DETERMINATION OF FLUNIXIN AND 5-HYDROXYFLUNIXIN IN BOVINE PLASMA WITH HPLC-UV-METHOD DEVELOPMENT, VALIDATION AND VERIFICATION

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Abstract

The method for quantitative determination of flunixin and its metabolite 5-hydroxyflunixin in bovine plasma has been developed and validated. Plasma samples were extracted with acetonitrile and analysed by liquid chromatography with ultraviolet detection. Extraction efficiency was tested and optimum pH conditions were established. Limit of the detection for both compounds was 0.03 µg/mL, limits of quantitation of flunixin and 5-hydroxyflunixin were 0.06 and 0.05 µg/mL, respectively. Both substances were determined with an accuracy below 12% (CV, %) and overall recovery in the range of 63-85%. The method was verified by the analyses of plasma samples from cows treated with flunixin meglumine.

Key words: flunixin, 5-hydroxyflunixin, HPLC.

Flunixin (FLU), a nicotinic acid derivative, is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the enzyme cyclooxygenase in the arachidonic acid cascade. Flunixin shows non-narcotic analgesic and anti-pyretic properties and is useful in veterinary medicine for treating a variety of disorders in various species. The drug can be applied to reduce inflammation and pain associated with musculoskeletal disorders and colic in horses, infectious diseases in cattle and as an aid in the treatment of mastitis-metritis-agalactia syndrome in sows (7).

In this paper, the method for the determination of flunixin and 5-hydroxyflunixin in bovine plasma has been described. The extraction’s efficiency was investigated and optimum pH conditions were established. The method was validated according to the procedure used in pharmacokinetics studies (9). The aim of the reported study was to develop a reliable HPLC method for flunixin and its metabolite 5-hydroxyflunixin determination in bovine plasma and to validate the method for pharmacokinetic study purposes. The method was verified in analyses of FLU and 5OHFLU in plasma samples from cows treated with flunixin meglumine.

Material and Methods

Reagents. The analytical standard of flunixin meglumine (FLU) was purchased from ISP Chemicals,
USA; 5-hydroxyflunixin (5OHFLU) was given by courtesy of the Community Reference Laboratory for Residues in Berlin. Acetonitrile (ACN, HPLC grade) was purchased from Merck (Germany). Methanol (MeOH, HPLC grade) and acetic acid (HPLC grade, <99%) was purchased from J.T. Baker (Germany). Sodium chloride (NaCl), ammonium acetate (CH₃COONH₄) and hydrochloric acid (HCl) were purchased from Polskie Odczynniki Chemiczne (Poland). Water was purified using Mili-Q system (Millipore, France).

**Control samples.** Plasma samples (free of flunixin residues) used for method development and validation were prepared from heparinised blood collected from cows in a slaughterhouse. After centrifugation (4 500 g, 30 min), the plasma was checked for possible presence of interferences by HPLC analysis, then transferred to screw-capped polypropylene tubes, and stored in a freezer at the temperature below -18°C until analysis. Before use, the required plasma portion was thawed overnight in a refrigerator (about 4°C).

**Biologically incurred plasma samples.** For experimental verification of the developed method, plasma samples with biologically incurred flunixin and 5-hydroxyflunixin were used. Two cows received a single intravenous dose of 2.2 mg/kg b.w. of flunixin meglumine (Flunimeg, Norbrook Laboratories). Blood samples were taken into heparinized tubes from the jugular vein prior to, and during 24 h after the drug’s administration at the following time-points: 0.5; 1; 1.5; 2; 2.5; 3; 3.5; 4; 5; 6; 7; 8; 10; 12; 16; 20 and 24 h. After centrifugation (4 500 g, 10 min) plasma samples were collected and stored in the freezer at the temperature below -18°C until analysis.

**Standard solutions.** All standard solutions were prepared in methanol. The stock standard solutions (1 mg/ml) were prepared by weighing 10.0 (± 0.1) mg of standard substances and dissolving in 10 ml volumetric flask. The stock solutions were stable for 12 months when stored at the temperature below -18°C. The standard solutions and dissolving in 10 ml volumetric flask. The stock standard solutions (1 mg/ml) were prepared by weighing 10.0 (± 0.1) mg of standard substances and dissolving in 10 ml volumetric flask. The stock solutions were stable for 12 months when stored at the temperature below -18°C. The working standard solutions (100 µg/ml) and mixed standard solutions (10 and 1 µg/ml of each compound), prepared by diluting suitable aliquot of stock standard, were stable for 6 months when stored at 2-8°C.

**Liquid chromatography.** The instrumental analysis was performed using Varian Prostar HPLC system equipped with quaternary pump, autosampler, column oven, and UV/Vis detector (290 nm), controlled by Galaxie Workstation software. Chromatographic analysis of compounds was performed on Inertsil ODS-3 column (150 mm × 4.6 mm, 5 µm, GL Science, Japan) connected with precolumn (4 mm × 3 mm, SecurityGuard, Phenomenex, USA). HPLC column was conditioned with mobile phase consisting of 10% of acetonitrile and 90% 0.05 M CH₃COONH₄ at pH 5.0 (adjusted with acetic acid) and 1.2 mL/min flow rate. The column oven temperature was controlled at 30°C. The following 30-min gradient elution programme was applied: 10% of ACN was pumped for 3 min, increased to 60% ACN at 15 min, and held for 2 min, then increased to 80% ACN at 20 min, and reduced to 10% of ACN at 25 min. For next 5 min, 10% of ACN was pumped. The 5 µl of final sample extract was injected into the column. In order to find the maximum wavelength of absorbance, the standards were analysed in the described chromatographic conditions using photodiode-array detector. The optimum wavelength of 290 nm was chosen.

**Sample extraction.** 0.5 ml of plasma was pipetted to the 20 ml glass centrifuge tube, 2 ml of ACN and 0.5 g of NaCl was added. The tube content was mixed for 1 min on vortex-mixer and centrifuged for 15 min (4 500 g, –5°C). The supernatant was transferred to another glass tube and evaporated to dryness under stream of nitrogen at 40°C. The residue was reconstituted in 0.5 ml of the volumetric mixture consisting of ACN, MeOH and 0.05 M CH₃COONH₄ at pH 5.0 (1+1+1), transferred to an autosampler vial, centrifuged for 5 min (4 500 g, 20°C) and analysed.

**Extraction efficiency study.** The incurred plasma sample containing flunixin and 5-hydroxyflunixin taken 1 h after injection was used to check extraction efficacy of the drugs. For this purpose, four 0.5 ml plasma samples were taken and three of them were acidified with 50 µl of 1 N, 3 N, and 6 N HCl. The samples were left to stand for 5 min and after that, all four samples were analysed according to the procedure. The results of analyses of acidified samples were compared to the result of analysis of the non-acidified plasma sample, regarded as 100%.

**Calibration and linearity.** Standard calibration curve was prepared by the injection of mixed standard solutions on five levels of FLU and 5OHFLU over the range 0.05–2 µg/mL and plotting the recorded peak areas versus concentration. The equation and regression coefficients, and linearity ranges were evaluated. The matrix calibration curves for FLU and 5OHFLU were prepared from the results of the analyses of the control plasma samples (n = 6) fortified with both substances at the levels of 0.1, 0.25, 0.5, and 2 µg/mL, and carried out throughout the whole procedure. For those, four-point curves equations, regression coefficients, and linearity ranges were evaluated.

**Validation experiment.** Specificity of the method was checked by chromatographic analysis of mixtures of NSAIDs, sulphonamides, nitroimidazoles, and tetracyclines. Additionally, equine and swine drug-free plasma were analysed to check possible species dependent matrix interferences. Recoveries were calculated for all fortification levels, by comparing peak areas of fortified samples to the corresponding standards. The precision of the assay was determined as the coefficient of variation calculated for three concentration levels (each in 6 replicates). Detection limits of the method were calculated on the basis of signal to noise ratio (S/N = 3 for limit of detection - LOD and S/N = 10 for limit of quantitation - LOQ).

**Pharmacokinetics analysis.** Two-compartment open method was used for pharmacokinetic analysis of FLU. Pharmacokinetic parameters of the bi-exponential
equation were obtained using Biokinetica 1.0 software (www.biokinetika.pl).

Results

Chromatograms of the control plasma sample and plasma sample from the cow treated with flunixin meglumine are shown in Fig. 1. No interference peaks were observed in the bovine plasma samples collected in the slaughterhouse, plasma of cows used in experiment as well as in samples of other species’ plasma.

Retention times of the analytes (12.5 min for 5OHFLU and 14.1 for FLU) were very stable during the whole experiment for the injected standard solutions and samples (± 0.5%).

The total time necessary for analysis of one sample was 90 min.

As it is presented in Fig. 2, recoveries of flunixin slightly increased with increasing HCl concentration, whereas recoveries of 5-hydroxyflunixin decreased significantly with the increasing acidification.

Results of validation are presented in Table 1. The average recoveries of FLU from plasma were 69-85% over a fortification range of 0.5-2.0 µg/mL, while average recoveries of 5OHFLU were 63-81%.

The within-day precision (represented by the coefficient of variation (CV, %) for three fortification levels) ranged from 3.3% to 8.4% and from 3.3% to 11.7% for FLU and 5OHFLU, respectively.

Fig. 1. Chromatogram of control plasma sample (A) and plasma sample collected from cow 6 h after i.v. administration of flunixin meglumine in the dose 2.2 mg/kg b.w. (B).

Fig. 2. Extractability of FLU and 5OHFLU from acidified incurred bovine plasma calculated as the percentage of results obtained with non-acidified plasma.
Fig. 3. Elimination profile curves of flunixin and 5-hydroxyflunixin after intravenous injection of flunixin meglumine (2.2 mg/kg b.w.) in two cows (A and B).

### Table 1
Results of method validation

<table>
<thead>
<tr>
<th>VALIDATION PARAMETERS</th>
<th>FLUNIXIN</th>
<th>5-HYDROXYFLUNIXIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection, µg/mL</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Limit of quantitation, µg/mL</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Standard calibration curve</td>
<td></td>
<td></td>
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<tr>
<td>$y = 17.353x + 194.65$</td>
<td>$y = 9152x + 95.995$</td>
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<tr>
<td>$R^2 = 0.9998$</td>
<td>$R^2 = 0.9997$</td>
<td></td>
</tr>
<tr>
<td>Linearity range, µg/mL</td>
<td>0.05-5</td>
<td>0.05-5</td>
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<tr>
<td>Matrix calibration curve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y = 13.193x – 225.67$</td>
<td>$y = 14343x – 521.27$</td>
<td></td>
</tr>
<tr>
<td>$R^2 = 0.9996$</td>
<td>$R^2 = 0.9992$</td>
<td></td>
</tr>
<tr>
<td>Linearity range, µg/mL</td>
<td>0.06-1.2</td>
<td>0.05-2.0</td>
</tr>
<tr>
<td>Precision, CV, % (n=6)</td>
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<tr>
<td>0.5 µg/mL</td>
<td>8.4</td>
<td>11.7</td>
</tr>
<tr>
<td>1.0 µg/mL</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>2.0 µg/mL</td>
<td>7.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Recovery, % (n=6)</td>
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<td></td>
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<tr>
<td>0.5 µg/mL</td>
<td>69</td>
<td>63</td>
</tr>
<tr>
<td>1.0 µg/mL</td>
<td>76</td>
<td>70</td>
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<tr>
<td>2.0 µg/mL</td>
<td>85</td>
<td>81</td>
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### Table 2
Pharmacokinetic data after single dose (2.2 mg/kg b.w.) of flunixin meglumine

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>AUC, mg/kg·h</th>
<th>$V_{DSS}$, L/kg</th>
<th>t$_{1/2}$, h</th>
<th>Cl, 1/h·kg</th>
<th>MRT, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow A</td>
<td>16.1</td>
<td>0.537</td>
<td>6.6</td>
<td>0.138</td>
<td>3.9</td>
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<tr>
<td>Cow B</td>
<td>14.4</td>
<td>0.69</td>
<td>5.8</td>
<td>0.157</td>
<td>4.5</td>
</tr>
<tr>
<td>Rantala (18)</td>
<td>17.1 ± 5.1</td>
<td>0.43 ± 0.1</td>
<td>5.7 ± 2.6</td>
<td>0.14 ± 0.04</td>
<td>3.5 ± 1.8</td>
</tr>
<tr>
<td>Odensvik (16)</td>
<td>14.2 - 16.8</td>
<td>0.297 – 0.419</td>
<td>3.8 – 4.3</td>
<td>0.122 – 0.142</td>
<td>-</td>
</tr>
</tbody>
</table>
Pharmacokinetic profiles of FLU and 5OHFLU in plasma of two cows after intravenous injection of flunixin meglumine are presented in Fig. 3. FLU and 5OHFLU were detected until 16 h after the administration in cow A, and 24 and 16 h, respectively, in cow B.

Pharmacokinetic parameters (AUC – area under curve, V_{DSS} – volume of distribution at steady state, \( t_{1/2} \) - elimination half-time, Cl - clearance, MRT – mean residence time) were calculated for both animals and compared to values obtained by other authors in Table 2.

Discussion

None of the methods for the determination of flunixin in plasma published in the literature enables to determine simultaneously flunixin and 5-hydroxyflunixin (12). This compound was identified as flunixin metabolite and was chosen to be a marker residue of flunixin in cow milk (7). Although 5OHFLU was detected in milk after flunixin administration to the cows, its presence in bovine plasma was not reported (3).

The method of sample preparation used in this study was based on the method published by Miksa et al. (13). These authors used ACN and NaCl solution for the deproteinisation and extraction of plasma sample. After mixing and centrifugation, the extract was analysed by LC-MS. We have decided to evaporate collected supernatant in order to obtain lower limits of detection. Use of ACN ensures good precipitation of plasma proteins and effective extraction of analytes, while NaCl enables complete separation of aqueous and organic phases.

Chromatographic conditions described in the procedure allowed good separation of analytes not only from matrix constituents but also from other drugs of NSAIDs group (carprofen, diclofenac, meloxicam, phenylbutazone, tolfenamic acid, vedaprofen) and some antimicrobials (nitroimidazoles, sulphonamides, and tetracyclines). It was concluded that the newly developed analytical method was specific enough for the determination of flunixin and 5-hydroxyflunixin in the same sample of bovine plasma.

Because of high protein binding of flunixin, the extraction efficiency study was important to obtain reliable results. Some authors evaluated the effectiveness of different solvents in combination with acidic and basic buffers. Odensvik et al. (16) checked recovery of flunixin isolated from plasma with diethyl ether and citrate buffer (pH 3.0, 3.5, 4.6), phosphate buffer (pH 3.5), sulphate buffer (pH 1.2) and dichloromethane with carbonate buffer (pH 9.8). Luo et al. (12) used phosphate buffer (pH 3.1) with different solvents: chlorobutane, dichloromethane, and petroleum ether. The authors of both articles tried to find the proper pH of a sample in order to release drug from protein and to facilitate isolation of protonated molecules using organic solvents. The reported recoveries were very high (over 90%), but results of these experiments could be overestimated because the authors used blank plasma spiked with flunixin, but not built-in samples.

In our assays, we wanted to verify the necessity of acidification of plasma sample before extraction. For this purpose, FLU and 5OHFLU recoveries from non-acidified and acidified plasma with naturally bound compounds were compared. The use of this incurred material ensured more reliable recoveries of analytes.

Influence of the concentration of hydrochloric acid used for acidifying plasma samples before extraction on the results of analysis was significant. Extraction efficiency study showed that acidifying plasma samples before extraction (which was expected to reduce protein binding) did not fulfill our expectation and was the reason of discrepancies in the results. Decreasing the 5-hydroxyflunixin concentration in acidified plasma samples was probably caused by compound degradation. Therefore, we have decided not to include this step into extraction procedure. This study proved that using of acetonitrile was sufficient for extraction of albumin-bound flunixin. It appeared that acidifying a sample is not necessary for FLU determination; moreover, this step can make simultaneous determination of FLU and 5OHFLU impossible.

The developed method is relatively simple and allows the detection of FLU and 5OH FLU in bovine plasma above the level of 0.03 µg/mL. Although sample preparation step was short, the obtained extracts were very clean. Method was linear within the range of concentrations in the experiment and showed good precision and recovery values.

To verify that the method was sensitive and suitable for the determination of FLU and 5OHFLU in the incurred bovine plasma, flunixin meglumine was intentionally administered to two cows and the plasma samples were collected and analysed. The plasma concentration-time curve showed a biphasic decline similar to those obtained by Anderson et al. (1) and Odensvik et al. (16). As presented in Table 2, some pharmacokinetic parameters of FLU calculated for those two cows were in good agreement with the data from the literature (16, 18). Second peak plasma concentration observed 3-4 h after treatment is characteristic for flunixin in cows. In opinion of some authors, such a peak is a result of enterohepatic recycling (10, 11, 16). In our study, a similar peak in the curve of 5OHFLU occurred at the same time.

The method for the determination of flunixin and 5-hydroxyflunixin in bovine plasma proved to be reliable, sensitive, and time-effective, and the results of validation and verification of the method show its usefulness.

References


