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Original manuscript

Flupirtine: preliminary pharmacokinetics in the donkey

M. Giorgi [a], F. Laus [b], V. De Vito [c]*, H. Owen [d], A. Poapolathep [e], E. Paggi [b], C. Vullo[f]

[a] *Department of Veterinary Sciences, University of Pisa, Via Livornese (lato monte), San Piero a Grado, Italy*

[b] *School of Biosciences and Veterinary Medicine, University Camerino, Matelica, Macerata, Italy*

[c] *Department of Veterinary Medicine, University of Sassari, Via Vienna 2, Sassari, Italy*

[d] *School of Veterinary Science, The University of Queensland, Gatton Campus, Gatton, Queensland 4343, Australia*

[e] *Department of Veterinary Pharmacology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand*

[f] *School of Pharmacy, University Camerino, Matelica, Macerata, Italy*

* Corresponding author: Tel.: +39 5022 10145

E-mail address: virgidevit@libero.it (V. De Vito)

Abstract

Flupirtine (FLU) is a non-opioid analgesic drug with no antipyretic or antiphlogistic effects labelled for humans. It does not induce the side effects associated with the classical drugs used as pain relievers (NSAIDs and opioids) in human beings. The aim of this study was to evaluate the pharmacokinetic profiles of FLU after IV and PO administration in healthy donkeys. Six Amiata breed adult jennies were randomly assigned to two treatment groups using an open, 2 x 2 Latin-square cross-over study design. Group 1 (n = 3) received a single dose of 1 mg/kg of FLU injected IV into the jugular vein. Group 2 (n = 3) received FLU (5 mg/kg) via nasogastric tube. The wash out period was 1-week. Blood samples (5 mL) were collected at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, 36 and 48 h and plasma was then analysed by a validated HPLC method. No adverse effects were noticed in either administration group. After IV and PO administrations, FLU was detectable in plasma for up to 24 h. The mean elimination half-life was longer after PO (10.81 h) than after IV (0.90 h) administration. The clearance was fast and the AUC was small, findings consistent with a low oral bioavailability of about 20%. The pharmacokinetic trend of FLU in donkeys was different from those earlier reported in cats and dogs. Further studies are needed to understand if this active ingredient may be used in donkeys.

Key words: donkeys; flupirtine; intravenous; oral; pharmacokinetics

1 Introduction

Flupirtine (FLU) is an aminopyridine drug (ethyl {2-amino-6-[(4-fluorobenzyl) amino] pyridin- 3-yl}carbamate) approved in Europe in 1984 for treatment of a wide range of pain states in human beings [1]. Flupirtine is a centrally acting analgesic with a mechanism of action unlike that of opiates and NSAIDs. It is active with a favourable tolerability and with no antipyretic or antiphlogistic effects in humans [2]. Flupirtine is the first drug to be recognised in the unique class of 'Selective Neuronal Potassium Channel Openers' (SNEPCO) [3]. It interacts with the G-protein-regulated, Inwardly Rectifying K^+ channels (GIRKs), a novel family of K^+ channels distinct from the voltage-dependent ones. They are regulated by neurotransmitters and are expressed in different parts of the brain. Flupirtine activates GIRKs and stabilizes the membrane resting potential by activating potassium channels KCNQ and thus generating a neuronal hyperpolarizing current (M-current). The increased M-current due to the action of FLU translates to decreased neuronal excitability [4]. Moreover, FLU inhibits the NMDA receptor indirectly by acting as an oxidizing agent at the redox site of the NMDA receptor, maintaining the Mg^{2+} block on the NMDA receptor [2].

In line with its mechanism of action promoting neuronal rest, it has proven useful in conditions involving neuronal hyperexcitability such as chronic pain (non-malignant and malignant), migraine and neurogenic pain [5-10]. Furthermore, its effect as a muscle relaxant represents added value in painful conditions associated with increased muscle tension, such as musculoskeletal back pain, myofascial pain and tension headaches [1,6,11-13]. Flupirtine has also been shown as beneficial in the short-term treatment of acute to moderate pain such as postoperative pain, trauma and dysmenorrhoea [14]. The approved indications of FLU differ between countries but mainly include the clinical management of musculoskeletal pain,

postoperative pain, headache, dysmenorrhoea, neuralgia and neuritis, post-traumatic pain (trauma and chemical burns) and pain associated with cancer [15-16]. It has possibly not been used to its full potential as an analgesic in the first decade of the 21st century, but in recent years, there has been a resurgence in FLU use after discovery of its powerful-synergistic effects when used with opioids [4,17-18] in addition to its properties when used alone [19].

While there is a substantial body of evidence on the efficacy of FLU in humans, only a single study on the analgesic effect of FLU in laboratory animals is present in the literature [20] and its pharmacokinetic profiles in cats [21] and dogs [22] have been recently described. Advanced studies (phase III) in dogs and horses are ongoing in the USA (http://www.kindredbio.com/#!pipeline/c1ktj). As no data on the pharmacokinetic profiles of FLU in donkeys exists, the aim of this study was to evaluate its pharmacokinetic after IV and PO administration in this species.

2 Materials and methods

2.1 Chemical and reagents

Pure FLU maleate salt and the Internal Standard trazodone (IS) powders (both >99.0% purity) were supplied by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN), methanol (MeOH), dichloromethane (CH_2Cl_2) and ethyl acetate (AcOEt) were purchased from Merck (Darmstadt, Germany). Ammonium acetate ($AcONH_4$) was purchased from Carlo Erba (Milano, Italy). Deionised water was produced by a Milli-Q Milli-pore Water System (Millipore, MA, USA). All other reagents and materials were of analytical grade and supplied from commercial sources. The LC mobile phase was filtered through 0.2 µm cellulose acetate membrane filters (Sartorius Stedim Biotech S.A., Aubagne Cedex, France) with a solvent filtration apparatus.

2.2 Animal and experimental design

The subjects were six Amiata jennies, aged 4 to 7 years and weighing 150 to 210 kg. The jennies were determined to be clinically healthy on physical examination, serum chemistry and haematological analyses. Animals were evaluated daily (for 1 week) for visible adverse effects by specialized personnel. Animal care and handling was performed according to the provision of the EC council Directive 86/609 EEC and also according to Institutional Animal Care and Use directives issued by the Animal Welfare Committee of the University of Camerino, which approved the study protocol.

Donkeys were randomly assigned to two treatment groups (six slips of paper marked with the numbers 1 to 6 in a box), using an open, single-dose, two-treatment, two-phase, paired, cross-over design (2 x 2 Latin-square). All subjects were fasted for 12 h overnight before each experiment.

In both periods, a jugular catheter was placed for the purpose of blood sample collection. In addition, in the IV group, a second jugular catheter was placed in the contralateral jugular vein for intravenous drug administration. Placement of the jugular catheter occurred approximately 12 h prior to the start of the investigation. Donkeys were restrained by a handler during the process of catheter placement. The area over the jugular vein was clipped and surgically prepared with alternating scrubs of 70% isopropyl alcohol and povidone iodine. The catheter site was infiltrated with 1 mL of 2.5% lidocaine/prilocain injection given subcutaneously (EMLA, AstraZeneca, Milan Italy). Using sterile technique, an 18 G x 55 mm intravenous catheter (Picco, Pulsion, Milan Italy) with injection plug was inserted into the vein and sutured to the skin using #3 nylon suture (Vetsuture, Sanitalia, Napoli, Italy). Catheter patency was maintained by flushing with 2 mL of a heparin saline solution containing ten international units heparin sodium/mL saline (Heparin

Sodium Injection, Baxter, Pisa, Italy). The catheter port was disinfected with an alcohol swab prior to sample collection.

During the first phase, each donkey in group 1 (n = 3) received a single dose of 1 mg/kg of FLU (Katadolon® 100 mg/3 mL vials, FLU D-gluconate AWD Pharma, Radebeul, Germany) injected IV at a flow rate of 3 mL/min. Group 2 (n = 3) received a dose of 5 mg/kg via the PO route (Efiret® 100 mg hard capsules, FLU maleate, Meda Pharma S.p.A. Milano, Italy). The oral formulation of FLU was given to all animals via nasogastric tube and consisted of capsules in 300 mL of distilled water. After administration, the nasogastric tube was rinsed with 300 mL of distilled water to ensure complete delivery of the drug into the stomach. A 1-week wash out period was observed between the phases, then the groups were rotated and the experiment was repeated. Blood samples (5 mL) were collected at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, 36 and 48 h after administration of FLU and placed in collection tubes containing lithium heparin. Samples were immediately centrifuged at 2000 *g* (10 min), and the harvested plasma was stored at -20 ° C until use within 30 days from collection.

2.3 High performance liquid chromatography

The analytical method was based on a previous method validated in dog plasma [22]. In brief, the HPLC system was an LC Jasco (Como, Italy) consisting of quaternary gradient system (PU 980) and an in line multilambda fluorescence detector (FP 1520). The chromatographic separation assay was performed with a Luna $C18_{(2)}$ analytical column (250 mm × 4.6 mm inner diameter, 5 µ particle size [Phenomenex, Bologna, Italy]) preceded by a security guard column with the same stationary phase ($C18_{(2)}$ [Phenomenex, Bologna, Italy]). The system was maintained at 25°C. The mobile phase consisted of ACN:$AcONH_4$ (20 mM) solution, pH 6.8 (60:40, v/v) at a flow rate of 1

mL/min. Excitation and emission wavelengths were set at 323 and 370 nm, respectively. The elution of the substances was carried out in isocratic mode.

2.3.1 Sample extraction

The procedure was performed in a 15 mL polypropylene vial. A 500 µL aliquot of plasma was added to 100 µL of IS (100 µg/mL) and vortexed for 60 sec. Four mL of AcOEt:CH_2Cl_2 (7:3 v/v) was added, then the sample was vortexed (30 sec), shaken (100 osc/min, 10 min) and centrifuged at 3000 *g* for 10 min at 10° C. Three mL of the supernatant was collected in a separate clean vial. The organic phase was evaporated under a gentle stream of nitrogen at 40 °C and reconstituted with 500 µL of the mobile phase. Twenty µL of this latter solution was injected onto the HPLC-FL.

2.4 Pharmacokinetic evaluation

Flupirtine plasma concentration vs. time curves were modeled for each subject using a mono- or a two-compartment open model [23]. Comparison between competing models was made using the residual plots, visual inspection of the goodness of fit curves and the Akaike's information criterion. The pharmacokinetic calculations were carried out using WinNonLin v 5.3.1 (Pharsight). The PO bioavailability was calculated from the ratio of the areas under the plasma FLU concentration curve after PO and IV administration, respectively, indexed to their respective dose:

$$F(\%) = (AUC_{PO} \times Dose_{IV})/(AUC_{IV} \times Dose_{PO}) \times 100$$

2.5 Statistical analysis

Pharmacokinetic variables were evaluated using the Student's *t* test to determine statistically significant differences between the treatment groups. Both pharmacokinetic parameters and FLU plasma concentrations are presented as means ± standard deviation (normality tested by Shapiro-

Wilk test). All analyses were conducted using GraphPad InStat (GraphPad Software). In all experiments, differences were considered significant if $P < 0.05$.

3 Results

The HPLC method was re-validated using donkey plasma. Briefly, FLU was linear (r^2 >0.99) in the range 10-1500 ng/mL. Limit of detection and quantification were 3 and 10 ng/mL, respectively. When samples exceeded the upper limit of the range, they were re-analysed after appropriate dilution. The intraday repeatability was measured as coefficient of variation and was lower than 4.9 %, whereas accuracy, measured as closeness to the concentration added on the same replicates, was lower than 7.1 %.

No behavioral changes or alterations in health parameters were observed in the IV and PO groups of animals during or after (up to 7 days) the drug administration. Physiological signs and parameters were normal.

A bi-compartmental model best fitted the plasma concentrations after IV and PO administrations in all the six donkeys. Two-compartment with bolus input and first-order output, micro-constants as primary parameters was used for the IV administration while a first-order input, first-order output, no lag time and micro-constants as primary parameters was used for the PO administration. The average plasma concentration vs. time curves after both the administrations are reported in Fig. 1. After IV administration the data at the first collection time point was extremely variable (range 1508-13296 ng/mL). Flupirtine was detectable in plasma up to 24 h, then the drug concentrations dropped down to the LOQ of the method (10 ng/mL). After oral administration (5 mg/kg), the FLU plasma concentrations were quite variable, and were detectable over the same

range of time. The average C_{max} (936 ng/mL) was shown at a T_{max} of 0.33 h. The oral bioavailability (F%) was 19.75 ± 12.16 %. The half-life of elimination (Beta_HL) value was 10 times higher in the PO compared to the IV group. The complete pharmacokinetic parameters are reported in Table 1.

4 Discussion

Flupirtine is a centrally acting, non-opioid analgesic that is available in a number of European countries for the treatment of a variety of pain states [15]. The therapeutic benefits seen with FLU relate to its unique pharmacological properties. Recently its potential for use in veterinary medicine has been explored [24]. Preclinical studies showed that FLU was more effective than paracetamol and as effective as pentazocine in the electrostimulated pain test in mice [25]. Flupirtine significantly prolonged the latency of the tail-flick test in rats [26]. Flupirtine produced an efficacy profile superior to that of tramadol for cancer-associated pain in rats and humans [4,5]. Flupirtine produced a significant increase in morphine antinociception when the two drugs were administered in combination in different rat models of pain [17,18]. If the opioid sparing effect is also evident in donkeys, this active ingredient could play an important role in combinatorial analgesic therapy in order to avoid moderately high regimens of opioids. Flupirtine might also be an attractive alternative for patients with a history of adverse drug reaction to NSAIDs [27]. Indeed it does not induce the gastrointestinal and renal side effects evoked by classical NSAIDs and COX-2 selective inhibitors [28].

Allometric scaling is an approach for dosage selection that can be used in the absence of either species-specific pharmacokinetic data or prior drug experience in the target species [29]. In the present study, an evidence-based approach rather than an allometric calculation of the dose was

preferred. Both the approaches share the assumption that species differences in pharmacodynamics are clinically negligible. The oral dose administered in the present study (5 mg/kg) was about 3 times higher than the minimum dose reported in human clinical practice (100 mg/subject/day). However, it was still within the recommended human clinical range (100-400 mg/subject/day) [15]. The rationale for oral dose selection of 5 mg/kg was based on earlier preclinical studies in dogs and cats. The ED_{50} of FLU after oral administration in the electrical tooth pulp stimulation test in dogs and cats was 3.5 mg/kg [25] and 3 mg/kg [20], respectively. Additionally, recent pharmacokinetic studies carried out with this dose regimen did not show any adverse effects after oral administration [21,22]. A recent study also indicated that the theoretical effective oral dose of FLU in horses is 2.6 mg/kg [30]. However, as according to earlier studies donkeys require higher drug dose than horses [31-35], a 5 mg/kg oral dose was preferred.

In variance to the PO route, 5 mg/kg of FLU administrated IV produced some adverse effects in dogs [22]. In the present study the IV dose was reduced to 1 mg/kg to minimise potential adverse effects. No side effects were visible in donkeys even though the highest plasma concentrations were higher than those detected in horses administered with the same IV dose (1mg/kg), where only some mild and transient adverse effects were visible [30]. However, FLU as an analgesic drug is expected to be administered in multiple doses. Toxicity might be potential at multiple dose regimen and should be tested in further studies.

No experimental information about the minimal effective concentration in humans or animal species is available for FLU. A recent study has calculated *in silico* that the theoretical plasma effective concentration of FLU in horses is 178 ng/mL [30]. If this data also holds true in donkeys,

238 FLU is not maintained in excess of that plasma level for long. Indeed in both the drug
239 administrations, FLU plasma concentrations are below that value after 1.5 hours.

240

241 Flupirtine is a water-soluble compound in the form of maleate salt (pKa 5.3) that is rapidly
242 absorbed from the human gastro intestinal tract [36]. The T_{max} found in this study (0.33 h) was
243 shorter than the T_{max} reported for dogs (1.42 h), humans (range 1.6-1.8 h), and cats (2.78 h)
244 showing a faster rate of absorption of the drug in donkeys. A number of factors may be responsible
245 for this difference: the large variation in this parameter in the donkey, different absorption, gastric
246 emptying, transit time or other species-specific factors

247

248 Following PO administration of FLU, donkeys showed mean terminal plasma elimination
249 half-lives in between those reported in cats (13.6 h) and dogs (7.1 h) [21-22]. The average clearance
250 value in donkeys was much larger than those reported in dogs (604 mL/h/kg) and in cats (195
251 mL/h/kg) [21-22]. Interestingly, oral bioavailability (F%) in donkeys has also been shown as half of
252 that reported for cats and dogs. This difference might be due to both larger clearance and rapid drug
253 metabolism. Large differences in F% between humans and animals and between animals
254 (carnivorous vs. herbivorous) have previously been demonstrated, indicating that F% values derived
255 in an animal species cannot always be extrapolated to humans or other animal species [37].
256 Remarkably, a recent study has shown an oral F% of about 70% in horses [30]; this difference
257 between the equine species is in line with earlier studies reporting a significantly reduced drug oral
258 F% in donkeys compared to horses [31-35].

259

260

Conclusion

This is the first study on FLU in donkeys. The pharmacokinetic profiles of FLU in donkeys were different compared to FLU disposition in humans, cats and dogs. Donkeys have shown a large clearance and a low oral bioavailability, which are consistent with relatively low plasma drug concentration profiles if compared to other animal species. Further studies need to be undertaken to confirm the pharmacokinetic profile and to evaluate the analgesic effect in this animal species.

Conflict of interest statement

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1. Pharmacokinetic parameters of flupirtine after IV (1 mg/kg) and PO (5 mg/kg) administrations in healthy donkeys (n = 6)

		IV			PO		
Parameters	**Units**	**Mean**		**SD**	**Mean**		**SD**
AUC	hr*ng/mL	1867.97	±	1138.93	1454.69	±	748.9953
K01_HL	hr	/	±	/	0.21648	±	0.097415
K10_HL	hr	0.13213		0.1005	0.49594	±	0.186669
Alpha	1/hr	11.9077	±	6.27947	2.85026	±	0.847724
Beta	1/hr	1.35979	±	0.60358	0.08926	±	0.048583
Alpha_HL	hr	0.0869	±	0.07795	0.25886	±	0.063732
Beta_HL	hr	0.9065	±	1.13075	10.8139	±	7.215173
Cmax	ng/mL	/	±	/	936.861	±	512.7385
Tmax	hr	/	±	/	0.33537	±	0.1168
CL‡	mL/hr/kg	679.419	±	312.95	4812.8	±	3721.237
V2‡	mL/kg	297.564	±	428.636	27870.9	±	22017.29
AUMC	hr*hr*ng/mL	702.329	±	378	/	±	/
MRT	hr	0.52	±	0.51	/	±	/
V1‡	mL/kg	157.81	±	179.621	3367.13	±	2777.976
K01	1/hr	/	±	/	4.63402	±	4.202936
K10	1/hr	7.91	±	5.62956	1.66127	±	0.922205
K12	1/hr	3.16	±	1.84219	1.10169	±	0.443344
K21	1/hr	2.19465	±	1.03867	0.17657	±	0.130243
F%	%				19.75	±	12.16

AUC, area under the plasma concentration–time curve; K01_HL, half-life of the absorption phase; K10_HL, half-life of the elimination phase; Alpha, rate constant associated with distribution; Beta, rate constant associated with elimination; Alpha_HL, distribution half-life; Beta_HL, elimination half-life; C_{max}, peak plasma concentration; T_{max}, time of peak; CL, clearance; V2, volume of compartment 2; AUMC, area under the first moment curve; MRT, mean residue time; V1, volume of compartment 1; K01, absorption rate; K10, elimination rate from compartment 1; K12, rate of movement from compartment 1 to 2; K21, rate of movement from compartment 2 to 1; F%, bioavailability. ‡ For the oral administration these parameters are divided for their bioavailability.

410 Figure captions

411 Fig. 1. Mean plasma concentrations of flupirtine vs. time curves following PO (5 mg⁄kg) (─●─) and
412 IV (--○--) (1 mg⁄kg) administrations in healthy donkeys (*n* = 6). The window graph focuses on the
413 FLU plasma concentrations detected in first 6h after treatment. Bars represent the standard
414 deviations.

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421 Fig 1

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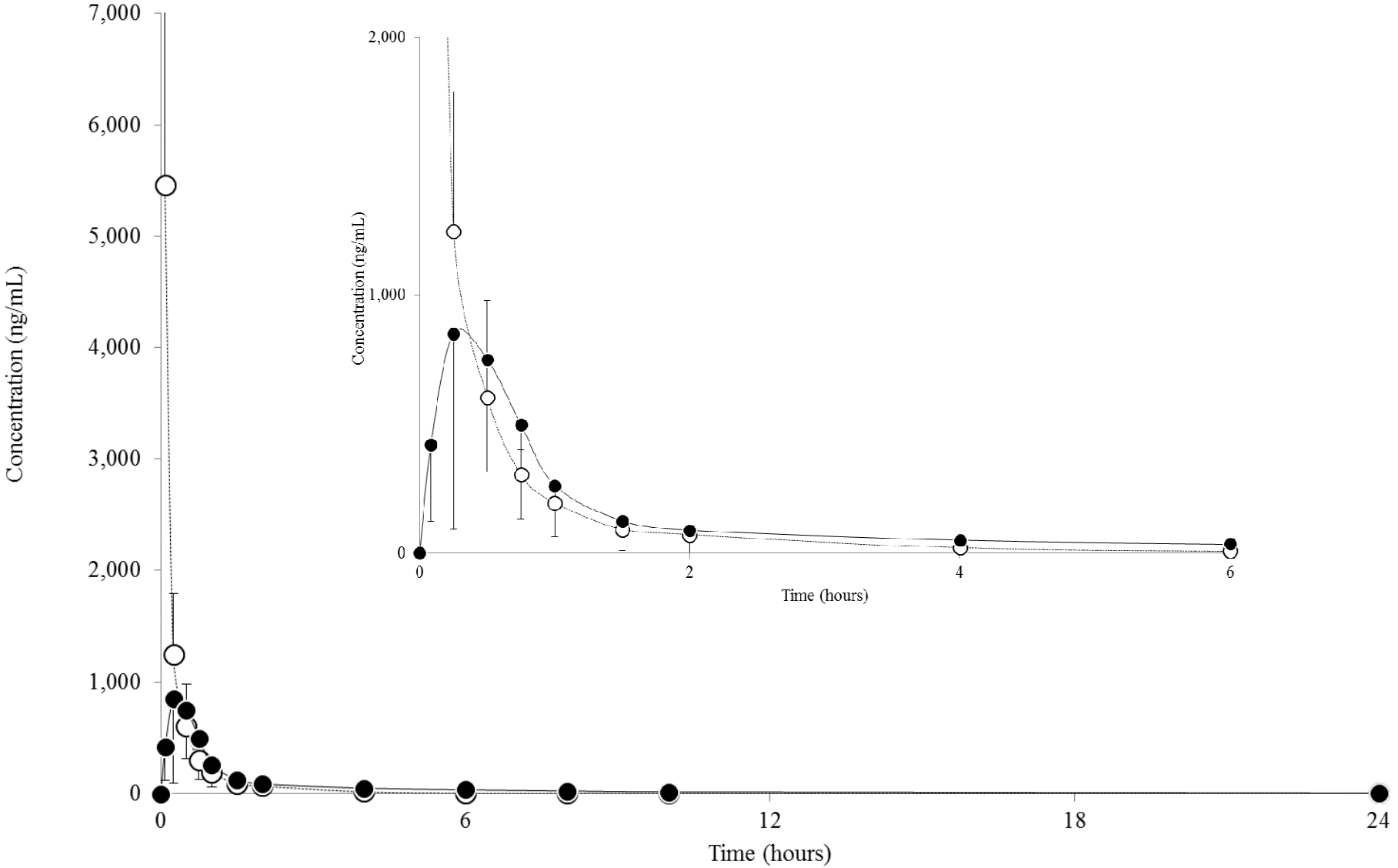

Highlights

Flupirtine IV (1 mg/kg) and oral (5 mg/kg) administered, did not show any adverse effect in donkeys

Flupirtine oral bioavailability was about quite low in donkeys (about 20%)

The pharmacokinetics of flupirtine in donkeys is different from those earlier reported in cats and dogs