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Quantification and confirmation of flunixin in equine plasma by liquid chromatography–quadrupole time-of-flight tandem mass spectrometry

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Abstract

The method describes quantification and confirmation of flunixin in equine plasma by liquid chromatography–quadrupole time-of-flight mass spectrometry (LC/Q-TOF/MS/MS). Samples were screened by enzyme-linked immunosorbent assay (ELISA) and only those samples presumptively declared positive were subjected to quantification and confirmation for the presence of flunixin by this method. The method is also readily adaptable to instrumental screening for the analyte. Flunixin was recovered from plasma by liquid–liquid extraction (LLE). The sample was diluted with 2 ml saturated phosphate buffer (pH 3.10) prior to LLE. The dried extract was reconstituted in acetonitrile:water:formic acid (50:50:0.1, v/v/v) and subsequently analyzed on a Q-TOF tandem mass spectrometer (Micromass) operated under electrospray ionization positive ion mode. The concentration of flunixin was determined by the internal standard (IS) calibration method using the peak area ratio with clonixin as the IS. The limits of detection (LOD) and quantification (LOQ) for flunixin in equine plasma were 0.1 and 1 ng/ml, respectively, whereas the limit of confirmation (LOC) was 2.5 ng/ml. The qualifying ions for the identification of flunixin were *m/z* 297 $[M + H]^+$, 279 (BP), 264, 259, 239 and those for clonixin (IS) were *m/z* 263 $[M + H]^+$, 245 (BP) and 210. The measurement uncertainty about the result was 8.7%. The method is simple, sensitive, robust and reliably fast in the quantification and confirmation of flunixin in equine plasma. Application of this method will assist racing authorities in the enforcement of tolerance plasma concentration of flunixin in the racehorse on race day.

Keyword: Flunixin

1. Introduction

Flunixin meglumine (Banamine®) is a non-steroidal anti-inflammatory agent (Fig. 1). Methods available for the quantification of flunixin in plasma include high performance liquid chromatography (HPLC) [1–6,24,25], thin-layer chromatographic (TLC) densitometry and gas chromatography–mass spectrometry (GC/MS) [7–11,24,25]. The use of LC/MS/MS in the determination of flunixin and its metabolite in bovine milk and meat was recently reported [12–14]. Results of previous studies reported by the authors [3–5] and other investigators [15–17] indicated that intravenous administration of flunixin (1.10 mg/kg) 24 h prior to race time resulted in a plasma concentration of less than 10 ng/ml of flunixin. Most importantly, the data suggested that such a low plasma concentration of flunixin was pharmacologically inactive because it was unable to inhibit thromboxane B_2 to pre-flunixin administration level. Inhibition of thromboxane B_2 was used as an index of pharmacological activity [4,15–17]. Other investigators [18] have suggested that 1 mg/kg dose produces a nearly maximal effect with a return to baseline value after a delay of 16 h. Based on the results of the study by Soma et al. [3–5], the Pennsylvania Racing Commissions established guidelines for the use of flunixin in racehorses. However, previous methods [3–5] used in establishing the guidelines for flunixin in racehorses in PA, and for the screening and quantification of flunixin involved the use of TLC and HPLC, respectively, with limit of quantification (LOQ) that was very close to the tolerance concentration of 10 ng/ml in plasma. To solve this problem, a more sensitive analytical method with an LOQ that is much lower than 10 ng/ml for

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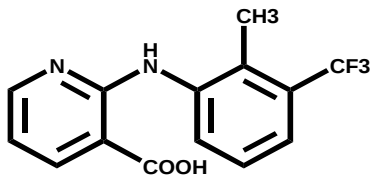
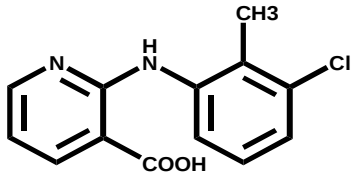


Flunixin $C_{14}H_{11}F_3N_2O_2$
C 56.76% H 3.74% F 19.24% N 9.46% O 10.80%
Molecular Weight 296.25
Exact Mass 296.0772



Clonixin $C_{13}H_{11}ClN_2O_2$
C 59.44% H 4.22% Cl 13.05% N 10.66% O 12.18%
Molecular Weight 262.70
Exact Mass 262.0587

Fig. 1. Chemical structures of flunixin and clonixin (IS).

flunixin in plasma was desired to increase the confidence in the threshold result.

The purpose of the study was to develop a sensitive method for the quantification and confirmation of flunixin in equine plasma using high performance liquid chromatography–quadrupole time-of-flight-tandem mass spectrometry (LC/Q-TOF-MS/MS). In addition, we wanted to emphasize the importance of measurement of uncertainty and demonstrate the estimation of uncertainty when quantitative measurement of an unknown concentration of an analyte in a sample is requested.

2. Experimental

2.1. Chemicals

All chemicals used in this study were commercially obtained. Unless otherwise stated, all reagents used, including water, were of analytical grade. Flunixin meglumine salt was obtained from US Pharmacopeia (US Pharmacopoeia Convention Inc., Rockville, MD, USA) and clonixin (IS; Fig. 1) was obtained from Schering Corporation (Schering Corporation, Kenilworth, NJ, USA). Acetonitrile, methanol and water of HPLC grade were obtained from J.T. Baker (J.T. Baker Chemicals, Phillipsburg, NJ, USA) and 1-chlorobutane (CBT) was obtained from EM Science (EM Science, Merck KGaA, Darmstadt, Germany). Ammonium Acetate was purchased from Fisher Scientific (Fisher Scientific, Fair Lawn, NJ, USA).

2.2. Preparation of reagents

Flunixin and clonixin (IS) stock solutions (1.0 mg/ml) were prepared in methanol and each solution was stored at 4 °C. The standard working solutions (0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0 μg/ml) were prepared by diluting the stock solution with acetonitrile:water:formic acid (50:50:0.1, v/v/v). The positive control standard working solutions were also prepared by diluting the stock solution with acetonitrile:water:formic acid (50:50:0.1, v/v/v) to obtain 1.0, 10.0 and 100.0 μg/ml. Phosphate buffer (pH 3.10) was prepared by continuously adding KH_2PO_4 to 950 ml water while stirring until the solution was saturated. The saturated solution was allowed to remain at room temperature for a minimum of 12 h before the clear supernatant was decanted into a 2.0 l beaker and the pH was adjusted to 3.10 using phosphoric acid. The final volume of 1000 ml was attained with HPLC water. Ammonium acetate (2 M, pH 5.0) was prepared by dissolving 154.2 g of ammonium acetate in 800 ml of water and adjusting the pH to 5.0 using acetic acid before bringing the final volume to 1000 ml with HPLC water. Clonixin was used as the internal standard (IS) and the stock solution was prepared by dissolving 100 μg of it in 4 ml acetonitrile:water:formic acid (50:50:0.1, v/v/v).

2.3. Preparation of calibrators and quality control samples

2.3.1. Standard calibrators and positive control samples (QC)

Standard calibrators and quality control (QC) samples were prepared using pooled equine plasma previously demonstrated to be flunixin-free by enzyme-linked immunosorbent assay (ELISA). A milliliter of plasma was used for preparing each concentration of either calibrator or positive control samples. A 10 μl volume of each standard working solution was added to each aliquot (1.0 ml) of negative plasma sample to prepare 1.0, 2.5, 5.0, 7.5, 10.0, 25.0, 50.0, 75.0, 100.0 and 150.0 ng/ml calibrators. Positive control (QC) samples were prepared by adding 10 μl of 1.0, 10.0 and 100.0 μg/ml QC working solution to each milliliter of blank plasma to prepare the final concentrations of 1.0, 10.0 and 100.0 ng/ml.

2.4. Sample preparation

The extraction efficiencies of flunixin (5, 50 and 500 ng/ml) from plasma at pH 3.30 by chlorobutane, dichloromethane and petroleum ether were evaluated. Each of the above concentrations of flunixin was spiked into 3 ml × 1 ml blank plasma samples in separately labeled test tubes. The extraction efficiency of the three solvents in extracting flunixin from plasma was evaluated by extracting each concentration of flunixin from plasma in which it was spiked by using each of the solvents. The organic layer was

recovered and dried, and the dried extract was reconstituted in the LC solvent and analyzed by measuring the peak area of the extracted plasma samples containing flunixin and comparing it with that of the same concentration of flunixin reference standard that had been added to three separate test tubes, dried and similarly reconstituted without prior extraction. For test sample extraction, the solvent with the highest extraction efficiency was chosen for use in the extraction of flunixin from plasma samples in this study. A milliliter of suspect sample (in triplicate) was dispensed into individual clean and labeled screw cap culture tubes (16 mm × 125 mm). To each calibrator, QC and unknown sample, 10 μl of 25 μg/ml stock solution of IS previously prepared (100 μg/4 ml) in acetonitrile:water:formic acid (50:50:0. 1, v/v/v) was added. To each sample, 2.0 ml of saturated phosphate buffer (pH 3.10) was added, mixed and the pH of the mixture was adjusted to 3.30 ± 0.10. A 5 ml aliquot of 1-chlorobutane was added to each sample tube and mixed on a rotorack for 10 min prior to centrifugation (3000 rpm × 10 min). The organic layer (top) was transferred, using Pasteur pipette, into a clean, labeled culture tube and evaporated to dryness at 55–65 °C (TechniDri-Block DB-3, Duxford, Cambridge, UK) under a steady flow of nitrogen or air. The dried extracts were reconstituted in 100 μl of acetonitrile:water:formic acid (50:50:0.1, v/v/v) and subjected to ultra-sonication (Brunson Ultrasonics Corp, Danbury, CT, USA) at 60 °C for 5 min. The reconstituted sample was used for analysis by LC/Q-TOF/MS/MS.

2.5. Validation of method

The method was validated under the guidelines presented by Shah et al. [19]. Fourteen assays for validation were performed; seven for between-run (inter-day assay) and seven for within-run (intra-day assay) to assess precision, accuracy and specificity.

Inter-assay accuracy and precision were assessed by analyzing 21 validation samples at three concentrations of flunixin (1, 10, and 100 ng/ml equine plasma) in seven separate experiments. These concentrations of flunixin corresponded to low, medium and high for constructing the standard curve. Intra-assay accuracy and precision were determined by analyzing six replicates of the three concentrations in each experiment. Accuracy was determined as the agreement between the concentration of flunixin detected and that spiked into blank plasma. Precision of the assay was determined as the relative standard deviation expressed as a percentage of the standard deviation divided by the mean of observed concentrations and was reported as percent coefficient of variation.

2.6. Stability of flunixin

Stability of flunixin in plasma at 1, 10 and 100 ng/ml was assessed following storage at room temperature, −70 °C and repeated freeze-thaw cycle. Analysis of samples at −70 °C also included the effect of freeze-thaw cycle. QC samples at the above concentrations were prepared and stored at room temperature (short-term) and at −70 °C (long-term). The samples were analyzed at 0, 5, 10, 15, 20 and 25 h for short-term storage and on 0, 10, 20, 30 and 40 days for long-term storage following preparation and storage to determine the effect of storage at room temperature, freezing and thawing cycles on the concentration of flunixin in the sample compared with the concentration that was spiked into the blank plasma on the day of preparation and storage. Zero hour in the case of short-term stability study refers to analysis of the samples immediately after flunixin had been spiked into blank plasma. Following preparation of the QC samples, three samples from each concentration was allowed to remain at room temperature for 10–12 h (zero-day) prior to analysis while the rest of the samples were stored at −70 °C in the case of long-term storage. Analysis of the rest of the samples was performed on various days as indicated above and in Fig. 2.

2.7. Analysis of plasma samples by LC/MS/MS

2.7.1. Instrumentation

Analysis was performed on a Q-TOF mass spectrometer equipped with electospray probe (Micromass) interfaced to a Series 1100 HPLC binary pump with an on-line degasser and an autosampler (Agilent Technologies, Wilmington, DE, USA). System control, data acquisition and processing were performed by Masslynx software (v 3.4) on Microsoft Windows NT.

2.7.2. Chromatographic conditions

Gradient chromatographic conditions were employed using an Agilent Zorbax XDB C8 (2.1 mm × 50 mm; 5 μm particle size) analytical column. The column temperature was set at 27 °C. The mobile phase gradient consisted of two pre-mixed components: A and B. Component A comprised 2 mM of pH 5.0 ammonium acetate:acetonitrile:ammonium hydroxide (95:5:0.01, v/v/v), and component B comprised 2 mM of pH 5.0 ammonium acetate:acetonitrile (5:95, v/v). The initial mobile phase gradient was from 95% A to 0% A (0–0.8 min), held 0% A for 1.5 min (0.8–2.3 min) and switched to the initial hold at 95% A (2.31–3.0 min). The flow rate was 0.2 ml/min and injection volume for each sample was 10 μl. Under these conditions, the retention time of flunixin and IS was 1.60 ± 0.15 and 1.45 ± 0.15 min, respectively. The total analysis time was 3.0 min.

2.7.3. MS conditions

The source temperature was set at 120 °C. Analysis was performed under positive ionization mode. Flunixin and IS were monitored at *m*/*z* 297 $[M + H]^+$ and 263 $[M + H]^+$, respectively. The optimized cone voltage and collision energy for flunixin were 30 and 25, respectively, and 30 and 20 for IS, respectively. The mass scan range was 100–300 Da. The scan time was 0.5 s. with inter-scan time of 0.05 s. The

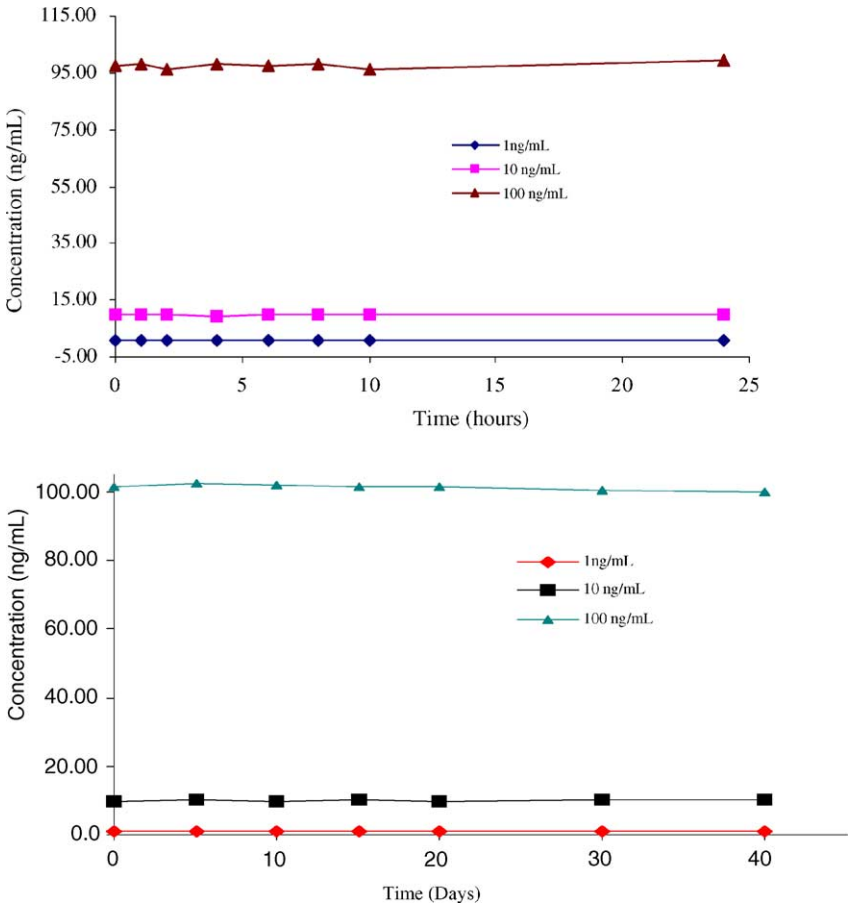


Fig. 2. Stability of flunixin at 25 C (top panel) and −70 °C (bottom panel).

qualifying ions for the identification of flunixin were *m*/*z* 297 $[M + H]^+$, 279 (BP), 264, 259, 239. The peak area ratio of flunixin to IS obtained from selected ion monitoring (*m*/*z* 279 for flunixin and *m*/*z* 245 for clonixin) was used to construct calibration curves by weighted (1/concentration) linear regression of standard concentration versus measured peak area ratio. Flunixin concentrations in unknown plasma samples were determined by interpolation from the calibration curve. Data collection and processing were performed using the Masslynx Quantify Software (Micromass).

2.8. Demonstration of absence of ionization suppression or enhancement effect

Since precursor-product ion in LC/MS/MS is target compound specific, the determination of interfering substances can only partially be based on the purity of the product ion full scan mass spectrum. Co-eluting substances with parent ions differing from the target precursor ion may still exert either enhancement or suppression of the ionization process, thus posing a great challenge to the validity of quantitative results. Thus, it became necessary for absence of ionization suppression or enhancement effect to be demonstrated. For this purpose, ionization stability was determined for the chromatographic and mass spectrometric conditions according to the method of Bonfiglio et al [20]. This determination was performed for the target compound, flunixin, as well as the IS. In this determination, post-column infusion delivery technique was used. Briefly, the test compound (flunixin or IS) was infused, post-column, through a Valco zero dead volume tee using a Harvard Apparatus syringe pump (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate of 0.2 ml/min into the LC effluent prior to entering the mass spectrometer. Blank plasma samples ($n = 4$) were then injected onto the Zorbax column. Effluent from the HPLC column combined with the infused analytes, entered the electrospray interface and was analyzed under the operating conditions for flunixin to measure the “matrix effect”, not only from one run, but also from late-eluting compounds that may not be detected until after several sequential analyses had been performed.

2.9. Estimation of measurement uncertainty

The method used in estimating measurement uncertainty (MU) of flunixin quantitation in this study was the laboratory control sample (LCS) or control charting according to Adams [21] and the American Association of Laboratory Accreditation Type 111 Policy [22]. For this estimation, we used 56 determinations of flunixin-augmented equine con-

trol plasma at 10 ng/ml as the laboratory control samples or quality control samples (QCS) from which the mean (x) and estimated standard deviation(s) of flunixin from plasma were calculated [22,23]. These initial determinations estimated the measurement uncertainty at 8.7%. Control charting of measurement uncertainty for samples over a period of 1 year demonstrated measurement uncertainty at ∼7.2%. This agreement in the estimated measurement uncertainty strongly supports the validity of the LCS or QCS approach to estimating measurement uncertainty. The use of 10 ng/ml of flunixin as the LCS was based on the tolerance concentration of flunixin in racehorses at race time in Pennsylvania. It should be noted that in using LCS or QCS for estimating MU, the method assumes a single measurement and, therefore, 1 is used as the square root of n. The same assumption should be made when analyzing a single sample where the number of calibrators used for reporting a single quantification result has 11 data points $n = 11$ with 10 d.f. ($k = 2.3$) instead of a larger "n" involving the use of LCS or control charting. From the estimated standard deviation of the LCS mean, the estimated standard uncertainty (u) was calculated ($u = s$ divided by the square root of $n = 1$). The combined standard uncertainty is the same as the estimated standard uncertainty. Estimated expanded uncertainty (U) was obtained as the product of combined standard uncertainty (u_c) and the coverage factor (k). Coverage factor ($k = 2$) in this case (d.f. = 55) denotes a 95% level of confidence in the result. The measurement uncertainty estimate is updated after each analysis. For a single sample analysis, individual determinations are performed in triplicate using duplicate (pre-sample and post-sample) calibrators and controls. Under such a situation, MU calculated by LCS or QCS is still valid and adequate for reporting the result for a single sample analysis.

3. Results

3.1. Identification of flunixin and clonixin

The diagnostic ions for the identification of flunixin were m/z 297 $[M + H]^+$, 279 (BP), 264, 259 (Fig. 3; top panel). All qualifying ions for flunixin were present in the full scan MS/MS spectrum. The retention time for flunixin was 1.60 ± 0.15 min (Fig. 4; top panel). The qualifying ions for IS were m/z 263 $[M + H]^+$, 245 (BP) and 210 (Fig. 3, bottom panel). All qualifying ions for IS were present in the full scan MS/MS spectrum, and the retention time for IS was 1.45 ± 0.15 min (Fig. 4; bottom panel). The limit of quantification of flunixin in equine plasma was 1.0 ng/ml.

3.2. Quantification of flunixin

3.2.1. Extraction of flunixin from equine plasma

At pH range of 3.0–5.0, the extraction efficiency of flunixin from plasma was >80% by 1-chlorobutane. As a result of this evaluation, pH 3.30 was used for the extraction of flunixin from equine plasma. The results presented in Table 1 showed that the extraction efficiency of flunixin from plasma by 1-chlorobutane was superior to that of either dichloromethane or petroleum ether for which reason 1-chlorobutane was chosen for use in the extraction of

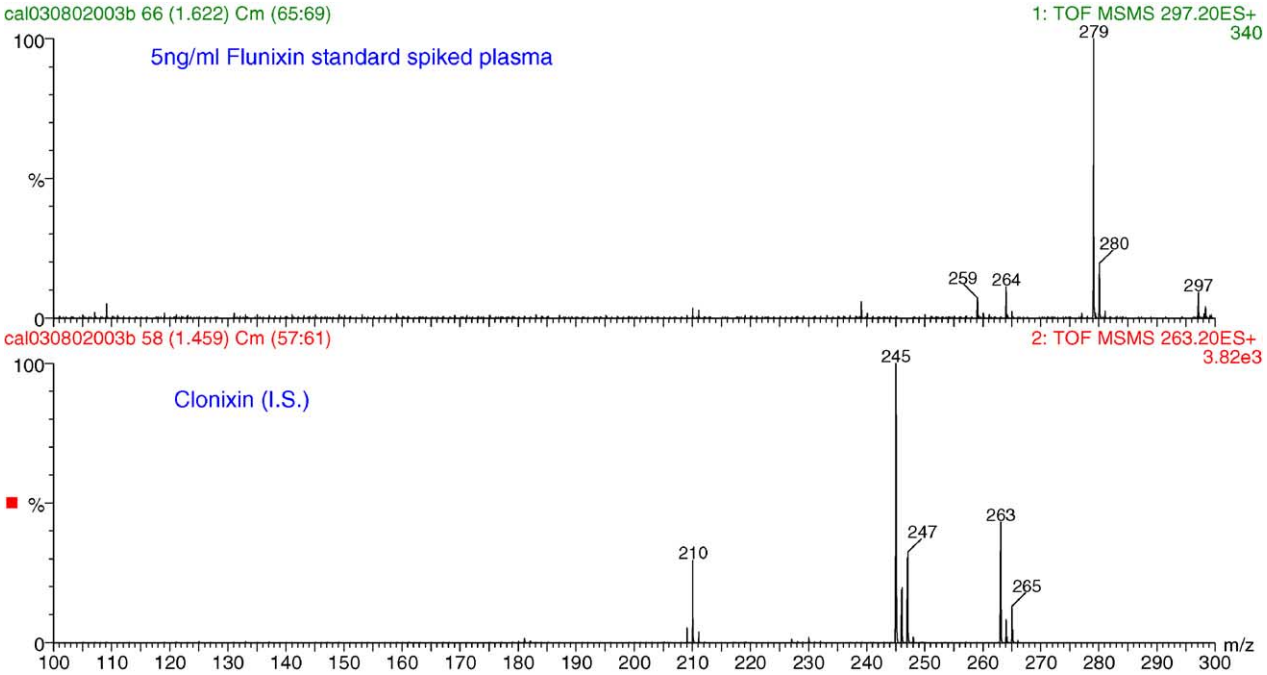


Fig. 3. Mass spectra of flunixin (top panel) and clonixin (IS, bottom panel) in equine plasma.

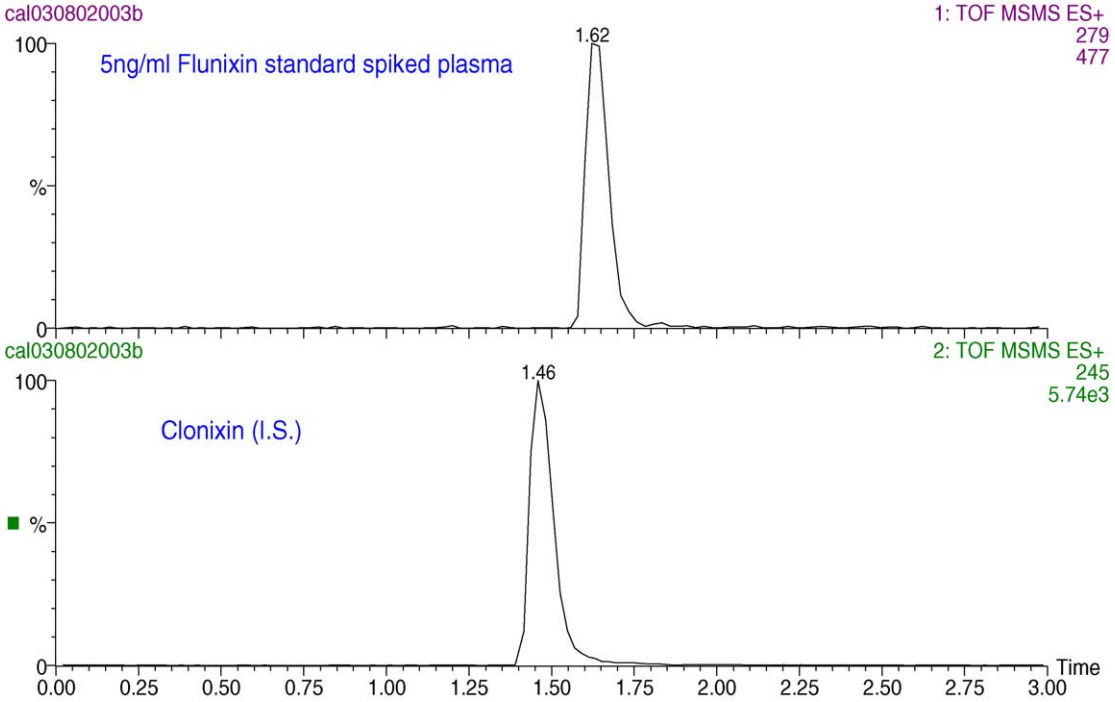


Fig. 4. Chromatograms of flunixin (top panel) and clonixin (bottom panel).

flunixin from plasma in this study. The extraction efficiency of flunixin by 1-chlorobutane was 82.3 ± 2.31% for 50 ng/ml ($n = 3$) and 97.33 ± 0.44% for 500 ng/ml ($n = 3$).

3.2.2. Calibration curve for flunixin and validation of method

Linearity of the method for quantification of flunixin was investigated over a concentration range of 1.0–150 ng/ml using flunixin-supplemented equine plasma samples. The plasma sample had previously been screened by ELISA and found to be flunixin-free. The calibration curve had a correlation coefficient of 0.999 (r^2) where $y = -1.22212e5 \times x^2 + 0.0141819 \times x + -0.00156850$; weighting ($1/x$) over the range of concentrations investigated. Reliable limit of detection (LOD) for flunixin was defined as the concentration at which the analyte produced a chromatographic peak with a signal-to-noise ratio (S/N) of >3 and the limit of quantification was defined as the lowest concentration at which the analyte can be quantitated with a precision and accuracy of greater than 15%. Thus, the LOQ for flunixin was 1.0 ng/ml. A summary of the intra-day and inter-day precision and accuracy data generated for the validation assay is presented in Table 2. The LOQ of 1.0 ng/ml met the acceptance criteria with an intra-day precision of 6.02% and an accuracy of 96.30%. For the inter-day determination, the precision for 1 ng/ml flunixin was 7.56% with an accuracy of 97.90%. Based on a S/N of >3, the LOD was 0.1 ng/ml in equine plasma. The precision and accuracy determined for the mid- and high concentrations of flunixin in plasma samples were also acceptable. Fig. 2 shows the stability of flunixin concentrations in plasma when stored at room temperature (top panel), −70 °C and during repeated freeze-thaw cycles (bottom panel). The various concentrations of flunixin in plasma were not diminished during storage at both room temperature and −70 °C. Thus, flunixin is stable during storage at room temperature for 25 h of observation and at −70 °C or repeated freeze-thaw cycles for at least 40 days, irrespective of the concentration.

Table 1
Extraction efficiency of flunixin by different solvents ($n = 3$)

Concentration of spiked (ng/ml)	Extraction efficiency (%)		
	1-Chlorobutane	Petroleum ether	Dichloromethane
5	55.00 ± 0.00	0.00	52.67 ± 1.15
50	82.33 ± 2.31	15.00 ± 1.00	71.67 ± 0.58
500	97.33 ± 0.44	25.33 ± 1.15	77.00 ± 0.58

3.2.3. Demonstration of the absence of ionization suppression or enhancement effects

Suppression or enhancement of ionization due to matrix effect on the ion intensity of flunixin was evaluated and the results are shown in Fig. 5a and b and that of clonixin as the internal standard in Fig. 6a and b. Fig. 5a shows the response obtained from the rate of infusion use during the determination (top panel) while the bottom panel shows the response from the 10 ng/ml threshold flunixin calibrator. Fig. 5b represents four randomly chosen blank plasma samples. Fig. 6a (top panel) shows the response for clonixin infusion rate whereas the bottom panel shows that of the internal standard (clonixin). Similar evaluations were performed on four

Table 2
Accuracy and precision of flunixin spiked into equine plasma samples ($n = 7$)

Flunixin added (ng/ml)	Intra-day			Inter-day		
	Flunixin detected (ng/ml)	CV[a] (%)	AR[b] (%)	Flunixin detected (ng/ml)	CV[a] (%)	AR[b] (%)
1.0	0.963 ± 0.058	6.02	96.30	0.979 ± 0.074	7.56	97.90
10	9.85 ± 0.645	6.54	98.50	9.87 ± 0.559	5.66	98.70
100	98.95 ± 0.645	4.55	98.95	98.49 ± 4.594	4.66	98.50

[a] Coefficient of variation (CV, %) = standard deviation of the concentration detected/mean concentration detected × 100.
[b] Accuracy (AR, %) = mean detected concentration/spiked concentration × 100.

randomly chosen blank plasma samples and the results are shown in Fig. 6b that demonstrated the absence of either relevant suppression or enhancement of clonixin ion intensity at the retention time of 1.46 min. Taken together, the results of these experiments demonstrated the absence of ionization suppression or enhancement in the retention time ranges of the compounds of interest (flunixin in Fig. 5a and clonixin in Fig. 6a) relevant to the experimental procedure described for flunixin by this method.

3.3. Determination of the presence of flunixin in racehorse samples-test of specificity

Equine plasma samples that showed presumptive presence of flunixin (>10 ng/ml) by ELISA were subjected to analysis for quantification and confirmation of the presence of flunixin by this method. For these samples to be declared positive, the following criteria were met. The accuracy of the concentration of flunixin in the QC samples should be

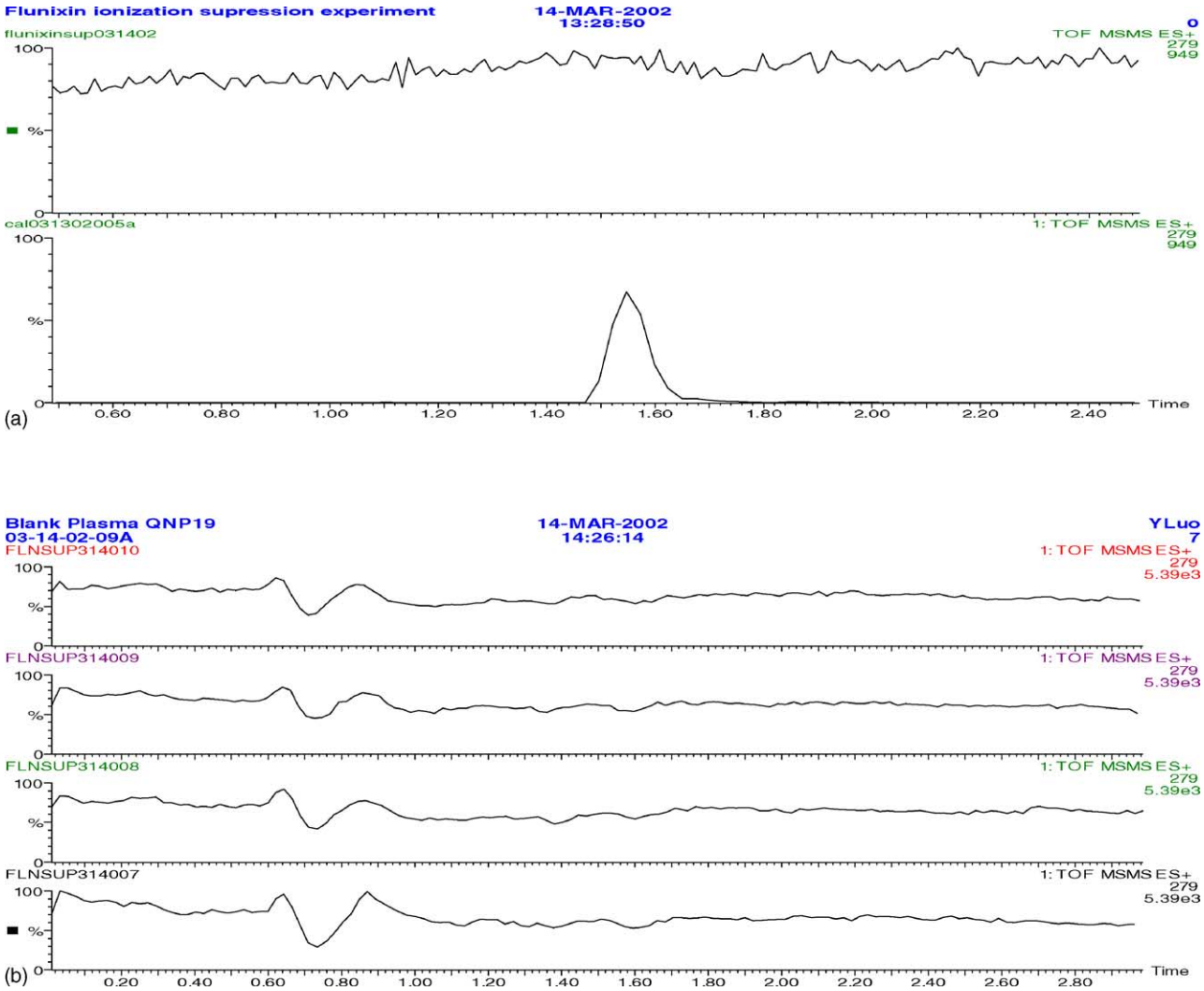


Fig. 5. (a) Flunixin infusion rate (top panel) approximating the 10 ng/ml threshold flunixin calibrator response (bottom panel). (b) Four successive different random blank equine plasma samples showing absence of either ionization suppression or enhancement at the retention time of flunixin.

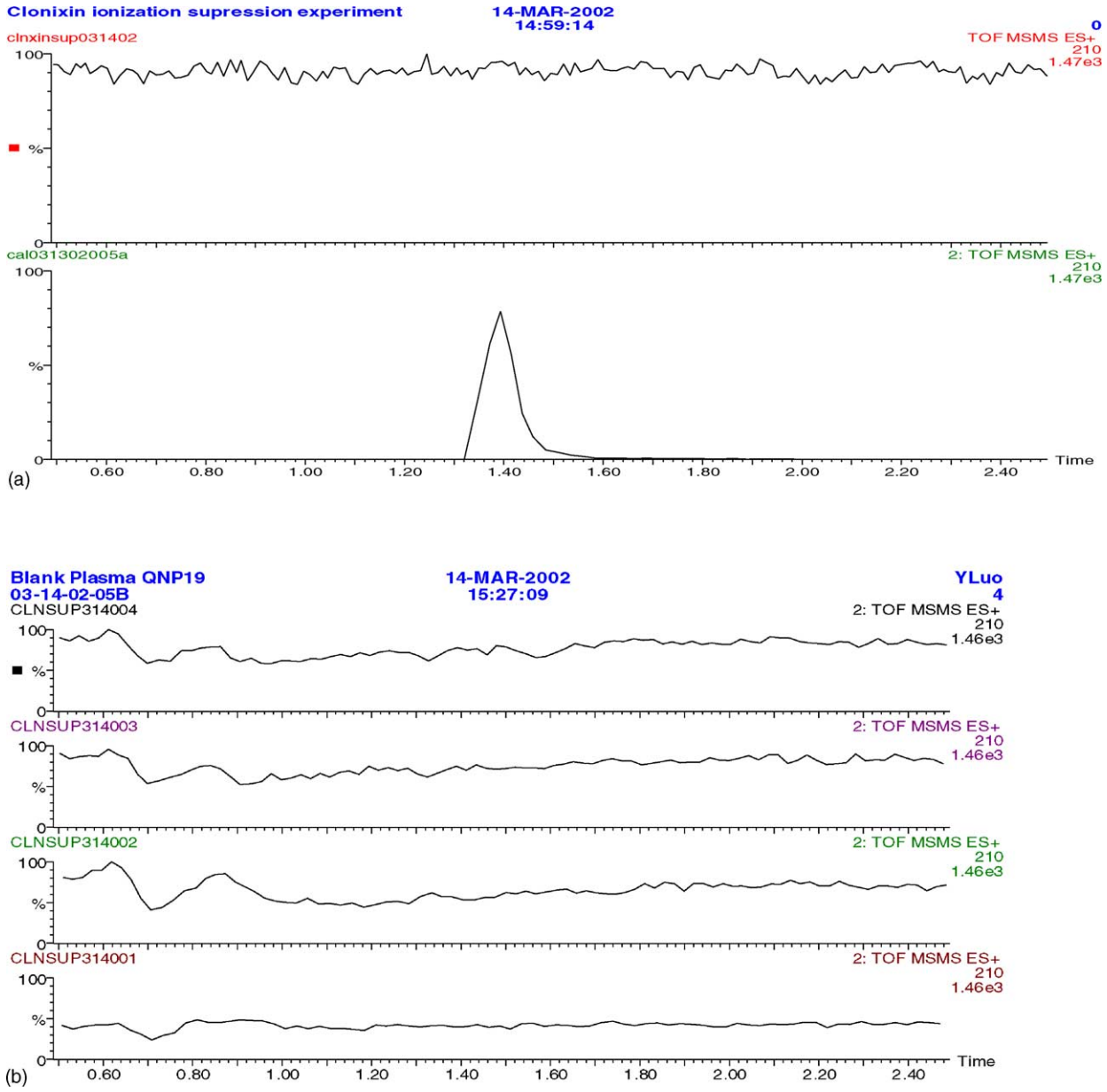
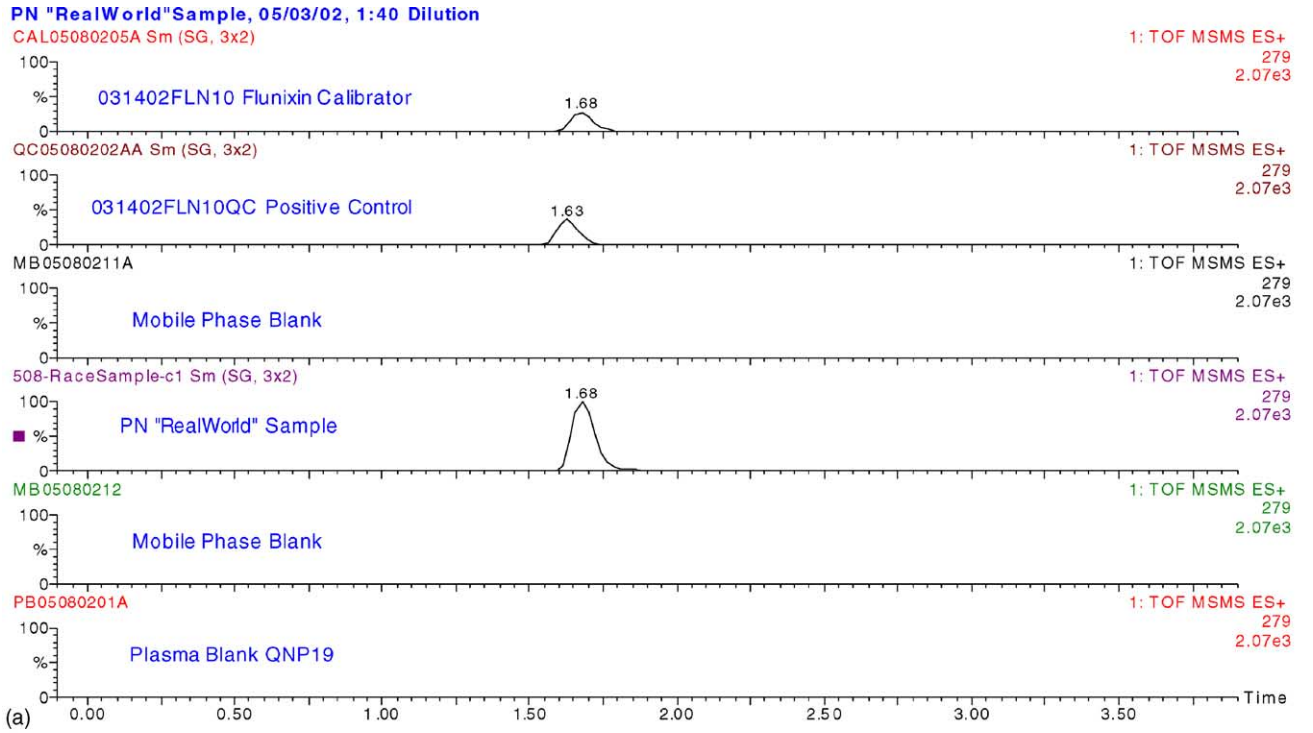


Fig. 6. (a) Clonixin infusion rate (top panel) approximating the internal standard clonixin (bottom panel). (b) Four successive different random blank equine plasma samples showing absence of either relevant suppression or enhancement at the retention time of clonixin.

80–120%. The sample must contain flunixin at a concentration greater than the established tolerance threshold of 10 ng/ml if: (a) The lower 95% confidence limit for the mean of the determined concentrations of the suspect sample ($n = 3$) was greater than the upper 95% confidence limit for the mean of all 10 ng/ml calibrators and 10 ng/ml QC samples ($n = 4$). (b) The LC retention times of the quantifying ion for flunixin in the sample, 10 ng/ml QC sample and the 10 ng/ml calibrators are within ±0.15 min. This requirement was met by inspection of the extracted ion chromatogram comparisons. The signal to noise ratio of the quantifying ions for flunixin and IS is greater than 20. Again, this was satisfied by inspection of the extracted ion chromatograms of the full scan MS/MS spectra. These spectra were averaged across the chromatographic peak at 20% peak height. (c) The MS/MS full scan spectra do not contain other mass spectral peaks greater than 25% of the quantifying ions for flunixin. This was determined by comparing the mass spectrum of the sample, standard, and positive control. The presence of such peaks indicated the possibility that the value determined for the integration of the quantifying ion chromatograms was skewed due to the presence of unknown co-eluting substances. (d) All negative samples demonstrated the absence of quantifiable flunixin concentration. The result of positive findings for the presence of flunixin in a “real world” sample is shown in Fig. 7a and b.



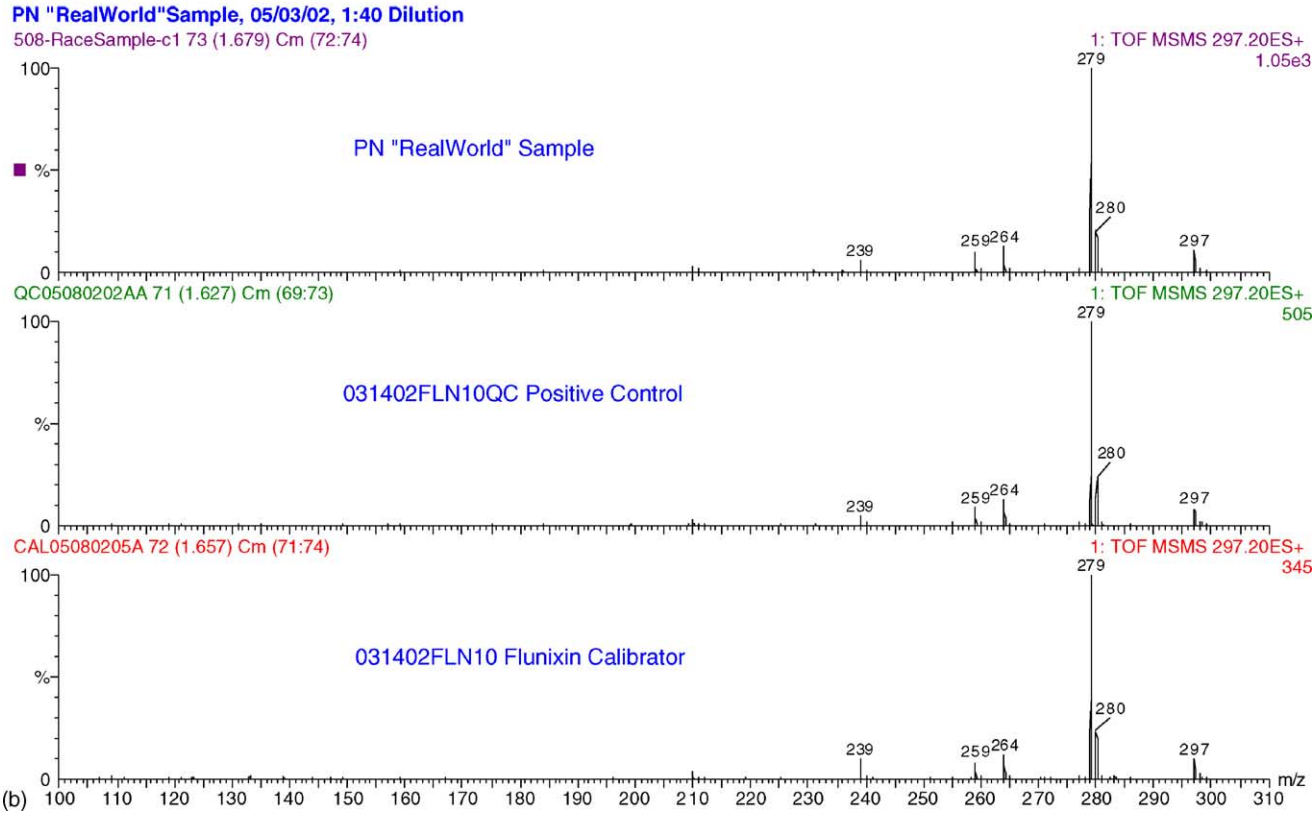


Fig. 7. (a) Selected ion chromatogram of flunixin in racehorse plasma samples. (b) Mass spectra of flunixin in equine plasma samples. The upper panel represents mass spectrum of flunixin in a plasma sample collected from a racehorse. The middle panel is the mass spectrum of a positive control (PC) plasma sample. The bottom panel is that of a flunixin standard spiked into a blank equine plasma sample.

Table 3
Measurement uncertainty budget by LCS or charting control

Symbol	Source of uncertainty	Value units (%)	Distribution	Divisor	Standard uncertainty	Degrees of freedom ($n-1$)	Other
U_1	Intermediate precision	4.33	N	1	433	55	Flunixin 10 ng/ml
Combined uncertainty	$(U_1^2)^{1/2} = 4.33$						
Expanded uncertainty ($k = 2$)	$(4.33 \times 2) = 8.7\%$ (result = ng/ml ± [ng/ml × 8.7%])						

Table 3 represents the uncertainty budget. The mean of the intermediate precision was 98.5% ($n = 56$) and the standard deviation was 4.33. Again, in comparing the estimated MU by LCS method with that of a single sample analysis the mean for a single analysis was 98.2% ($n = 11$) and the standard deviation was 4.25 suggesting that the two methods of estimation were very close (±0.87 ng/ml versus ± 0.95 ng/ml). Standard Uncertainty was the same as the standard deviation because the square root of "n" in the case of using LCS for estimating MU was taken as 1 and, therefore, the standard deviation (4.33) was divided by 1. The degrees of freedom was 55 ($n - 1$) for LCS and 10 for single sample analysis (Table 3). Table 4 shows the recovery of flunixin and clonixin (IS) from equine plasma.

4. Discussion

Previous studies [7] showed that following an intravenous administration of 1.10 mg/kg flunixin to horses, flunixin and the 5-hydroxylated metabolite were detected up to 175 and 54 h, respectively. When 1.10 mg/kg flunixin was administered in five consecutive daily doses by the intramuscular or intravenous route, flunixin was detected in urine for as long as 15 days [4].

Flunixin is rapidly excreted in urine over 2–4 h. At a dose of 2.2 mg/kg, the α, β, and λ half lives ($t_{1/2}$) of flunixin were 0.61, 1.5, and 6.0 h, respectively [4]. With a lower dose of 1.1 mg/kg the third compartment could not be well defined due to the limited LOQ of HPLC method of quantification [4]. Administration of flunixin by intramuscular route prolonged its detection in plasma due to the slower absorption [24,25].

Previous studies in which flunixin concentration in urine was reported using HPLC and/or GC/MS indicated limits of detection of 50 ng/ml by HPLC and of 5 ng/ml by GC/MS [11,7,24]. From the results obtained, it can be noted that these methods (HPLC and GC/MS) are limited by low sensitivity. Unlike GC/MS and HPLC, LC/Q-TOF/MS/MS provides enhancement in sensitivity, with LOD of 0.10 ng/ml that is nearly 50 times more sensitive for flunixin in plasma than that by GC/MS or HPLC [24]. Other investigators [12–14] using LC/MS/MS have demonstrated increased sensitivity in the determination of flunixin and 5-hydroxyflunixin in milk and meat products. Although different matrices (milk versus plasma) were involved, the LOD (0.1 ng/ml) and LOQ (1.0 ng/ml) results of this study in plasma compared favorably with those in bovine milk [12], suggesting that the limiting factors in improved sensitivity are the extent of analyte recovery and instrumentation (LC/MS/MS versus GC/MS). Thus, the increased sensitivity resulting in the improved limit of detection and quantification of flunixin in plasma would help in the regulation of the use of flunixin in racehorses. Determination of analyte stability in plasma is of particular interest to the primary laboratory and the secondary laboratory for independent analysis of the split sample. Test samples are stored at −20 °C at the racetracks prior to shipment to the laboratory. However, presumptive positive plasma samples are stored at −70 °C in the laboratory prior to performing quantitative analysis. It should be noted that all blood samples are centrifuged and plasma collected and stored (−20 °C) at the racetracks prior to shipment to the laboratory. The split samples are stored at −20 °C under the custody of the Commission at the racetrack. Since it takes an average of 2–3 days for the laboratory to receive samples from the racetracks in PA, it is important to separate red blood cells from plasma to avoid hemolysis and thus, preserve the quality of the sample shipped to the laboratory.

Conformance to the rules and regulations of racing required by the regulators for the use of flunixin in racehorse is based on strict adherence to the dose, time and a specified tolerance concentration or acceptance limit of the measurand in a post-race plasma sample. For this reason, there are narrow limits on which administrative decisions on tolerance are made from the quantitative results and, therefore, MU about the result must also be reported. Every measurement is subject to some uncertainty or doubt about the result of the measurement [21,23]. MU is an attempt to quantify the extent of the uncertainty or doubt that exists about the measurement result relative to the "known" or "true value"

Table 4
Recovery of flunixin and clonixin from equine plasma ($n = 6$)

Name of drugs	Spiked concentration (ng/ml)	Determined concentration (ng/ml)	Recovery (%)	CV (%)
Flunixin	1	1.054 ± 0.017	105.40 ± 1.70	1.61
	10	10.50 ± 0.39	104.95 ± 3.89	3.71
	100	100.52 ± 3.00	100.52 ± 3.00	2.98
Clonixn	1	0.56 ± 0.040	55.88 ± 4.02	7.19
	10	8.28 ± 0.36	82.76 ± 3.56	4.30
	100	99.32 ± 3.16	99.32 ± 3.16	3.18

of the measurand [23]. When a quantitative measurement is made using a specific method developed for the purpose of providing quantitative results, information about the uncertainty of the result must be treated as an integral part of the final result of the measurand. A measurement result is considered to be complete only when it is accompanied by a statement of the uncertainty surrounding the result of the measurand [21]. For any measurement to be adequately evaluated, the fitness of purpose of the measurement must be stated [21] so that meaningful interpretation of the result can be made. To avoid any confusion and the temptation of using statistics and uncertainty analysis interchangeably, it is important to note that statistics is not the same as uncertainty analysis because statistics can be used to draw conclusions that do not provide any information about uncertainty of the measurement [21,23,26]. A given coverage factor (k) represents a particular confidence level in combination with the degrees of freedom, for example, a coverage factor of 2 with 50 d.f. denotes a confidence level of 95%. Thus, coverage factor (k) or t-statistic is based on the degrees of freedom associated with the number of samples tested and a desired level of confidence. MU should be reported as standard uncertainty multiplied by the coverage factor at a desired confidence level [21,23]. The divisor for repeated analysis as in the use of LCS in estimating MU is 1 even though the probability distribution is normal. For other sources of uncertainty where the probability of distribution is normal, triangular or rectangular, the divisor is the square root of 2, 6 or 3, respectively [21,23]. Thus, the use of LCS in estimating measurement of uncertainty is valid, fast, simple and less cumbersome than the extended method of The Guide to the Expression of Uncertainty of Measurement (GUM) [23]. Most importantly, the method of LCS is adequate if it satisfies the client's requirements for acceptable concentration of flunixin in plasma. It is important to emphasize caution and reasonableness in any method used and in interpreting the result because there is nothing that can be considered the "final word" on the uncertainty of a given measurement [21]. By using LCS or QCS in estimating MU for flunixin quantification by this study, it is certain that the method satisfies the needs of the user and that the uncertainty result is correctly reported without misrepresenting its reliability, and is reported with sufficient information to inform the user of the uncertainty and how it was estimated [21,23]. The quantitative result was expressed as the concentration of flunixin (ng/ml) plus or minus the expanded uncertainty (ng/ml).

5. Conclusion

By this method, the LOQ of flunixin in plasma was at least an order of magnitude lower than the tolerance concentration (10 ng/ml) of flunixin in racehorses at race time. The use of LC/Q-TOF-MS/MS greatly improves the sensitivity and increases accuracy and precision in the quantification as well as simultaneous confirmation for the presence of flunixin in equine plasma by full scan tandem-mass spectrometry. The method is rapid, simple and sensitive for qualitative and quantitative determination of flunixin in equine plasma. The method was successfully applied to "real world" samples. With the use of laboratory control sample method, the estimated MU at the 95% confidence level was ±0.87 ng of flunixin/ml of plasma. MU must be reported with the quantitative result of the measurand because it provides information about the extent of doubt expressed about the result of the measurand. Application of this method will assist racing authorities in the enforcement of the decision or tolerance concentration of flunixin in racehorses at race time.

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