Quantification of flunixin in equine plasma by ultra-high performance liquid chromatography electrospray ionization tandem mass spectrometry (UHPLC-ESI/MS/MS)

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Abstract

An UHPLC-ESI/MS/MS method was developed for simultaneous quantification and confirmation of flunixin in equine plasma. Flunixin was recovered from equine plasma by strong anion exchange solid phase extraction (SAX-SPE). The concentration of flunixin was determined between 5 and 250 ng/mL by internal standard calibration using flunixin-d_3 as the internal standard. The limits of detection (LOD) were determined to be 1 ng/mL. The measurement precision and accuracy were 4.8% and 101.9%, respectively. The method is not interfered by any other NSAIDs that are regulated by the United State Equestrian Federation (USEF). The method can assist USEF to control doping in horse racing.

Introduction

Flunixin is a non-steroidal anti-inflammatory drug (NSAID). It is possibly the most effective NSAID for the treatment of colic and associated endotoxemia. Experimentally, flunixin has been shown to be effective in reducing the acute systemic side effects and preventing clinical signs of endotoxemia, including cardiovascular and hemodynamic alterations, hypoxemia, and lactic acidosis [1]. In racehorses, however, flunixin has the capacity to affect racing performance, thus its usage is subject to doping control regulations. In order to discriminate between doping and therapeutic use of flunixin, a threshold of 1.0 µg/mL in equine plasma has been adapted in samples collected immediately after the competition by the United States Equestrian Federation (USEF).

Post-race analysis of flunixin in equine plasma usually begins with a presumptive test using an enzyme-linked immunosorbent assay (ELISA). Positive samples are further submitted for quantification and confirmation of flunixin by chromatographic based methods. For the quantification of flunixin in equine plasma, we have developed a liquid chromatography ultraviolet detection (LC-UV) method after flunixin was recovered from equine plasma by SAX-SPE. However, LC-UV lacks defensible confirmation of analytes, so it will not withstand legal challenges in court. In contrast, liquid chromatography mass spectrometry (LC-MS) can extract molecular fingerprints from very low concentrations of analytes, so it can provide legally defensible confirmation and will withstand legal challenges in court [2]. In this study, we would like to develop a LC-MS method for the simultaneous quantification and confirmation of flunixin in equine plasma.

Experimental

SAX-SPE procedure

1. **Sample pretreatment:** Dilute 200 µL equine plasma and 200 µL internal standard solution with 1.6 mL of 0.5% (V/V) ammonia in water.
2. **Column conditioning**: Use gravity flow to apply 1 mL methanol, followed by 1 ml HPLC water through the column.
3. **Sample loading**: Use gravity flow to load the sample.
4. **Column wash**: Use gravity flow to apply 2×500 µL water, followed by 2×500 µL methanol. Finally, use a gentle vacuum to dry the column.
5. **Analyte elution**: Use gravity flow to apply 2×500 µL 5% (V/V) formic acid in methanol.
6. **Eluate drying**: Use gentle nitrogen stream to dry the eluates.
7. **Sample resuspension**: Add 200 µL mobile phase.

Table 1. Agilent 1260 Infinity II LC conditions

Parameter	Value
Column	Agilent Zorbax Eclipse C18 50 mm × 2.1 mm, 1.8 µm
Column temperature	40 °C
Injection volume	10 µL
Mobile phase	A: Water/acetonitrile 95/5 + 0.1 % formic acid
	B: Methanol/acetonitrile 95/5
Flow rate	0.300 mL/min
Gradient program	
0.0 minute	40% B
2.0 minute	90% B
6.0 minute	90% B
6.5 minute	40% B
Stop time	6.5 minute
Post time	5.5 minutes

Table 2. Agilent 6545 Q-TOF MS and MS/MS parameters

Parameter	Value
System tune	Standard 3200 m/z; 2 GHz Extended dynamic range; high resolution slicer mode
Transmission tune	50–750 m/z; 2 GHz Extended dynamic range; high sensitivity slicer mode
Mass calibration	50–750 m/z; 2 GHz Extended dynamic range; high sensitivity slicer mode
Ion source	Dual AJS ESI
MS acquisition mass range	100–1000 m/z
MS acquisition rate	10 spectra/s
MS/MS acquisition mass range	50–350 m/z
MS/MS acquisition rate	10 spectra/s
Drying gas temperature	325 °C
Drying gas flow	10 L/min
Nebulizer pressure	20 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Ionization mode	Positive
Capillary voltage	3500 V
Nozzle voltage	800 V
Fragmentor	120 V
Skimmer	45 V
Oct1 RF Vpp	750 V
MS reference mass ions	121.0509, 922.0098

Table 3. Agilent 6545 Q-TOF MS and MS/MS parameters

Parameter	Precursor (m/z)	RT (min)	Δ_{RT} (min)	Isolation width	CE	Quantifier ion	Quanlifier ion
Flunixin	297.085	4.88	0.5	~1.3 m/z	25	279.074	264.050
Flunixin-d_3	300.103	4.88	0.5	~1.3 m/z	25	282.293	264.050

Results

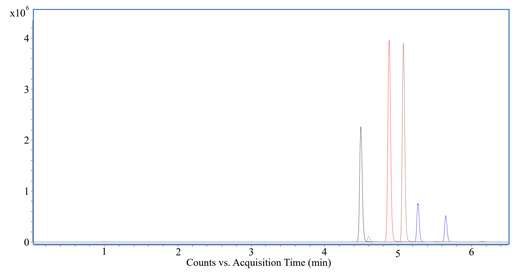

Figure 1. UHPLC-ESI/MS baseline separation of flunixin from other NSAIDs regulated by USEF. The peaks were identified by their retention time as listed in **Table 4**. Each peak was extracted using the $[M+H]^+$ ion of the analyte (**Table 4**) with ±20 ppm mass window

Table 4. UHPLC-ESI/MS interference study: retention time, theoretically calculated *m/z*, and experimentally measured *m/z* of NSAIDs regulated by USEF

NSAID	Retention time (minutes)	$[M + H]^+$ (Calculated)	$[M + H]^+$ (Measured)
Ketoprofen	4.49	255.1015	255.1016
Naproxen	4.60	231.1016	231.1016
Flunixin	4.88	297.0841	297.0845
Phenylbutazone	5.07	309.1595	309.1598
Diclofenac	5.27	296.0240	296.0240
Meclofenamic acid	5.65	296.0238	296.0240

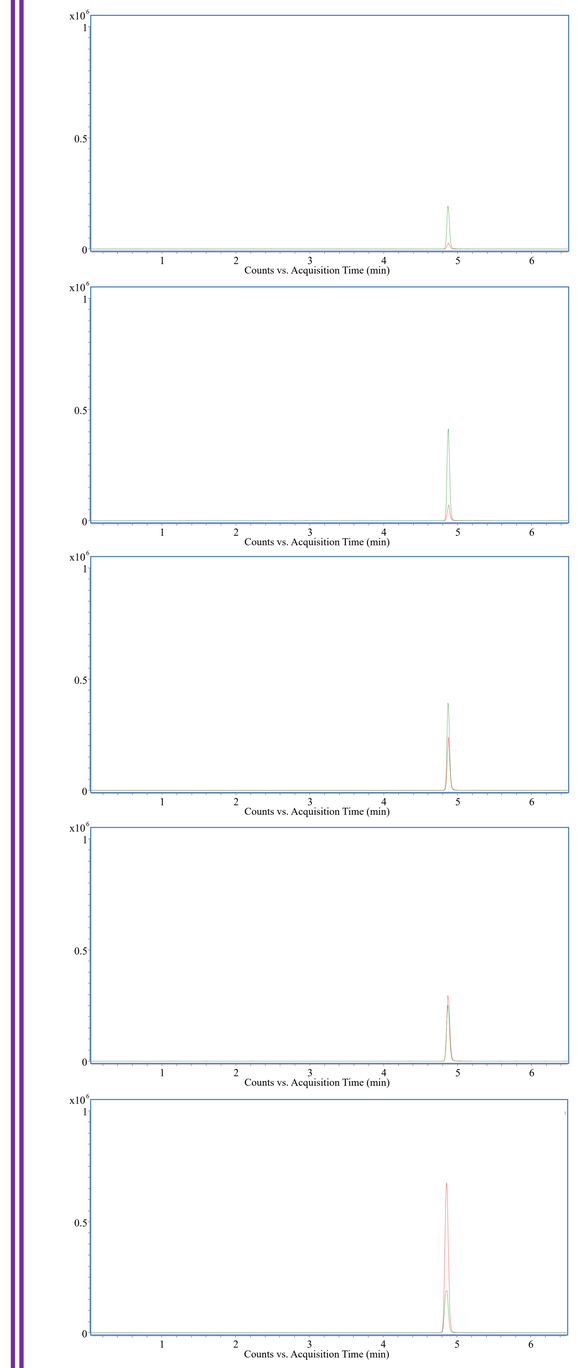

Figure 2. UHPLC-ESI/MS/MS chromatogram of 5, 10, 25, 50, and 125 (from top to bottom) ng/mL flunixin (red) and 25 ng/mL flunixin -d_3 (green).

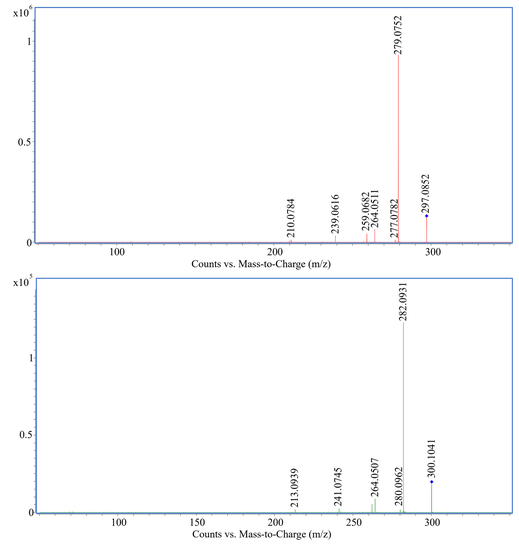

Figure 3. UHPLC-ESI/MS/MS spectra of flunixin (red) and flunixin -d_3 (green).

Figure 4. Flunixin internal standard calibration curve

Table 5. Precision and accuracy

QC	Spiked Conc.(ng/mL)	Measured Conc. (µg/mL)	% Accuracy
1	25.0	24.4	97.5
2	25.0	26.8	107.1
3	25.0	25.3	101.0
Average		25.5	101.9
% RSD		4.8	4.8

Conclusions

- An UHPLC-ESI/MS/MS method for simultaneous quantification and confirmation of flunixin in equine plasma has been developed.
- The limits of detection (LOD) was 1 ng/mL.
- The precision and accuracy were 4.8% and 101.9%, respectively.
- The method is not interfered by any other NSAIDs that are regulated by the United State Equestrian Federation (USEF).
- The method can assist USEF to control doping in horse racing.

References

1. Knych, H.K. Vet. Clin. N. Am.-Equine Pract. 33, 1-15 (2017).
2. B. Heffron et al. J. Anal. Toxicol., 37 (2013) 600-604.

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