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Determination of flunixin in equine plasma by reversed-phase liquid chromatography

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Abstract: Flunixin is determined in equine plasma by liquid chromatography on LiChrosorb RP-18 with 70% methanol in phosphate buffer pH 3.1 as the eluent, with detection at 284 nm. Plasma is deproteinized with methanol and the supernatant is then injected directly into the system. With a short pre-column (5 × 3 mm i.d.), which is replaced after 25–40 injections of sample, 420 plasma samples could be analysed on one analytical column. The detection limit in plasma is 0.30 μmol/l (89 ng/ml) and the method can be used in pharmacokinetic studies.

Keywords: *Direct injection; equine; flunixin; pre-column; reversed-phase liquid chromatography.*

Introduction

Flunixin meglumine is a non-steroidal analgesic agent with anti-inflammatory and antipyretic activity [1, 2]. The drug has been approved for use in horses and is included in a study on effects of drugs on performance in the horse [3]. The aim of the present investigation was to develop a simple assay for the analysis of flunixin in equine plasma for use in pharmacokinetic studies.

Flunixin has previously been determined in equine urine and plasma by gas chromatography [4, 5]. Derivatization of flunixin with pentafluorobenzyl bromide or *N,O*-bis(trimethylsilyl)-acetamide in combination with the electron capture detector was sensitive enough for the analysis of flunixin in equine plasma, although a non-linear standard curve was reported [5]. The high-performance liquid chromatographic (HPLC) method was expected to be simpler.

Several reports have appeared in the literature where plasma or serum proteins are precipitated with organic solvents [6–14] or strong acids [15–18]. After centrifugation the supernatant is injected onto a reversed-phase column. The method is applicable to substances that are present in rather high concentrations in blood samples and have high molar absorptivity or fluorescence, e.g. nicotinic acid [6], furosemide [7–9], quinidine [10], *p*-aminosalicylic acid [11], theophylline [12], chloramphenicol [13], penicillins [15],

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acetylsalicylic acid and metabolites [16, 17], pyridine derivatives [18] and some anti-inflammatory drugs [14].

In the present method the plasma proteins are precipitated with methanol. The choice of liquid chromatographic system used to isolate flunixin in the supernatant is discussed with respect to the pH of the phosphate buffer and the concentration of methanol in the eluent. The method is similar to one recently reported by Hardee *et al.* [19], but is easier to perform as no evaporation of the sample is required and no internal standard is needed.

Experimental

Apparatus

The liquid chromatograph consisted of a Constametric I pump (Milton Roy Company, Riviera Beach, USA), a Rheodyne injector (Berkeley, CA, USA) with a 20-μl or a 50-μl loop and an LDC Spectromonitor III UV detector with variable wavelength detection at 284 nm. For area calculations an integrator, Shimadzu Chromatopac C-RIB (Shimadzu Corporation, Kyoto, Japan), was used. The chromatographic columns (150 × 4.5 mm i.d.) were made of 316 stainless steel equipped with zero volume Swagelok unions and column end fittings and 2 μm stainless steel frits from Altex Scientific. A short pre-column (5 × 3 mm i.d.) was used in some of the experiments. The pre-column was made after the design of Wahlund *et al.* [20], which is an improvement of the pre-column used for on-line trace enrichment [21]. Both the analytical column and the precolumn were slurry-packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, FRG).

In the preparation of plasma samples, a 100-μl Labpipette (Labsystems Oy, Helsinki, Finland) and an Eppendorf Multipipette® 4780 with Combitip 5.0 ml (Eppendorf Gerätgebau, Netheler und Hinz GmbH, Hamburg, FRG) were used to measure volumes of 100 and 200 μl. These were calibrated and the volumes found to be 99.8 ± 0.4 μl and 199.8 ± 0.7 μl (n = 10), respectively. The samples were prepared in 1.5 ml Eppendorf centrifuge tubes and centrifuged in an Eppendorf Centrifuge 5412. All experiments were carried out at ambient temperature, *ca* 23°C.

Chemicals

The structure of flunixin, 2(2′-methyl-3′-trifluoromethylanilino)-nicotinic acid (MW 296.3), is illustrated in Fig. 1. Flunixin meglumine was kindly supplied by Schering Corporation (Kenilworth, NJ, USA).

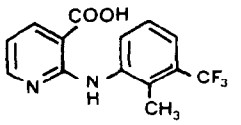

Figure 1
Structure of flunixin.

Methanol (analytical reagent grade), was obtained from May & Baker Ltd, (Dagenham, UK). Phosphate buffers were prepared in distilled water, using phosphoric acid, sodium dihydrogen phosphate and disodium hydrogen phosphate with an ionic strength of 0.05.

Chromatographic procedures

The eluents were prepared by mixing known volumes of phosphate buffers and methanol. The mixtures were allowed to stand overnight and were degassed in an

ultrasonic bath before use. Sulfathiazole was used to determine the void volume of the column, V_m, which was used to calculate the capacity ratios, k'.

The analytical column (150 × 4.5 mm i.d.) was packed by a balanced-density slurry technique and was tested as described earlier [22, 23]. At the beginning of this study the support at the top of the analytical column was changed regularly and the column was washed with 50 ml methanol every second day. Subsequently a short pre-column (5 × 3 mm i.d.) was used to increase the life of the separation column. The pre-column was prepared with the aid of a water suction pump. Approximately 0.05 g support was suspended in 2 ml methanol. The slurry was added in portions to the column and when filled, the column was washed with 3 ml methanol. After connection to the liquid chromatographic system, 40–50 ml of eluent was allowed to pass before injection. The pre-column was regularly replaced after the injection of 25–40 plasma samples.

Analytical procedure

Plasma samples. Blood samples were collected in heparinized vacuum tubes, Venoject®. Plasma was prepared and stored frozen at −20°C before being analysed.

Analytical method. Plasma 100 μl was pipetted into micro-centrifuge test tubes and 200 μl methanol was added to precipitate the proteins. The tubes were capped, shaken by hand and then centrifuged for 2 min. The injector loop was rinsed with 200 μl supernatant and 20 μl or 50 μl was injected onto the chromatographic column. A 50-μl loop was used for plasma concentrations of flunixin below 5 μmol/l (1.4 μg/ml) in plasma.

Standard preparation. Drug-free plasma was mixed with a solution of flunixin in 0.025 M disodium phosphate buffer, pH 9.2 (50 μl/ml of plasma). Both pooled plasma and plasma from individual horses was used to measure the accuracy of the analytical method.

Quantitative evaluation. At the beginning of the study, the peak heights for flunixin in the chromatogram were measured manually and the concentration of flunixin was calculated by comparison with external standards prepared in eluent. Subsequently an integrator was used to measure the peak areas. Flunixin was added to plasma at two concentrations, 5.18 and 51.8 μmol/l for 20-μl injections and 1.18 and 7.76 μmol/l for 50-μl injections of samples. These were used to calibrate the integrator and the response factor calculated was used for quantitation.

Results and Discussion

Chromatography

An advantage of the present method is that flunixin can be analysed without extraction. By this procedure, linear calibration curves were obtained, whereas the derivatization method did not yield satisfactory calibration curves [5], although the extractability of flunixin into organic solvents seems to be good.

The retention time of flunixin on LiChrosorb RP-18 was investigated to find a suitable concentration of methanol and pH of the phosphate buffer in the eluent. The influence on the retention of the methanol concentration at a constant pH of 3.1, is demonstrated in Fig. 2. As expected, the retention of flunixin decreased with increasing concentration of methanol in the eluent.

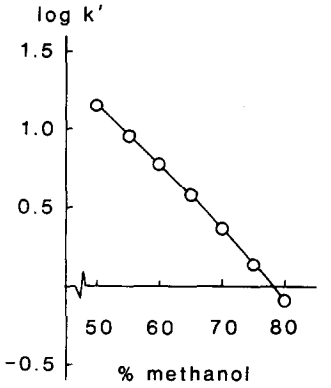

Figure 2
Relationship of capacity ratio (as log k') of flunixin with the concentration of methanol in the eluent. Eluent: phosphate buffer pH 3.1–methanol.

To establish the effect of pH the methanol concentration was kept constant (60% or 70% v/v) and pH of the phosphate buffer in the eluent was changed, as shown in Fig. 3. The decrease of retention at higher pH is probably due to protolysis of the acid in the eluent (cf. [24]).

A suitable retention time of flunixin was obtained at pH 5 with 60% v/v methanol in the eluent, or at pH 3 with 70% v/v methanol in the eluent. In the method the plasma proteins are precipitated with methanol. The use of 200 µl methanol to 100 µl plasma will precipitate the plasma proteins to 99% [25]. As the supernatant injected on the column contains about 66% v/v methanol and the concentration of methanol in the eluent should not be lower to maintain good column performance, the analysis was performed at pH 3 using 70% v/v methanol in the eluent. The retention time of flunixin under these conditions was 6 min at a flow-rate of 0.8 ml/min. Sample injection can only be made every 13 min, since a small interfering peak representing an unidentified plasma component appears after 16 min. Figure 4 shows chromatograms of two plasma samples taken from a horse before and 5 h after an intravenous injection of flunixin.

Column stability

At the beginning of the study, quantitative determination was made by peak height measurements. It was noticed that the peak obtained became a little broader and the retention time decreased during the analysis. The peak shape was restored by replacing the packing at the top of the analytical column. This had to be done after the injection of 20 plasma samples to maintain good column performance. The column was also flushed with 50 ml methanol each time the support was replaced. The column could, however, only be restored in this way 5 times; thus 100 plasma samples could be injected onto one column, after which a new column had to be prepared.

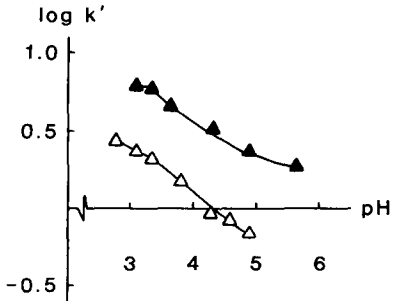

Figure 3
Relationship of retention (as log k') of flunixin with pH of the phosphate buffer in the eluent. Eluent: phosphate buffer–methanol; methanol concentration: △ 70% v/v, ▲ 60% v/v.

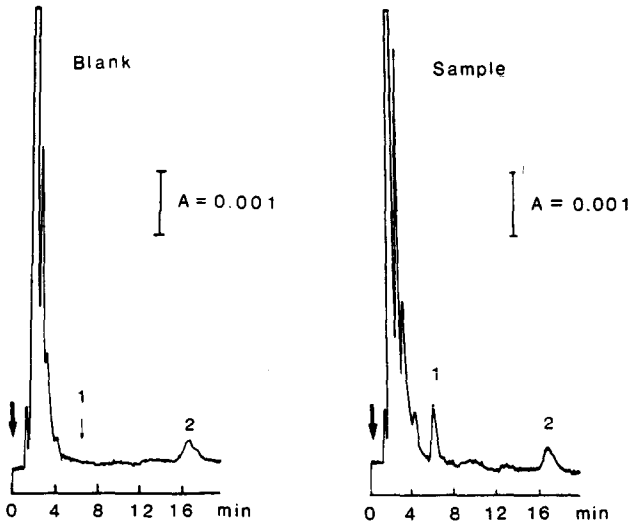

Figure 4
Isolation of flunixin from equine plasma. Plasma samples taken before and 5 h after an intravenous injection of flunixin (1 mg/kg body weight) to a horse. Plasma concentration of flunixin = 1.79 μmol/l (0.53 μg/ml); injection volume, 50 μl; eluent, phosphate buffer (pH 3.1)–methanol (3:7 v/v); flow-rate, 0.8 ml/min, pressure: 6.5 MPa; support, LiChrosorb RP-18 (10 μm). Peaks: 1 = flunixin; 2 = endogenous compound.

An improvement was made by the use of a short 5 mm pre-column. The pre-column was easily packed by hand in 30 min, as discussed above. Further improvement was obtained when peak area were measured instead of peak height. As a rule the pre-column was changed every 40 injections for 20-μl injection volumes and every 25 injections when the injection volume was 50 μl. In this way, 450 injections of plasma samples were made on one analytical column, with only a minor decrease in column performance.

Quantitative determinations

Recovery and precision. Flunixin was added to the drug-free plasma from each of five horses to give a final concentration of 4.93 μmol/l (1.46 μg/ml) in plasma. The samples were analysed as described above and the recoveries, calculated from direct injection of flunixin dissolved in the eluent, were: 96.3, 94.8, 98.9, 99.0 and 96.3% respectively (n = 3). The same concentration of flunixin in phosphate buffer taken through the same analytical method gave 99.7% recovery.

The recovery and within-run precision of flunixin were estimated from repeated injections of samples prepared in plasma at three different concentrations. The relative standard deviation (RSD) was somewhat higher when measuring the peak areas compared to the peak heights, as indicated in Table 1. The between-run RSD was comparable with that found during a run.

Calibration data. A linear standard curve for flunixin was obtained in the range 1.03–103.5 μmol/l (0.306–30.7 μg/ml) in plasma (Fig. 5). The injection volume was 20 μl. The standard curve obtained by analysis of flunixin added to pooled plasma has a small intercept, attributable to a small disturbing peak eluting just before the flunixin peak in the chromatogram. It was observed that not all horse plasma samples contained

Table 1
Recovery and precision of the method

Amount		RSD (%)	
Added (μmol/l)	Found* (%)	Peak area	Peak height
Within-run precision (n = 8)			
51.8	97.8	2.25	—
7.77	98.5	3.23	1.88
1.48†	99.6	6.42	4.55
Between-run precision‡			
7.77	97.5	3.04 (n = 8)	—
1.48†	97.6	5.47 (n = 7)	—

* Estimations were made from repeated injections of spiked equine plasma. Quantitation was performed by peak area measurements and the precision expressed as relative standard deviation (RSD; %).
† Injection volume: 50 μl.
‡ Analysis performed on different days.

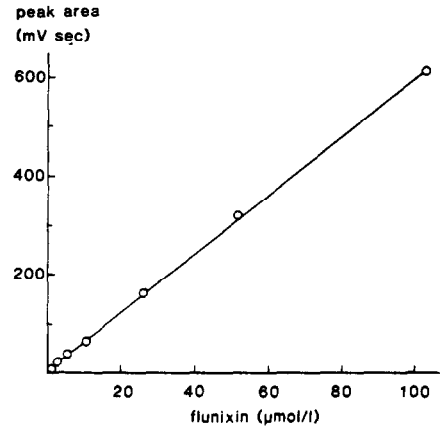

Figure 5
Standard curve of flunixin in plasma. Injection volume: 20 μl.

this interfering endogenous compound. Standards should be prepared in appropriate blank plasma for the quantitative determination of flunixin. The regression data for the calibration curve were: $y = 5.925\ x + 7.039$; SE of slope = 0.0659; SE of intercept = 3.169; $r = 0.9994$; $n = 12$.

Detection sensitivity. The detection limit for flunixin in plasma, defined as the concentration which gives a signal equal to twice the baseline noise, was 0.74 μmol/l (0.22 μg/ml) for flunixin when the injection volume was 20 μl.

A 50 μl loop was used for low plasma concentrations of flunixin to increase the sensitivity. The practical detection limit for flunixin in plasma was thereby improved to 0.30 μmol/l (89 ng/ml).

Plasma samples from horses

The plasma levels in a horse after one intravenous or one intramuscular injection of flunixin (1 mg/kg body weight) are presented in Fig. 6. The curve after intravenous administration of flunixin is similar to that reported by Hardee *et al*, [19] in a study where flunixin and phenylbutazone, another anti-inflammatory drug, were given simultaneously.

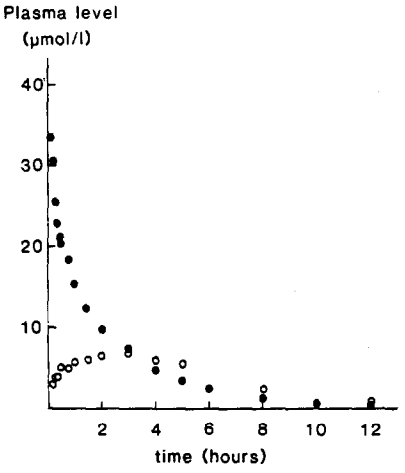

Figure 6
Concentrations of flunixin in equine plasma. Dose: 1 mg/kg body weight; ● intravenous administration; ○ intramuscular administration.

With the method developed the plasma concentration of flunixin can be determined up to 12 h after administration of a therapeutic dose to the horse. The pharmacokinetics of flunixin will be the subject of a further study.

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