ELISA ASSAY FOR FLUNIXIN

T. Edgar, T. Tobin*, H.H. Tai†, S. Bass, J. T. Ice and D. E. Schroedter

*Neogen Corporation, ELISA Technologies Division, Lexington, Kentucky 40505; *Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40506; †College of Pharmacy, University of Kentucky, Lexington, Kentucky 40506, USA*

ABSTRACT

Flunixin (2-[2-methyl-3-(triflouromethyl)phenyl] amino-nicotinic acid, Banamine) is a non-steroidal anti-inflammatory drug used widely in veterinary medicine, often to relieve inflammation in joints and muscles. Unfortunately, this anti-inflammatory effect can be abused in the racing industry. Currently, the presence of flunixin in a racing sample is forbidden in all United States jurisdictions. State-of-the-art testing for flunixin involves screening samples with thin layer chromatography (TLC, limits of detection ranging from 250–1,000 ng/ml) with confirmation by high performance liquid chromatography (HPLC, limit of detection of 50 ng/ml) or gas chromatography coupled with mass spectroscopy (GC/MS, limit of detection of 10 ng/ml).

Flunixin specific antisera was developed in rabbits. A flunixin hapten was linked to horseradish peroxidase to form a drug:enzyme conjugate. These reagents were optimised to form a standard curve. Standard curves were established in equine urine, equine plasma, equine serum and canine urine.

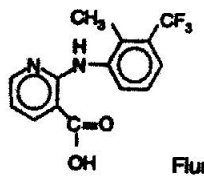
A background study was performed with a large number of negative equine racing urine samples. Some interference was seen with unknown endogenous urine components. This effect was abolished by a 1:10 dilution of the sample in assay buffer. Cross-reactivity studies were performed with a variety of illegal drugs, therapeutic drugs, potential masking agents and drug vehicles. Significant cross-reactivity was seen only with niflumic acid (3.6% cross-reactivity).

Post administration urine samples were assayed by TLC, HPLC and enzyme linked immunosorbent assay (ELISA). Flunixin was detected by the ELISA 15 days after the last administration. Equivalent data were seen with HPLC. TLC failed to detect flunixin in these samples after the first day.

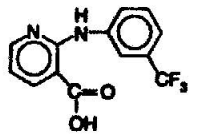
The I_{50} of the assay (a measure of sensitivity) with equine urine is 15 ng/ml, significantly less than TLC. The limit of detection of the assay is approximately 70 pg/ml. Recognising that flunixin is a legitimate therapeutic drug, and that some jurisdictions may not want a screen that is more sensitive than TLC, a study was performed to determine what sample dilutions would yield I_{50}s more closely matching TLC sensitivity. Sample dilutions of 1:500 produced an I_{50} of approximately 1,000 ng/ml. Dilutions of the administration samples produced much better agreement between the ELISA and TLC. The I_{50} of the assay with equine plasma is 350 ng/ml (parts/million). This result is roughly equivalent to the sensitivity of current screening method (TLC).

INTRODUCTION

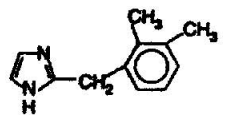
Flunixin (Fig 1) is a non-steroidal anti-inflammatory drug (NSAID). As with other NSAIDs, flunixin inhibits the activity of cyclooxygenase, reducing the production of prostaglandins. Flunixin-meglumine



Flunixin



Niflumic acid



Detomidine

Fig 1: Flunixin and cross-reactant structures.

TABLE 1: Cross-reactivity study

Cross-reactant	% cross-reactivity	Cross-reactant	% cross-reactivity
Niflumic acid	3.63	Hydrocortisone	< 0.01
Detomidine	0.86	Ibuprofen	< 0.01
Meclofenamic acid	0.05	Indomethacin	< 0.01
Phenothiazine	0.03	Indoprofen	< 0.01
Diflunisal	0.02	Isoxicam	< 0.01
Etodolac	0.02	Isoxsuprine	< 0.01
Reserpine	0.02	Ketoprofen	< 0.01
Stanozolol	0.01	Ketorolac	< 0.01
Xylazine	0.01	Lidocaine	< 0.01
6-α Methylprednisolone	< 0.01	Mefenamic acid	< 0.01
Acepromazine	< 0.01	Meperidine	< 0.01
Acetominophen	< 0.01	Mepivacaine	< 0.01
Amcinonide	< 0.01	Metaproterenol	< 0.01
Aminophylline	< 0.01	Methocarbamol	< 0.01
Amiprilose	< 0.01	Methotrimeprazine	< 0.01
Ascorbic Acid	< 0.01	Methylene Blue	< 0.01
Aspirin	< 0.01	Nabumetone	< 0.01
Atropine	< 0.01	Nalbuphine	< 0.01
Benzydamine	< 0.01	Nandrolone	< 0.01
Betamethasone	< 0.01	Naproxen	< 0.01
Boldenone	< 0.01	Nefopam	< 0.01
Budesonide	< 0.01	Niacinamide	< 0.01
Buprenorphine	< 0.01	Orphenadrine	< 0.01
Butorphanol	< 0.01	Oxyphenbutazone	< 0.01
Carbamazepine	< 0.01	Pentazocine	< 0.01
Carprofen	< 0.01	Pentoxifylline	< 0.01
Chlorzoxazone	< 0.01	Phencyclidine	< 0.01
Clenbuterol	< 0.01	Phenylbutazone	< 0.01
Clobetasol propionate	< 0.01	Piroxicam	< 0.01
Clobetasone butyrate	< 0.01	Polyethylene glycol	< 0.01
Cromolyn	< 0.01	Prednisolone	< 0.01
Dantrolene	< 0.01	Prednisone	< 0.01
Desoximetasone	< 0.01	Procaine	< 0.01
Dexamethasone	< 0.01	Promazine	< 0.01
Dezocine	< 0.01	Propoxyphene	< 0.01
Diazepam	< 0.01	Pyrantel	< 0.01
Diclofenac	< 0.01	Pyrilamine	< 0.01
Dipyrone	< 0.01	Salbutamol	< 0.01
ε-amino caproic acid	< 0.01	Salicylamide	< 0.01
Ethyl *p*-aminobenzoate	< 0.01	Salicylic acid	< 0.01
Fenbufen	< 0.01	Sanguinarium chloride	< 0.01
Fenoprofen	< 0.01	Sufentanil	< 0.01
Flufenamic acid	< 0.01	Sulindac	< 0.01
Flumethasone	< 0.01	Suprofen	< 0.01
Flunisolide	< 0.01	Terbutaline	< 0.01
Fluphenazine	< 0.01	Thiamine	< 0.01
Flurazepam	< 0.01	Thiosalicylic acid	< 0.01
Flurbiprofen	< 0.01	Tiaprofenic acid	< 0.01
Furosemide	< 0.01	Tolmetin	< 0.01
Glycopyrrolate	< 0.01	Triamcinolone	< 0.01
Guaifenesin	< 0.01	Trichlormethiazide	< 0.01
Hordenine	< 0.01	Zomepirac	< 0.01

(Banamine; Schering Plough, New Jersey, USA) is an effective therapeutic medication and is used widely in veterinary medicine. The drug exhibits a unique analgesic property in reducing visceral pain associated with colic. As an anti-inflammatory, flunixin has also been used to treat endotoxic shock and severe diarrhoea (Anon 1991). The drug's anti-inflammatory actions are also used to relieve inflammation in joints and tissues. However, as an anti-inflammatory, flunixin can be abused in the racing industry.

Enzyme linked immunosorbent assays (ELISA) have been shown to have great value as screening tools for drugs in race samples (Tobin *et al.* 1988). The development of such a test for flunixin is described.

A competitive ELISA format was chosen for this test. In this format, antibodies specific to flunixin

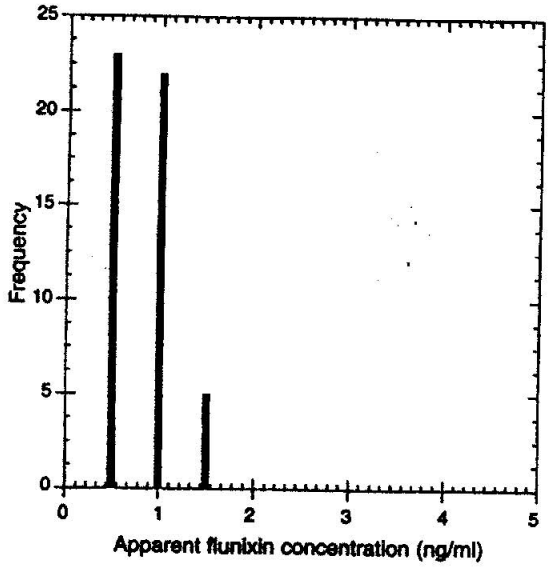


Fig 2: Diluted equine urine matrix effects (equine urine samples were diluted 1:10 in EIA buffer).

are immobilised onto microtitre plate wells. The sample is added along with a flunixin:enzyme conjugate. Flunixin in the sample or control competes with the conjugate for antibody binding sites during an incubation phase. Following this incubation, unbound drug and conjugate are removed by washing. An enzyme substrate and chromophore are then added. Bound enzyme conjugate activity produces a blue colour, the intensity of which is inversely proportional to the concentration of flunixin in the sample.

Reagents

NHS (N-hydroxysuccinimide), EDC (1-ethyl-3-(diaminopropyl)carbodiimide), bovine serum albumin (BSA) and common buffer salts were obtained from the Sigma Chemical Company (Missouri, USA). Horseradish peroxidase (HRP) was purchased from the Boehringer Mannheim Corporation (Indiana, USA). Conjugate purification was performed by liquid chromatography with Kwik Columns from Pierce (Illinois, USA). Protein A was purchased from Repligen (Massachusetts, USA) and goat anti-rabbit IgG antisera was obtained from Zymed (California, USA).

A carboxylic acid derivative of flunixin was synthesised. This derivative was used to make both immunogens and enzyme conjugates. Active ester chemistry using N-hydroxy succinimide and 1-ethyl-3-(diaminopropyl) carbodiimide (Grabarek and Gergely 1990) was used to link the hapten to BSA (immunogen) or HRP (enzyme conjugate). Anti-flunixin antisera was developed in New Zealand White rabbits. Rabbits were boosted monthly and blood samples collected at least once a month. The serum was harvested and stored at <-20°C.

Antisera selection

Anti-flunixin antisera immobilisation was compared using 3 methods. The antisera were diluted and allowed to bind directly to the wells through hydrophobic interactions, to goat anti-rabbit IgG antisera pre-coated wells, or to Protein A pre-coated wells. Protein A pre-coated wells produced superior results and were used for further assay development. Anti-flunixin antisera were titred. Titre results were used to form mini-pools which were evaluated for standard curve performance. The I_{50} for all the minipools of one rabbit were very similar. The antisera represented in the largest minipool were combined, aliquoted and frozen. The new antisera pool and conjugate levels were optimised to produce the best standard curve performance.

Assay format

Anti-flunixin assay plates are supplied ready to use. Samples are diluted in enzyme immunoassay (EIA) buffer and 20 µl added to the wells. To maximise conjugate stability, the flunixin:enzyme conjugate is provided as a concentrate. The conjugate is diluted 1:180 in EIA buffer and 180 µl are added to each well. The reaction is incubated for 1 h at room temperature. The wells are washed with wash buffer (provided with the kit). A one-step peroxidase substrate is added to the wells (150 µl per well). Colour is allowed to develop for 30 min at room temperature. The absorbance of each well is measured on a microtitre plate reader.

Cross-reactivity

Cross-reactivity to a variety of drugs was determined by spiking the drug into EIA and determining the apparent flunixin concentration in the spiked samples. Those drugs that demonstrated some competition with the conjugate for antibody binding were assayed again. In the second assay, a standard curve was produced for the cross-reactant. Percent cross-reactivity was determined by dividing the I_{50} concentration of flunixin by the I_{50} of the cross-reactant.

Results are shown in Table 1. Slight cross-reactivity was seen with niflumic acid (I_{50} = 82 ng/ml) and detomidine (I_{50} = 345 ng/ml). The structures of flunixin and the 2 cross-reactants can be seen in Figure 1.

TABLE 2: Duration of detection following flunixin administration (administration samples were diluted 1:500 in EIA buffer and quantitated)

Horse no	Route of administration	h post withdrawal	TLC +/-	ELISA +/-
A	iv	24	+	+
A	iv	48	-	+
B	iv	24	+	+
B	iv	48	-	-
C	iv	24	-	+
C	iv	48	-	-
D	iv	24	+	+
D	iv	48	-	-
E	im	24	+	+
E	im	48	+	+
F	im	24	+	+
F	im	48	+	+
G	im	24	+	+
G	im	48	+	-
H	im	24	+	+
H	im	48	+	+

Sample matrix effects

Negative track samples were assayed in the flunixin assay. No false positives were seen with equine plasma samples. Significant matrix effects were seen with equine urine samples. However, the matrix effects are reversible by dilution in EIA buffer. A 1:10 dilution of equine urine samples with EIA buffer reduced the matrix effects to an acceptable level (Fig 2).

Assay sensitivity

The I_{50} was determined for flunixin standard curves in equine urine, equine plasma and equine serum. The I_{50}s of plasma and serum curves were similar, ranging from 9–15 ng/ml. A urine dilution of 1:10 dilution increased the I_{50} of this curve to 15 ng/ml.

Current thin layer chromatography (TLC) screening has a limit of detection of approximately 1,000 ng/ml (R. A. Sams, personal communication). As flunixin is a legitimate therapeutic drug, some racing jurisdictions may not desire an ELISA screening assay with sensitivity so much lower than TLC. Therefore, a sample dilution study was performed. Flunixin standards were made in an equine urine sample. These standards were then diluted in EIA buffer and assayed. The normal 1:10 dilution produced an I_{50} of 15 ng/ml. A 1:500 dilution increased the I_{50} to 1,100 ng/ml, and a 1:2,500 dilution gave an I_{50} of 7 µg/ml. Therefore, a laboratory that does not wish to confirm urine samples with less than 1 µg/ml should dilute all urine specimens 1:500 in EIA buffer.

Duration of detection

Horses were given flunixin iv or im for 5 days. Urine samples were collected, assayed and flunixin concentrations were determined from standard curves. The ELISA samples were diluted 1:10 as recommended to overcome matrix effects. ELISA samples were also diluted 1:500, the recommended dilution for achieving an I_{50} of 1,000 ng/ml. When samples were diluted only 1:10, flunixin could be detected for up to 15 days after withdrawal of the drug. The larger dilution produced good agreement with TLC results (Table 2). A sample was judged positive in the ELISA if absorbance was less than the I_{50} of the standard curve. Flunixin was detected in all 24 h samples, one of which was negative by TLC. Horses receiving flunixin iv were negative by TLC after 48 h. One of these samples was positive by ELISA. Horses receiving flunixin im were positive in the TLC assay after 24 and 48 h. One 48 h sample was negative by ELISA.

Conclusion

An ELISA assay which is specific and sensitive has been developed for flunixin. The assay may be more sensitive than is desired for an approved therapeutic drug, but this problem can be circumvented easily by sample dilution. Correlation with TLC has been demonstrated.

Acknowledgement

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