters for the FF and FFA determination in pig CSF (n=6)

Validation criteria	Parameter	Results		
		FF		FFA
Linearity	range (µg/mL)	0.05 - 5.0		
	slope	2.109		0.992
	intercept	- 0.020		- 0.007
	R^2	0.993		0.992
Precision	intraday (% RSD)	0.26		0.37
	intermediate (n = 12; % RSD)	0.38		0.67
Accuracy	50% recovery (mean% ± SD)	111.76 ± 1.20		98.75 ± 7.68
	100% recovery (mean% ± SD)	100.95 ± 1.15		91.92 ± 3.62
	150% recovery (mean% ± SD)	86.58 ± 0.64		91.74 ± 17.12
Sensitivity	LOD (µg/mL)	0.0023		0.0100
	LOQ (µg/mL)	0.0071		0.0303

RSD - relative standard deviation; SD - standard deviation

FF (FFA) peak response extracted from the standard solution. Quantitative measurement of the matrix effect was evaluated in the range from 0.05 to 5.0 µg/mL. The matrix effect was eliminated by comparing the results of spiked CSF sample after extraction (R^2: 0.999 for FF, 0.996 for FFA) with the standard solution (R^2: 0.998 for FF, 0.993 for FFA) at the same concentrations. Carryover effect was also observed by injecting blank pig CSF solution after injection of the 0.05 µg/mL standard. No carryover was quantified. Stability of FF, FFA and CAP in CSF was assessed in triplicate on spiked samples at concentrations of 0.5, 1.0 and 1.5 µg/mL after five freeze-thaw cycles during 7 days. The results were compared with those obtained from freshly prepared samples. The deviation from the initial concentrations of the FF, FFA and CAP was below 1.7% for all stability experiments.

To ensure correct interpretation and comparison of the results, the measurement uncertainty of the SPE-HPLC-DAD methods for FF and FFA in pig CSF was evaluated. Nine individual sources of uncertainty were taken into account (Table 3). They were associated with standard purity, uncertainty of the balance, volume of flasks and pipettes, and the calibration curve, repeatability, and accuracy of the method. A coverage factor of 2 was decided to be used at the confidence level of 95% to estimate the expanded uncertainty for FF and FFA (Eurachem, 2012). Significant contributions to the combined standard uncertainty were associated with recoveries for FF and FFA. This was
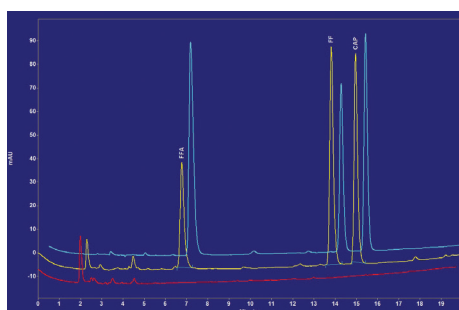

Figure 2. Typical SPE-HPLC-DAD chromatograms of blank CSF (■), spiked CSF solution (■) and standard solution (■)

Table 3. Measurement uncertainty of the SPE-HPLC-DAD method

Type of evaluation	Sources of uncertainty	Range or value	Distribution	Degrees of freedom	Standard uncertainty	Bias	Relative standard uncertainty
B	Standard purity	± 0.5%	rectangular	∞	0.0029	-	0.0029
	Balance	± 1.8E-07 g*	normal	∞	8.7E-06	-	0.0009
	Volume, 10 mL	± 0.04 mL	rectangular	∞	0.023	-	0.0023
	Pipette 0.1 mL	± 0.02%	rectangular	∞	0.00012	-	0.0012
	Pipette 0.5 mL	± 0.1%	rectangular	∞	0.0006	-	0.0012
	Pipette 1 mL	± 0.2%	rectangular	∞	0.0012	-	0.0012
A	Calibration curve						
	FF	1.0915 µg/mL	normal	17	-	0.02	0.004
	FFA	1.0499 µg/mL	normal	17	-	0.01	0.002
	Repeatability						
	FF	0.7684 µg/mL	normal	5	-	0.008	0.004
	FFA	0.5866 µg/mL	normal	5	-	0.004	0.003
	Accuracy						
	FF	99.8%	normal	8	-	1.0	0.003
	FFA	94.1%		8	-	8.9	0.03
SPE-HPLC-DAD for FF	Combine standard uncertainty type B				0.007		
	Combine standard uncertainty type A				0.004		
	Expanded uncertainty				0.02 µg/mL		
SPE-HPLC-DAD for FFA	Combine standard uncertainty type B				0.030		
	Combine standard uncertainty type A				0.004		
	Expanded uncertainty				0.07 µg/mL		

* $U_{balance}$ = 1.8E-05+ 1.4E-6 x W (g); where W is the nominal value of weight (0.010 g).

expected, given the challenges involved in the selection of SPE eluent for polar and non-polar amphenicols.

In our preliminary study of FF availability in pig plasma after repeated intramuscular administration of reference and generic VMP (Šandor et al., 2012), FF was detected at 216 hours. The mean plasma concentrations of FF were quantified in three pigs treated with reference VMP (0.033 ± 0.003 µg/mL) and in four pigs treated with generic VMP (0.082 ± 0.007 µg/mL). In addition to our preliminary FF results in pig plasma and considering the CSF composition and formation, the purpose of the present study was to explore FF disposition in pig CSF at 216 hours after the first application of both VMPs. The disposition of FFA was also investigated

to determine the elimination route of FF metabolites (FFA, FF alcohol, FF oxamide monochloride FF) via blood. Figure 3 shows the CSF chromatograms of the treated pigs. The SPE-HPLC-DAD results showed no FF or FFA disposition in CSF samples collected from pigs treated with a reference or generic VMP in the period from 216 to 384 hours after the first drug administration.
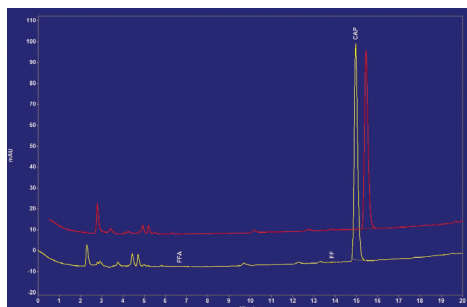

Figure 3. Representative chromatograms of pig CSF at 216 hours after intramuscular administration of reference (■) and generic VMP (■)

The blood-brain barrier formed by brain capillary endothelial cells in the central nervous system (CNS) could prevent the passage of FF and FFA into the pig CNS from the circulating blood after application of the recommended dose. However, de Craene et al. (1997) reported the maximum FF concentration in calves CSF at 2 hours after intravenous treatment. This is different than in our study, though those data are not fully comparable due to the different target species, time of CSF sampling, dose and route of administration. Nevertheless, additional studies are necessary for a better understanding of FF and FFA distribution. New studies should be conducted on CSF samples obtained from pigs a short time after the first drug administration. The data obtained in the present study could be valuable for further studies of FF and FFA in pig CSF after intramuscular administration of VMP.

Conclusions

Optimization and validation demonstrated, and the measurement uncertainty confirmed, that the SPE-HPLC-DAD method for FF and FFA determination in pig CSF ensures good repeatability and reproducibility. Considering the nature of biological fluids, this method could be suitable for further PK studies of FF in pig plasma and serum after intramuscular administration of VMPs.

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Razvoj SPE-HPLC-DAD metode za određivanje florfenikola i florfenikol amina u cerebrospinalnoj tekućini svinja

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Raspodjela florfenikola (FF) i njegovog metabolita florfenikol amina (FFA) u cerebrospinalnoj tekućini svinja istražena je nakon dvokratne primjene u mišić s 48-satnim razmakom originalnog i generičkog veterinarsko-medicinskog proizvoda (VMP) iste terapijske doze (20 mg FF/kg tjelesne mase) u istim uvjetima pokusa. Oba VMP-a imaju odobrenje za stavljanje u promet u Hrvatskoj te su po farmaceutskom obliku otopine za injekcije i sadržavaju 300 mg FF/mL. Pokus je proveden na klinički zdravim svinjama raspoređenim u dvije pokusne i jednu kontrolnu skupinu. Prvoj pokusnoj skupini životinja primijenjen je originalni, drugoj skupini generički lijek, dok je treća skupina bila kontrolna. Žrtvovanje životinja uslijedilo je nakon 216, 288 i 384 sata od prvog davanja VMP-a. Uzorci cerebrospinalne tekućine prikupljeni su u trenutku žrtvovanja i analizirani su optimiranom i validiranom tekućinskokromatografskom metodom uz detekciju UV-detektorom s nizom fotodioda (HPLC-DAD). Obrada uzoraka cerebrospinalne tekućine provedena je ekstrakcijom na čvrstoj fazi C18 (SPE). Primjenom SPE-HPLC-DAD metode ustvrđeno je da matrica ne utje-če na linearnost FF i FFA u radnom području od 0,05 do 5,00 µg/mL te je postignuta granica detekcije od 0,0023 µg/mL za FF i od 0,0100 µg/mL za FFA. Vrijednosti analitičkih povrata kreću se od 86,6 % do 111,8 % za FF, odnosno od 91,7 % do 98,8 % za FFA. U ovom radu je dokazano da je SPE-HPLC-DAD metoda selektivna, osjetljiva i pouzdana analitička metoda za određivanje FF i FFA u cerebrospinalnoj tekućini. S obzirom na rezultate našeg preliminarnog istraživanja FF u plazmi svinja tretiranih originalnim, odnosno generičkim VMP-om, cilj ovog istraživanja bila je i usporedba distribucije FF u uzorcima plazme i cerebrospinalne tekućine svinja nakon 216. sata od primjene VMP-a. Međutim, u uzorcima cerebrospinalne tekućine svinja žrtvovanih u navedenom pokusnom razdoblju nisu detektirani ni FF ni FFA. Zbog sličnosti matrica, SPE-HPLC-DAD metoda mogla bi poslužiti u budućim farmakokinetičkim studijama FF u uzorcima plazme i seruma dobivenim od svinja nakon primjene u mišić.

Ključne riječi: *florfenikol, florfenikol amin, svinje, cerebrospinalna tekućina, veterinarsko-medicinski proizvodi, HPLC-DAD*