ELSEVIER

Journal of Chromatography B, 692 (1997) 187–198

JOURNAL OF CHROMATOGRAPHY B

Determination of flunixin in equine urine and serum by capillary electrophoresis[1]

Xuelin Gu[a], Mona Meleka-Boules[a], Chao-Ling Chen[a,*], Dagmar M. Ceska[b], David M. Tiffany[b]

[a]*Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610-0136, USA*

[b]*Division of Pari-Mutuel Wagering, Department of Business and Professional Regulation of Florida, Tallahassee, FL 32399, USA*

Received 16 January 1996; revised 13 August 1996; accepted 19 August 1996

Abstract

A capillary electrophoresis (CE) and a solid-phase extraction method was developed for the determination of flunixin in equine urine and serum. The suitable CE run conditions were described. The factors affecting flunixin recovery rates were investigated and optimum solid-phase extraction conditions for flunixin in equine urine and serum were established. Limits of detection and quantitation were 3.4 and 5.6 ng/ml for serum and 16.9 and 33.1 ng/ml for urine, respectively. The recoveries exceeded 96% for urine and 79% for serum. Urine samples from race horses and urine and serum samples from a mare administrated with flunixin were analyzed with this procedure.

Keywords: Flunixin

1. Introduction

Flunixin, 3-pyridine-carboxylic acid 2-[(2-methyl-3-trifluoromethyl) phenyl]amine (Fig. 1), is a potent non-steroidal anti-inflammatory drug with analgesic activity [1,2]. Several studies have shown that flunixin can be used effectively to prevent endotoxin-induced prostaglandin mediated changes in experimentally induced endotoxic shock [3,4], in the management of pain in equine colic [5] and as an anti-inflammatory drug for equine musculoskeletal disorder [2,6]. Flunixin is used widely in horses as a therapeutic medicine or as a doping agent [7,8]. Therefore, a simple, rapid, reliable analytical method for determining flunixin in horse urine and serum may help in therapeutic management and for regulation in the racing industry. Gas chromatography (GC) [7–9] and high-performance liquid chromatography (HPLC) [10–14] methods have been developed to analyze flunixin in horse urine or serum. However, the polar acidic group of flunixin must be derivatized to obtain good chromatographic behavior

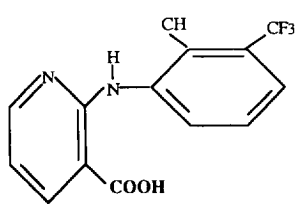

Fig. 1. Molecular structure of flunixin.

*Corresponding author.

[1] Published as College of Veterinary Medicine Journal Series No.

PII S0378-4347(96)00393-3

in GC methods. The low reproducibility and a non-linear standard curve [9] may be caused by the derivatization step of flunixin. Furthermore, both HPLC and GC methods reported to date used liquid–liquid extraction as a sample clean-up method. The liquid–liquid extraction methods are laborious and generally involve large amounts of hazardous organic solvent. In order to overcome the disadvantages of current methods, a capillary electrophoretic (CE) method combined with solid-phase extraction for the determination of flunixin in horse urine and serum was developed and described in this paper.

2. Experimental

2.1. Reagents and materials

Flunixin meglumine reference standard was kindly donated by the Schering-Plough Research Institute (Kenilworth, NJ, USA). Sodium dodecyl sulfate (SDS), sodium borate, glacial acetic acid, sodium acetate, methylene chloride, HPLC grade ethanol and HPLC grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Banamine (flunixin injectable form) was obtained from Schering-Plough Animal Health (Kenilworth, NJ, USA). Bond Elut Certify C_{18} columns were supplied by Varian (Harbor city, CA, USA). Silica capillaries were purchased from J&W Scientific (Folsom, CA, USA)

2.1.1. Solutions

CE run buffer: 75 m*M* SDS, 50 m*M* sodium borate, 2% ethanol, pH 9.2. Flunixin dissolving buffer: 75 m*M* SDS, 50 m*M* acetic acid, 2% ethanol, pH adjusted to 4.5 with 1 *M* NaOH. Sample dilution buffer and Bond Elut Certify column wash buffer: 1 *M* acetic acid, 1 *M* sodium acetate, pH adjusted to 4.5 with 6 *M* HCl.

2.2. Apparatus

The CE system used in this study was a Beckman P/ACE 5510 equipped with a diode-array detector (Beckman Instruments, Fullerton, CA, USA). A Vac Elut Sample processing station (Varian Associates, Sunnyvale, CA, USA) was used to perform sample extraction. A MultiVAP analytical evaporator (Organomation Associates, South Berlin, MA, USA) was used to dry samples.

2.2.1. CE run conditions

The run buffer contained 75 m*M* SDS, 50 m*M* sodium borate, 2% ethanol with pH 9.2. An uncoated silica capillary tubing (57 cm total length (50 cm to detecting aperture)×75 μm I.D., 375 μm O.D. was used. Voltage was set at 20 kV and the temperature at 23°C. UV absorbance was measured at 286 nm and scan range was set from 200 to 400 nm. The capillary was rinsed with run buffer for 2 min before the sample injection. The sample was injected for 10 s with low pressure (0.5 p.s.i. (1 p.s.i.=6894.76 Pa)). The run time was 20 min and the capillary was post-rinsed with 0.01 *M* sodium hydroxide solution and water for 2 min each. The capillary was regenerated by rinsing with 1 *M* HCl for 5 min, 0.01 *M* NaOH for 10 min and water for 5 min before the first run daily.

2.3. Analysis of free flunixin in horse urine and serum

A 2 ml sample of horse urine or serum was diluted with 4 ml of sample dilution buffer and then applied to a Bond Elut Certify C_{18} column preconditioned with 5 ml of ethanol and 10 ml of water. The sample was passed through the column by vacuum, and then the column was washed with 5 ml of wash buffer, followed with 1 ml of water. The column was dried under full vacuum (15 p.s.i.) for 2 min and tip of the Vac Elut delivery needle was wiped. Finally, flunixin was eluted from the column with 5 ml of methylene chloride. The eluate was evaporated at 56°C water bath and the residue was redissolved in 0.5 ml of ethanol. The ethanol was removed with the multiple evaporator under a gentle stream of nitrogen gas. The residue was reconstituted in the flunixin dissolving buffer, 75 m*M* SDS, 50 m*M* acetic acid, 2% ethanol, pH 4.5 before CE analysis.

2.4. Analysis of free and conjugate flunixin in horse urine

The urine sample was hydrolyzed according to the method of Craig et al. [15]. A 2-ml volume of horse urine was mixed with 400 μl of 1 *M* NaOH and

incubated at 63°C for 4 min. The pH was adjusted to 3–4 by adding 120 μl of 6 *M* HCl. A 4-ml volume of acetic acid buffer was added and the sample was extracted and analyzed as described above (Section 2.3).

2.5. Peak identification

The unknown peak in the samples was preliminarily identified by comparing CE migration time with flunixin standard and then the sample extract was co-injected with the standard (sample solution was injected first, and then flunixin standard solution was injected within the same run) to further identify an unknown peak. The peak identity was confirmed by comparing its UV spectrum with that of flunixin standard.

2.6. Quantitative analysis

The concentrations of flunixin in samples were calculated based on the peak area from a standard curve which was established monthly and correlated daily by 5, 20, 50 μg/ml flunixin standards. A stock solution of flunixin standard was prepared at the concentrations of 100 μg/ml, aliquoted and stored at −20°C. The different concentrations of standards were prepared freshly by diluting the stock solution with the dissolving buffer (75 m*M* SDS, 50 m*M* acetic acid, 2% ethanol, pH 4.5).

2.7. Determination of recovery

Blank horse urine and horse serum were fortified with flunixin standards to final concentrations of 50, 100, 250 and 500 ng/ml, respectively. Samples of 2 ml were extracted and analyzed as descibed previously. The amounts of flunixin in urine or serum were calculated using the area under the curve as described in Section 2.6. The recovery and standard deviation of recovery were then calculated from the amounts of flunixin added.

2.8. Flunixin administration trial

A 500-mg amount of flunixin (Banamine, 50 mg/ml) was injected intramuscularly to an exercise-conditioned Thoroughbred mare of approximately 500 kg body weight. Urine and blood were collected at 2, 4, 6, 8, 24 and 48 h. Urine was collected by free catch, since the mare was trained to urinate on command by a signal from its handler. Urine samples were placed in 250 ml polypropylene bottles with plastic lids and frozen at −20°C until assayed. The blood samples were collected in glass Vacutainer tubes, allowed to clot overnight at 4°C and were then centrifuged at 2800 *g* for 20 min to separate serum. The serum samples were stored at −20°C freezer until assayed. Blank urine and blood were collected before administration of flunixin.

2.9. Urine samples of racing horses

Ten urine samples of racing horses were obtained from the Division of Pari-mutuel Wagering, Department of Business and Professional Regulation of Florida. The samples were preliminarily determined to be flunixin positive by enzyme immunoassay screening procedure. Urine samples were extracted before and after sodium hydroxide hydrolysis.

3. Results and discussion

3.1. CE separation

The peak shape of flunixin was affected by the pH of buffer in which flunixin was dissolved. A very flat and wide peak was observed when flunixin was dissolved in CE run buffer of 75 m*M* SDS, 2% ethanol, 50 m*M* sodium borate, pH 9.2 (Fig. 2A). On the other hand, a very sharp peak was obtained when flunixin was dissolved in the acidic buffer of 75 m*M* SDS, 2% ethanol, 50 m*M* acetic acid, pH 4.5 (Fig. 2B). A sharp and narrow peak is desirable because it not only enhanced sensitivity of analysis, but also increased the efficiency of separation. Since the mechanism of CE separation is very complicated, we have no explanation for this electrophoretic behavior.

The electrophoregrams of flunixin standard, extract of blank horse urine and blank horse urine fortified with flunixin standard to final concentration of 100 ng/ml were shown in Fig. 3. Although many endogeneous compounds were co-extracted with flunixin from horse urine (Fig. 3B), flunixin was separated from major endogenous compound peaks

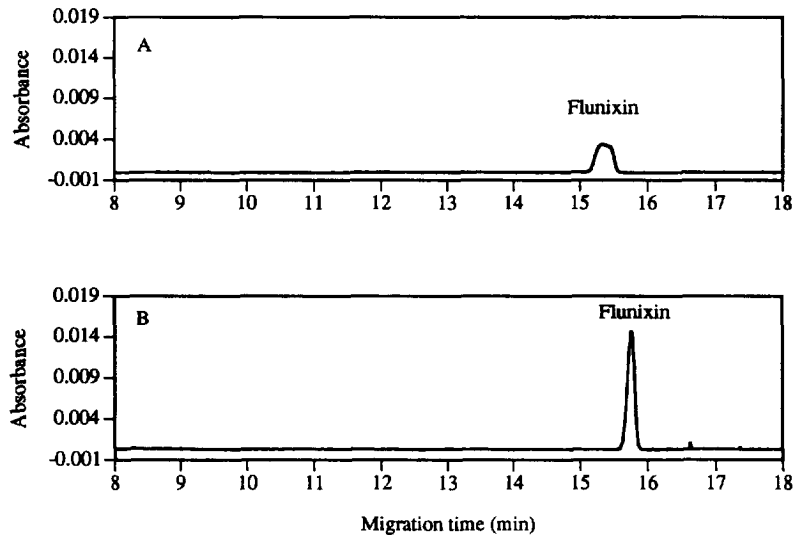

Fig. 2. Electrophoregrams of flunixin standard dissolved in different buffers. (A) 75 m*M* SDS, 50 m*M* sodium borate, 2% ethanol, pH 9.2; (B) 75 m*M* SDS, 50 m*M* acetic acid, 2% ethanol, pH 4.5.

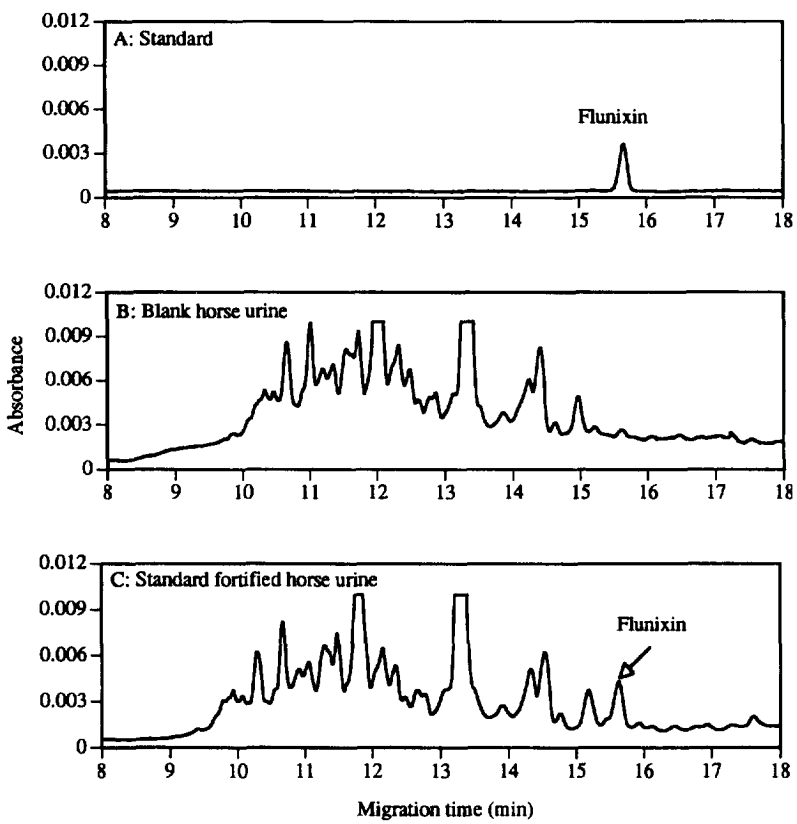

Fig. 3. Electrophoregrams of extracts of horse urine. (A) 40 μg/ml of flunixin standard; (B) 50 μl of extract from 2 ml of blank horse urine; and (C) 50 μl of extract from 2 ml of blank horse urine fortified with 200 ng of flunixin.

(Fig. 3C). However, a small endogenous compound peak could not be completely separated from the flunixin peak (Fig. 3B,C). This peak interfered with the detection of flunixin at low concentration level. That explains why the limits of detection and quantification of flunixin are higher in urine samples than in serum samples. Much less endogeneous compounds were co-extracted with flunixin from horse serum (Fig. 4) as compared to horse urine (Fig. 3). The separation of flunixin from co-extracted endogenous compounds from serum is far easier.

3.2. Recovery

Flunixin is a weak acidic compound (Fig. 1) with an apparent acid dissociation constant about 1.5×10^{-6} [8]. Therefore, acidic condition is superior to the flunixin in binding to the reversed-phase C_{18} column. At first, we used 0.01 *M* HCl solution to acidify horse urine and serum samples. However, too many matrix compounds were co-extracted with flunixin from horse urine and serum. This resulted in difficulty in the dissolving the final extraction residue in 50 μl of CE dissolving buffer. A weaker acid with a pK_a lower than that of flunixin was then used. The weaker acid created more selective extraction conditions for flunixin. Thus, we used acetic acid buffer (pH 4.5) to acidify and to dilute urine and serum samples before they were applied to Bond Elut Certify C_{18} column. It showed some selectivity for flunixin extraction. However, the recovery of flunixin depends on the amount of the acid added. A 2 ml sample of flunixin fortified horse urine and serum samples (100 ng/ml) was diluted with 4 ml of 0.5, 1, 2 *M* acetic acid buffer and were extracted with Bond Elut Certify C_{18} column as described previously (Section 2.3). The recovery rate of flunixin in serum was increased rapidly with increasing the concentration of acetic acid from 0.5 *M* to 1 *M*. Acetic acid buffer 1 *M*, was sufficient to obtain high recovery of

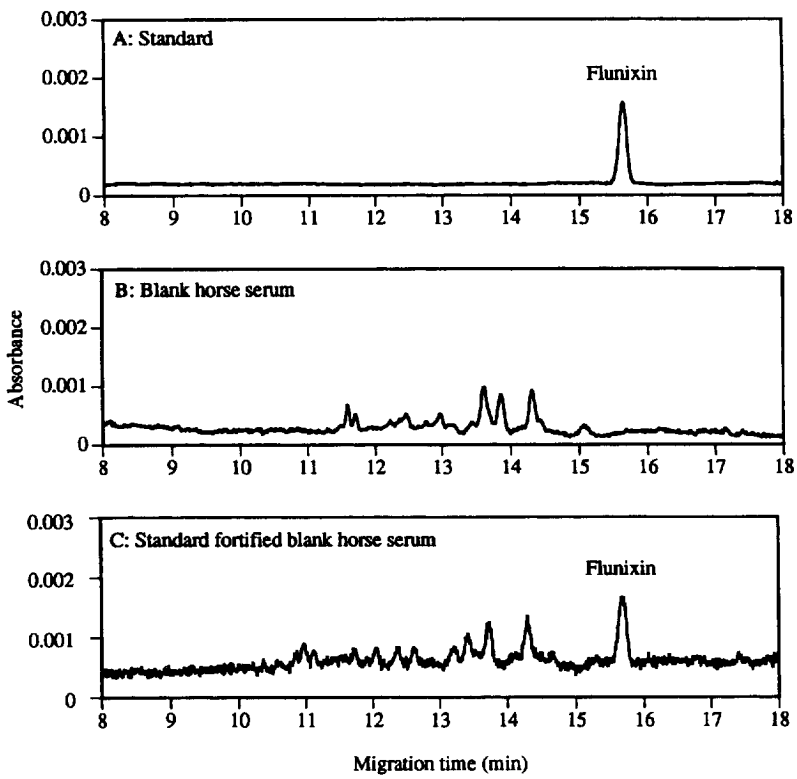

Fig. 4. Electrophoregrams of extracts of horse sera. (A) 20 μg/ml of flunixin standard; (B) 50 μl of extract from 2 ml of blank horse serum; and (C) 50 μl of extract from 2 ml of blank horse serum fortified with 100 ng of flunixin.

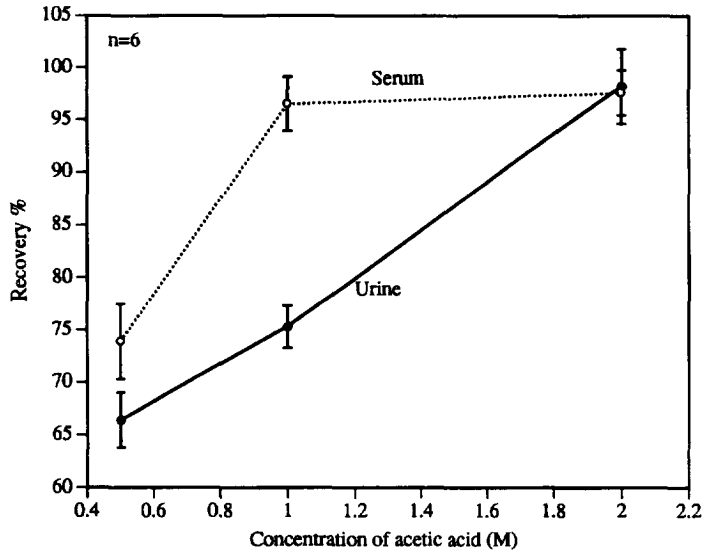

Fig. 5. Effect of the concentration of acetic acid buffer on the binding of flunixin to the Bond Elut Certify C_{18} column in horse urine and serum. Vertical bars represent the standard errors of means.

flunixin from horse serum using Bond Elut Certify C_{18} column (Fig. 5). However, 2 *M* acetic acid buffer was needed to obtain high recovery from horse urine (Fig. 5). For convenience of buffer preparation, 2 *M* acetic acid buffer was used for diluting both urine and serum samples in this study.

After extraction of urine and serum samples, the column was washed with 5 ml of 2 *M* acetic acid buffer to remove unbound materials. The salts trapped in the column were removed before elution, because salts adversely affect the migration behavior of the compounds within the CE capillary. A 1-ml volume of water was used to remove the trapped salts. On the other hand, using a relatively large volume of water, reduced the recovery rate of flunixin.

The use of methylene chloride for elution of flunixin from the Bond Elut Certify C_{18} column produced urine and serum extracts that contained less co-extracted compounds than the use of methanol or ethanol. However, some white powders remained on the inter-wall of tube after removing methylene chloride which resulted in low recovery of flunixin, especially for serum samples. This problem was overcome by redissolving residues of urine or serum extracts in 0.5 ml of ethanol. After removing ethanol by evaporation, the residues were reconstituted with 50 μl of 75 m*M* SDS, 50 m*M* acetic acid, 2% ethanol, pH 4.5 for CE analysis.

Using this extraction procedure, the recovery rate of flunixin in fortified horse urine and serum exceeded 94% for all levels of fortified samples except in serum fortified with 50 ng/ml of flunixin (Table 1).

3.3. Reproducibilities of quantitation and migration times data

The qualitative and quantitive reproducibilies of CE are still problematic in some cases. The binding of matrix proteins onto the wall of uncoated silica capillary usually causes migration time change from run to run. Recently, Wätzig and Lloyd [16] discovered that SDS wash solution is much more effective to remove protein from the capillary than sodium hydroxide solution. They observed very consistent migration times by washing capillary with 50 m*M* SDS solution between runs even with direct injection of plasma samples. In our experiment, most of the proteins were removed from horse urine and serum samples in pretreatment step and the run buffer of 75 m*M* SDS at pH 9.2 also prevented protein from binding to the capillary wall. Therefore, the contamination of capillary was minimized or eliminated

Table 1
Recovery and coefficient of variation of flunixin in horse urine and serum

	Fortified sample concentration (ng/ml)	n	Recovery (%)	Coefficient of variation (%)
Urine	50	5	97.7	4.08
	100	5	98.2	3.58
	250	5	101.0	4.44
	500	6	96.1	6.03
Serum	50	5	79.3	8.80
	100	6	97.6	4.83
	250	5	94.3	6.04
	500	6	96.0	2.39

using our experimental conditions. Consistent migration times of flunixin standards were achieved (the coefficient variations of migration times were 1.31% in day 1 and 1.20% in day 2).

In pressure injection mode, the P/ACE 5510 system calculates a (pressure×time) value, using 0.5 p.s.i. for the amount of pressure and time value entered. The viscosity of sample, the temperature setting and the amount of fluid present in the exit vial affect the sample injection. Less than 5% coefficient of variation of quantitation was achieved since all the above factors remained constant in our study.

3.4. Limits of detection and quantitation

Flunixin was detected at maximum absorbance wavelength of 286 nm. The baselines from 6 separate blank horse urine and serum samples were measured at wavelength of 286 nm. From these blank baselines, mean backgrounds and standard deviations (S.D.) of backgrounds were calculated. Using the environmental measurement guideline of the American Chemical Society [17], the limits of detection (LOD=mean background+3 S.D.) and the limits of quantitation (LOQ=mean background+10 S.D.) of flunixin in horse urine and serum were calculated and are summarized in Table 2.

3.5. Calibration curve

The linear regression equation of peak area and concentration of flunixin was $y=0.09286x-0.0507$. A high correlation coefficient ($r^2=0.999$) was obtained for flunixin standards ranging from 1 to 80 μg/ml (Fig. 6). Since 2 ml of horse urine and serum samples were extracted and were subsequently concentrated to the final volume of 50 μl, this standard range would be equivalent to that of 25 ng to 2 μg/ml of flunixin in horse urine and serum. However, when flunixin concentration exceeded 2 μg/ml in samples or 80 μg/ml in final concentrated solution, the standard curve was no longer linear (Fig. 6). Therefore, the samples need to be diluted before they can be measured when flunixin concentrations in samples are higher than 2 μg/ml.

3.6. Analysis of flunixin in racing horse urine and serum

Ten urine samples from racing horses, that were preliminarily determined to be flunixin positive by

Table 2
Limit of detection (LOD) and quantification (LOQ) of flunixin ($n=6$)

	Background[a] (ng/ml)	Standard deviation (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)
Urine	9.99	2.31	16.9	33.1
Serum	2.4	0.317	3.35	5.57

[a] The baselines of blank horse urine or serum samples were converted into the concentration of flunixin in horse urine or serum samples at described experimental conditions (see Section 2).

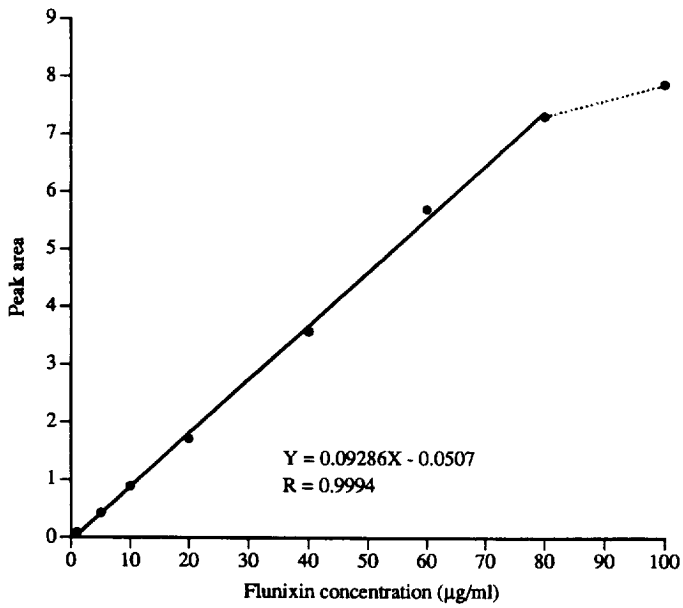

Fig. 6. Calibration curve of flunixin.

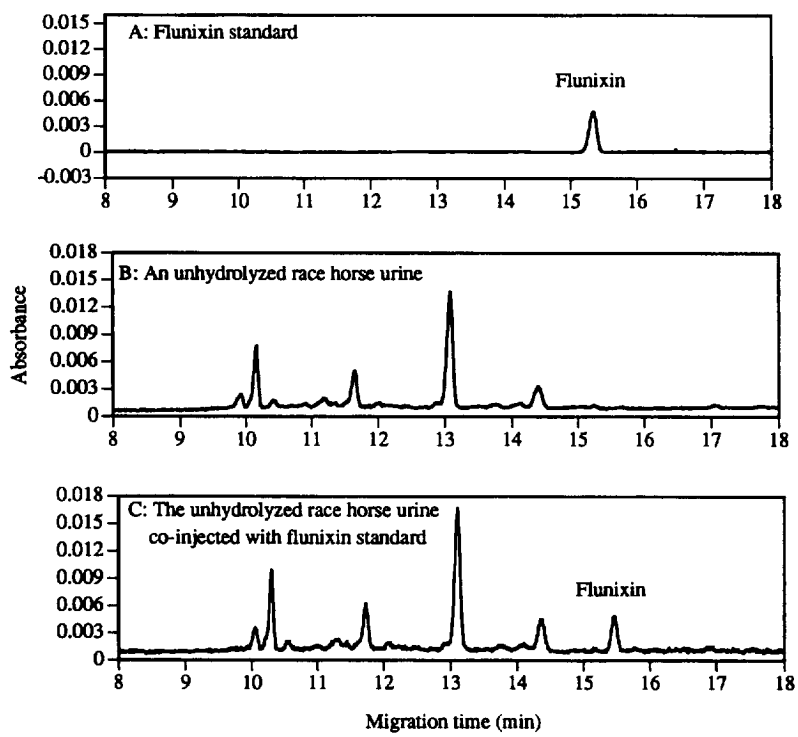

Fig. 7. Electrophoregrams of extract of unhydrolyzed race horse urine. (A) 40 μg/ml of flunixin standard; (B) 50 μl of extract from 2 ml of unhydrolyzed race horse urine; and (C) the extract of unhydrolyzed race horse urine co-injected with 40 μg/ml of flunixin standard.

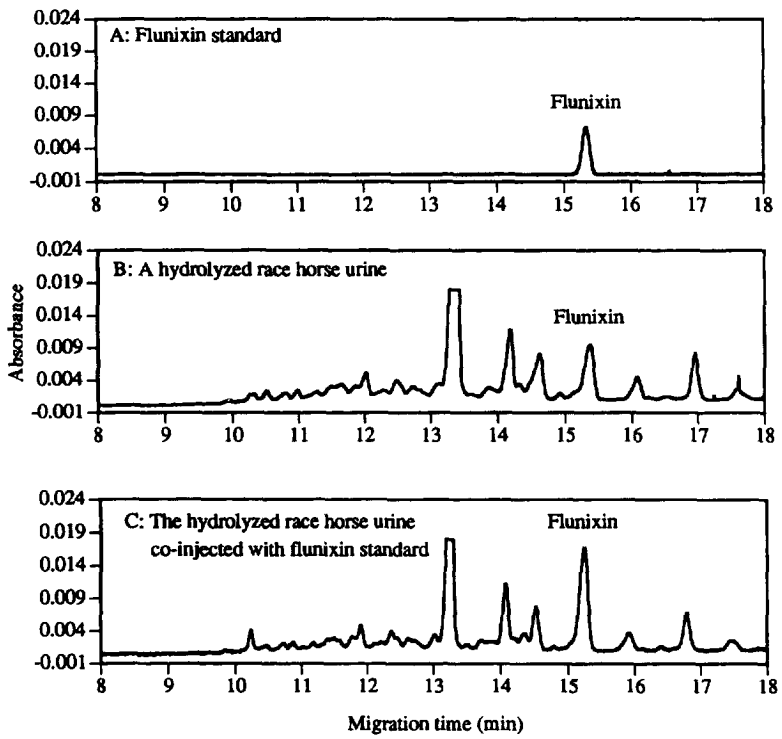

Fig. 8. Electrophoregrams of extract of hydrolyzed race horse urine. (A) 40 μg/ml of flunixin standard; (B) 50 μl of extract from 2 ml of hydrolyzed race horse urine; and (C) the extract of hydrolyzed race horse urine co-injected with 40 μg/ml of flunixin standard.

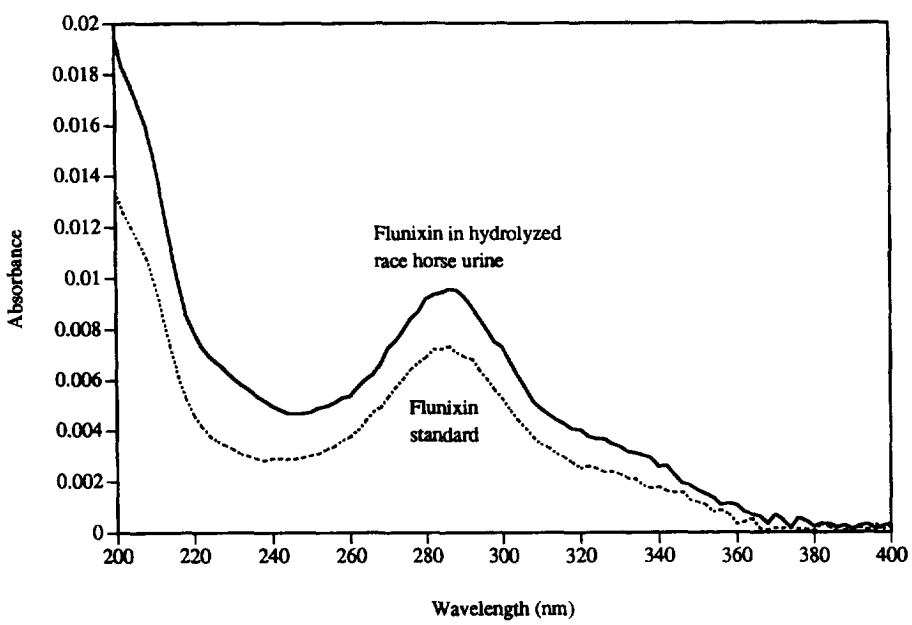

Fig. 9. Spectra of CE peak in hydrolyzed race horse urine assigned to flunixin and in flunixin standard solution.

enzyme immunoassay, were analyzed by this CE procedure. No flunixin peak was found in any of the ten unhydrolyzed horse urine samples as compared to the electrophoregrams of flunixin standard and to the extract of the unhydrolyzed racing horse urine co-injected with flunixin standard (Fig. 7). However, when the horse urine samples were hydrolyzed, a peak was observed at the migration time of flunixin standard in nine of the ten hydrolyzed horse urine samples (Fig. 8A,B). The height and area of this peak was increased when flunixin standard was co-injected (Fig. 8C). Therefore, this peak was determined to be flunixin in the horse urine. This was further confirmed by UV spectra. The peak assigned to be flunixin in the extract from hydrolyzed race horse urine has the same UV spectrum pattern as flunixin standard (Fig. 9).

The electrophoregraphic peak pattern was changed because of hydrolysis of horse urine (comparing Fig. 7B and Fig. 8B). However, no interfering matrix peak was found in hydrolyzed blank horse urine sample (Fig. 10)

The concentrations of total flunixin in race horse urine samples were quantitatively calculated and the results were summarized in Table 3.

3.7. Analysis of flunixin in urine and serum from a horse after flunixin administration

In order to determine the ratio of free flunixin to the conjugated flunixin in horse urine, a Thoroughbred mare was intramuscularly administrated with a therapeutic dose (1.1 mg/kg) of flunixin. Urine samples collected at different times following flunixin administration were divided into two groups. Unhydrolyzed horse urine was extracted and analyzed for free flunixin. The urine for the other group was hydrolyzed before extraction and

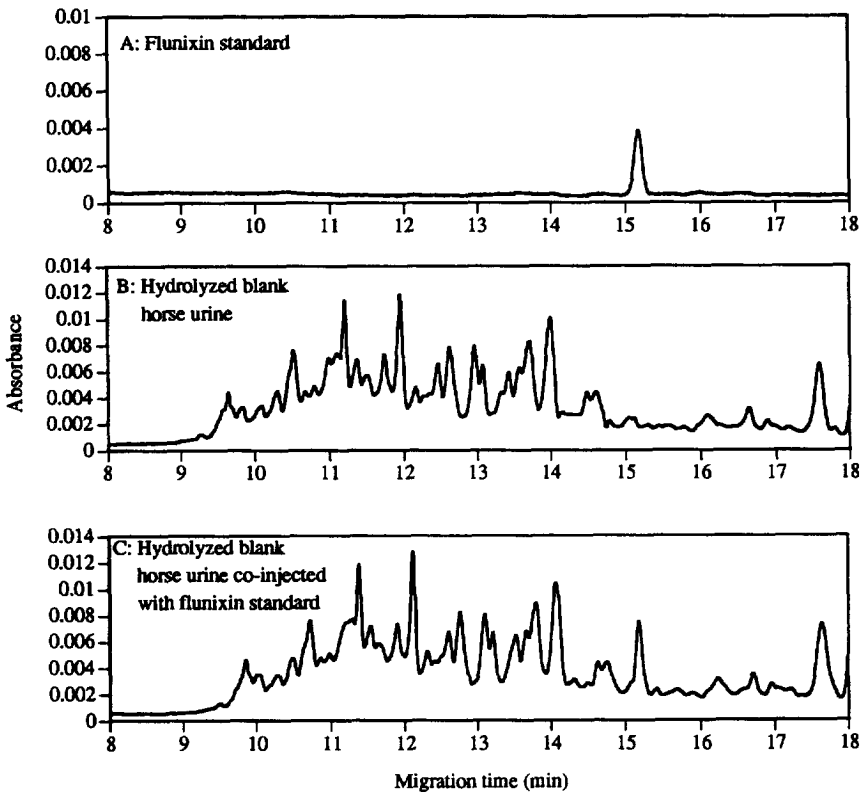

Fig. 10. Electrophoregrams of extract of hydrolyzed blank horse urine. (A) 4 μg/ml of flunixin standard; (B) 50 μl of extract from 2 ml of hydrolyzed blank horse urine; and (C) the extract of hydrolyzed blank horse urine co-injected with 4 μg/ml of flunixin standard.

Table 3
Concentration of flunixin in ten hydrolyzed race horse urine samples

Sample	Concentration (ng/ml)	Coefficient of variation (%)
1	193.5	4.57
2	–[a]	
3	367.3	6.03
4	171.2	7.46
5	947.3	3.28
6	61.7	8.50
7	95.8	6.67
8	85.6	6.54
9	72.0	9.03
10	33.7	13.30

The results represent average of triplicate assay for each sample.
[a] The concentration is lower than the limit of quantitation.

Table 4
Concentration of flunixin in urine and serum from a horse that received intramuscular administraion of 1.1 mg/kg flunixin

Time of sampling (h)	Urine (C.V.[a])		Serum (C.V.[a])
	Free flunixin (μg/ml)	Total flunixin (μg/ml)	Free flunixin (μg/ml)
2	0.57 (0.47)	22.8 (5.1)	0.85 (4.7)
4	0.67 (8.3)	95.1 (5.0)	0.83 (5.5)
6	0.47 (4.5)	21.2 (1.1)	0.59 (4.6)
8	0.46 (4.5)	54.5 (4.4)	0.27 (2.2)
24	–[b]	1.48 (3.5)	0.060 (6.5)
48	–[b]	1.67 (8.5)	0.024 (14.4)

The results represent average of 4 assays.
[a] C.V.: Coefficient of variation.
[b] The concentration is lower than the limit of quantitation.

analysis. The amount of flunixin in hydrolyzed horse urine reflected total flunixin (free+conjugate). The results showed that about 98.5% of flunixin was excreted into urine as conjugate form (Table 4). Without hydrolysis, flunixin in the urine was detected only up to 8 h post administration even though the total amount of flunixin was high in 48 h in hydrolyzed urine samples. Therefore, horse urine should be subjected to a simple one-step hydrolysis for detection of flunixin. On the other hand, free flunixin in serum can be easily detected for up to 48 h (Table 4). Therefore, it is unnecessary to hydrolyze horse serum samples.

Acknowledgments

This project was supported by the Division of Pari-Mutuel Wagering, Florida State Department of Business and Professional Regulation. We also thank Schering-Plough Research Institute for kindly supplying flunixin meglumine reference standard.

References

[1] V. Ciofalo, M. Latranyi, J. Patel and R. Taber, J. Pharmacol. Exp. Ther., 200 (1977) 501.
[2] J.W. Houdeshell and P.W. Hennessey, J. Equine Med. Surg., 1 (1977) 57.
[3] G.D. Bottoms, J.F. Fessler and F. Roesel, Am. J. Vet. Res., 42 (1981) 1514.
[4] J.F. Fessler, G.D. Bottoms and F. Roesel, Am. J. Vet. Res., 43 (1982) 140.
[5] G.D. Vernimb and B.S. Hennessey, J. Equine Med. Surg., 1 (1977) 111.
[6] K.L. Anderson, H. Kindahl and A.R. Smith, Am. J. Vet. Res., 47 (1986) 1366.
[7] Ph. Jaussaud, D. Courtot, J.L. Guyot and J. Paris, J. Chromatogr., 423 (1987) 123.

[8] M. Johansson and E.L. Anler, J. Chromatogr., 427 (1988) 55.
[9] S. Chay, W.E. Woods, T. Nugent, J.W. Blake and T. Tobin, Equine Proct., 4 (1982) 16.
[10] A.J. Higgins, P. Lees, S.C. Sharma and J.B. Taylor, Equine Vet. J., 19 (4) (1987) 303.
[11] A.K. Singh, Y. Jang, U. Mishra and K. Granley, J. Chromatogr., 568 (1991) 351.
[12] L.R. Soma, E. Behrend, J. Rudy and R.W. Sweeney, Am. J. Vet. Res., 49 (1988) 1894
[13] G.E. Hardee, J.W. Lai and J.N. Moor, J. Liq. Chromatogr., 5 (1982) 1991.
[14] S. Chay, W.E. Woods, T. Nugent, J.W. Blake and T. Tobin, Equine Practice, 4 (10) (1982) 16.
[15] A.M. Craig, L.L. Blythe, L.H. Appell and M.L. Slizeski, J. Vet. Pharmacol. Therap., 10 (1987) 298.
[16] H. Wätzig and D.K. Lloyd, Electrophoresis, 16 (1995) 57–63.
[17] L. Keith, W. Crummett, J. Deegan, R. Libby, J. Taylor and G. Wentler, Anal. Chem., 55 (1983) 2210–2218.