



Ultra-performance liquid chromatography–tandem mass spectrometry determination and depletion profile of flunixin residues in tissues after single oral administration in rabbits



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ABSTRACT

An ultra-performance liquid chromatography with tandem mass spectrometric detection (UPLC–MS/MS) method was developed for the detection of flunixin residues in rabbit tissues. The samples were extracted with acidic acetonitrile, defatted with n-hexane, and then purified by HLB solid-phase extraction cartridge. Analysis was carried out on UPLC–ESI-MS/MS working with multiple reaction monitoring (MRM) mode. The limits of detection (LODs) of the method were 0.3–0.8 $\mu\text{g kg}^{-1}$ and limits of quantification (LOQs) were 1.0–3.0 $\mu\text{g kg}^{-1}$ in rabbit tissues, respectively. In all fortified samples at a concentration range of 1.0–300.0 $\mu\text{g kg}^{-1}$, mean recoveries were 61.7–115.7% with relative standard deviations (RSDs) below 16%. Residue depletion of flunixin in rabbit was conducted after oral administration at a dose of 5 mg kg^{-1} of body weight. The average concentrations for flunixin measured 2 h post-administration in kidney and intestine were significantly higher than in liver, heart and muscle. The concentrations for flunixin in all rabbit tissues were below the LOD or not detected in all tissues after 96 h administration of drug. A minimum withdrawal time of 21 h was indicated for residue levels in heart, liver, kidney, intestine and muscle below the maximum residue limits (MRLs).

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1. Introduction

Flunixin (2-[[2-methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic acid, CAS: 38677-85-9; Fig. 1) is a non-steroid anti-inflammatory drugs (NSAIDs), which can inhibit the synthesis of cyclooxygenase, resulting in decreased production of prostaglandin and thromboxane A₂ [1,2]. It is widely used to treat the inflammation disorders of animals in many countries such as acute inflammation, mastitis metritis agalactia syndrome, musculo-skeletal disorders and colic [3,4]. Many researches indicated that flunixin has potential risks such as gastro-intestinal damages, hematopoietic and renal systems toxicities, hepatotoxicity, aseptic meningitis, and so on [5–9]. Flunixin was classified as the pharmacologically active substances and its maximum residue limits (MRLs) or tolerance were established in animal tissue and milk by Japan (JPN) [10], European Union (EU) [11], Canada [12] and USA [13] in the range of 2 $\mu\text{g kg}^{-1}$ –200 $\mu\text{g kg}^{-1}$.

Currently, methods for determination of flunixin and related residues, such as high performance liquid chromatography (HPLC) [14,15], gas chromatography(GC) [16], gas chromatography–mass spectrometry (GC–MS) [17,18], and capillary electrophoresis (CE) [19], have been developed. As is well known, LC–MS or LC–MS/MS is an analysis method which is widely used in labs, due to its good separation, sensitivity and selectivity. LC–MS/MS was also applied to analyze flunixin and related residues in urine [20], plasma [21], milk [22–25] and tissues [26]. Boner et al. [26] described a method for the determination of flunixin in bovine edible tissues by LC–MS/MS. Hu et al. [27] reported a multi-residue method for the determination of thirty NSAIDs in swine muscle by UPLC–MS/MS. In recent years, as food culture and consumption level increase, and some special disease, such as mad cow disease, foot-and-mouth disease are popular in edible animals, rabbit edible tissues and other pollution-free edible tissues are urgently demanded by consumers, especially in China, Japan and other regions. And rabbit meat possesses high quality, second only to the ostrich meat. Furthermore, flunixin residue is possible to remain in rabbit tissues. Hence, it is necessary to establish a rapid and reliable analysis method for flunixin in rabbit edible tissues based on LC–MS/MS.

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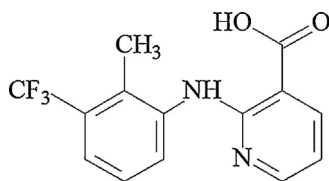


Fig. 1. Structure of flunixin.

The kinetic behavior of flunixin, with good bioavailability from oral, intramuscular and subcutaneous administration routes, a high degree of binding to plasma protein, penetration into and slow clearance from acute inflammatory exudates [28], has investigated in many species, including cattle [29,30], sheep [31], camel [32], chicken [33], swine [34], horse [35] and rabbit [15,36,37]. Characteristics of flunixin in Angora rabbits including short elimination half-life, high clearance, large volume of distribution and dose independent in the dose range of 1.1–2.2 mg kg⁻¹ were described and both doses were well tolerated by all Angora rabbits [15]. However, to our knowledge, there are no published investigations on residue depletion of flunixin in rabbit tissues (heart, liver, kidney, intestine and muscle). The depletion data in rabbit tissues would be helpful for safety evaluation.

The purposes of the present study were to develop a rapid and reliable analytical method for the determination of flunixin in rabbit tissues using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) and to evaluate the residue depletion of flunixin in rabbit edible tissues (heart, muscle, liver, kidney and intestine). The method was then applied to monitor residue depletion of flunixin from healthy rabbits after a single oral administration of 5 mg kg⁻¹. Moreover, the concentrations of flunixin from rabbit tissues were compared to MRLs established by JPN and used to calculate the withdrawal time.

2. Materials and methods

2.1. Chemicals and reagents

Flunixin meglumine was supplied by Sigma–Aldrich (MO, USA). LC-grade acetonitrile and methanol were obtained from Fisher Scientific (New Jersey, USA). Formic acid and ammonium acetate of HPLC grade were purchased from Acros Organics (Brussels, Belgium). Analytical grade ammonium hydroxide (25–28%), hexane, phosphoric acid and anhydrous sodium sulfate were provided by Beihuaqingxi Corp. (Beijing, China). Water was purified using a Milli-Q Synthesis system from Millipore (Bedford, USA). Oasis HLB (150 mg) extraction cartridges were supplied by Waters (Milford, USA).

2.2. Standard solution

Flunixin standard solutions were prepared in acetonitrile at 100 µg mL⁻¹ (corrected for purity), and stored at 4 °C, which are stable for at least 6 months (data not shown). The stock solution was diluted with appropriate volumes of acetonitrile to prepare working solutions (1.0 µg mL⁻¹). Flunixin meglumine solution (500 mg mL⁻¹) was purchased from Zhejiang Haizheng Pharmaceutical Corp. (Taizhou, Zhejiang, China). The working solution (5.0 mg mL⁻¹) was prepared by diluting original solution (500 mg mL⁻¹) with normal saline.

2.3. Animal treatment

Twenty-one healthy New Zealand rabbits of both sexes weighing 3.0 ± 0.4 kg were placed individually in cages in the university

animal house and acclimated for 14 days prior to the study under common farm conditions, with continuous ventilation and heating systems. Flunixin free commercial feed and water were supplied *ad libitum*. They were fasted for 12 h before being given the test treatments. The rabbits were randomly assigned to two groups. The test group received a single oral dose of 5 mg kg⁻¹ bw of flunixin meglumine, control animals did not receive any treatment and were used to determine the validation criteria of the analytical method. One control and three medicated animals were sacrificed at 2, 8, 12, 24, 48 and 96 h post-administration. Tissue specimens of heart, liver, kidney, intestine and inner thigh muscle were collected, homogenized and stored at –20 °C until analysis.

2.4. Sample preparation

Tissue samples (2.0 g), 2.0 g of anhydrous sodium sulfate and 15 mL of acidic acetonitrile (acetonitrile–phosphoric acid, 80 + 1, V/V) were placed in a 50 mL centrifuge tube. Then the tube was shaken for 20 min, ultrasonic for 10 min and centrifuged at 2810 × g for 5 min. The supernatant was transferred to another 50 mL tube. The residue was extracted once more as above. The supernatants were combined and 15 mL hexane saturated with acetonitrile was added. The tube was vortexed followed by centrifugation at 2810 × g for 5 min and the hexane layer was removed. The lower layer was subsequently evaporated to dryness using rotary evaporator at 40 °C. The residue was reconstituted by 10 mL of methanol–0.02 M phosphoric acid (5 + 95, V/V) and 10 mL hexane. The supernatant was removed and then the lower layer was loaded on HLB cartridge which was preconditioned with 10 mL methanol, water and 0.02 M phosphoric acid sequentially. The cartridge was washed with 5 mL water and eluted with 4 mL ammonium hydroxide–acetonitrile (5 + 95, V/V). The collected eluate was evaporated to dryness under gentle stream of nitrogen at 40 °C. The residue was reconstituted with 1 mL acetonitrile–0.1% formic acid (1 + 9, V/V) containing 0.5 mM ammonium acetate and filtered through 0.22 µm membrane filter before UPLC–MS/MS analysis.

2.5. UPLC–MS/MS analysis

The LC–MS/MS system consisted of a Waters Acquity UPLC apparatus coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray ionization (ESI) interface. The instrument control, data acquisition and data treatment were performed with Masslynx 4.1 Analyst software. Chromatographic separation was achieved using an Acquity BEH C18 column (50 mm × 2.1 mm, 1.7 µm). The column was kept at 30 °C. The mobile phase consisting of acetonitrile (solution A) and 0.1% formic acid in water containing 5 mM ammonium acetate (solution B) was adopted at a flow rate of 0.2 mL min⁻¹. A gradient elution was applied for: 0–2 min 90% B, 2 min 70% B, 3 min 50% B, 4 min, 30% B, 5 min, 10% B, 6 min, 90% B. The injection volume was 10 µL.

The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. The parameters for the mass spectrometer were as follows: ESI source block and desolvation temperature: 120 and 350 °C; capillary voltage: 3.0 kV; cone voltage: 45 V; argon collision gas pressure: 2.4 × 10⁻⁶ Pa; cone nitrogen and desolvation gas flows: 100 and 600 L h⁻¹, direct infusion was performed to optimize multiple reaction monitor (MRM) transitions and associated acquisition parameters. The optimized conditions were as follows: *m/z* 297.2 → 279.2 (quantitative ion, collision energy, 23 eV), *m/z* 297.3 → 264.2 (qualitative ion, collision energy, 34 eV).

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