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Research Articles

Bioanalytical Method Validation and Quantification of Flupirtine in Canine Plasma by HPLC with Spectrofluorimetric Detection

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Abstract: Flupirtine (FLU) is a non-opioid analgesic drug belongs to the class of N-Methyl-D-Aspartate (NMDA) antagonist without antipyretic or antiphlogistic properties. No analytical method to detect FLU in canine plasma samples through a fluorimetric detector has been published to date. The analytical method described in this work provides a selective and accurate quantification of FLU. The mobile phase consisted of ACN:$AcONH_4$ (20 mM) pH 6.8 (60:40, v/v) at a flow rate of 1 mL min^{-1} in isocratic mode. Excitation and emission wavelengths were set at 323 and 370 nm, respectively. The recoveries of FLU and IS (trazodone) were about 89 and 77%. Typical retention times for FLU and IS was 4.6±0.2 and 5.8±0.2 min, respectively. Limits of quantification and detection were 1 and 0.3 ng/mL, respectively. The described method was validated according to international guidelines on the bioanalytical method validation. The applicability of this method was verified by determining FLU in canine plasma after single oral treatment with 5 mg kg^{-1} of Efiret®. The low LOQ showed that the present method could be useful for the FLU measurement even when administered in sub-clinical doses.

Keywords: Flupirtine HPLC-FL, Bioanalytical Method, Dog, Plasma Concentration

Introduction

Flupirtine (FLU), ethyl-*N*-[2-amino-6-(4-fluorophenylmethylamino) pyridin-3-yl]carbamate, is a triaminopyridine derivative (Fig. 1). It is commercially available as a maleate salt because FLU itself is poorly water soluble. It is a weak base (pKa 5.3) with weak lipophilic features (Klawe and Maschke, 2009).

FLU is a non-opioid analgesic drug without antipyretic or antiphlogistic properties. It is the first drug in this unique class within the World Health Organization analgesics groupings: The 'Selective Neuronal Potassium Channel Openers' (SNEPCO) (Kornhuber *et al*., 1999). FLU was first approved in Germany 1989 (Singal *et al*., 2012). The main applications of FLU in human medicine are for the management of pain following surgery, trauma, dental extraction, pain associated with muscle spasms, cancer, degenerative joint diseases and conditions such us headache and dysmenorrhoea (Harish *et al*., 2012). FLU interacts with a G-protein-regulated channel, namely the Inwardly Rectifying K^+ channels (GIRKs). The GIRKs represent a newly recognised family of K^+ channels distinct from the voltage-dependent ones. They are regulated by neurotransmitters, occur as different subtypes and are variously expressed in different parts of the brain. FLU activates GIRKs and stabilizes the membrane resting potential by activating potassium channels KCNQ, which in turn generates neuronal hyperpolarizing current (M-current) (Giorgi and Owen, 2012). By increasing the M-current, FLU is able to decrease neuronal excitability (Kolosov *et al*., 2012). Moreover, FLU inhibits the NMDA receptor indirectly by acting as an oxidizing agent at the redox site of the NMDA receptor (Singal *et al*., 2012), while the Mg^{2+} block on the NMDA receptor remains unchanged (Kornhuber *et al*., 1999).

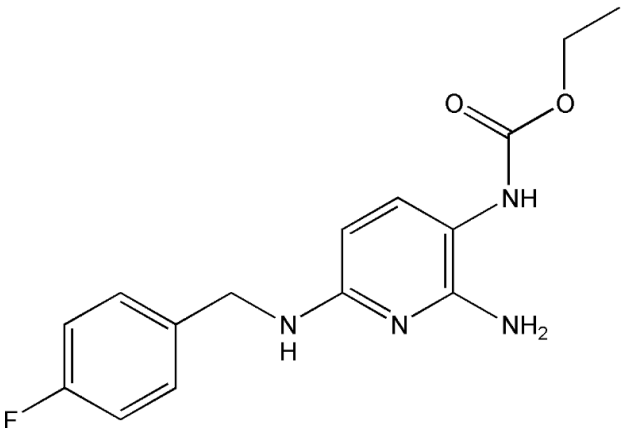

Fig. 1. Chemical structure of flupirtine: ethyl-N-[2-amino-6-(4-fluorophenylmethylamino)pyridin-3-yl]carbamate

The unique pharmacological properties of FLU contributes to its therapeutic benefits, without undesirable adverse effects such as respiratory depression, tolerance and dependence that are typical of opioids, or the gastrointestinal and renal problems associated with non-steroidal antiinflammatory drugs (Devulder, 2010). A number of studies also report an important superadditive (synergistic) effect between FLU and opioid drugs (Kolosov *et al*., 2012; Capuano *et al*., 2011, Lee *et al*., 2015).

FLU is rapidly absorbed from the gastrointestinal tract in humans (Friedel and Fitton, 1993). It does not undergo cytochrome P450 enzyme mediated metabolism to a significant extent. The oral and rectal bioavailabilities are about 90% and 72.5%, respectively. Plasma half-life is in the range 8.5-10.7 h (Niebch *et al*., 1992). A single study is present in the literature concerning its pharmacokinetics in laboratory species (Obermeier *et al*., 1985).

Various analytical methods have been developed to quantify FLU in biological matrices such as plasma and urine from rats and dogs by HPLC-UV and radio-immunoassay (Obermeier *et al*., 1985), human plasma and urine by HPLC-FL (Niebch *et al*., 1992; Narang *et al*., 1984) and human or rat plasma by LC-MS-MS (Chen *et al*., 2001; Zhong *et al*., 2001; Kandasamy *et al*., 2011). This latter device has also been used for the investigation of the *in-vitro* metabolism of FLU (Methling *et al*., 2009). Recently other methods using HPLC (Kathirvel *et al*., 2013) and LC-MS-MS (Zhang *et al*., 2014) have also been developed in FLU based pharmaceutical formulations in order to evaluate impurities.

There is a substantial body of evidence on the efficacy of FLU in humans however this is insufficient to recommend its off-label use in veterinary clinical practice (Giorgi and Owen, 2012). Moreover, nowadays, animals (especially pets) are treated as members of the family and pet owners demand the same level of care they expect for themselves. This change in attitude has resulted in a push for the development of more effective and innovative veterinary therapy (Giorgi *et al*., 2012; Giorgi and Yun, 2012). Since only one HPLC-UV study has been described to detect FLU in canine plasma (Obermeier *et al*., 1985), the aim of the present study was to validate a simple, accurate and rapid HPLC-FL method for the measurement of FLU concentration in canine plasma.

Materials and Methods

HPLC-FL

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 (250×4.6 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. A range of buffer pH (4.0, 5.0, 6.0 and 6.8) was assayed to optimize the chromatographic separation. Excitation and emission wavelengths were set at 323 and 370 nm, respectively. The elution of the substances was carried out in isocratic mode.

HPLC-MS

HPLC-MS Chromatographic separation was performed by an Agilent Technologies (Santa Clara, CA, USA) 1290 HPLC system which consisted of a high pressure pump, auto-sampler and column oven, coupled to an Applied Biosystems/Sciex (Foster City, CA, USA) API 4000 triple quadrupole mass spectrometer, equipped with Turbo V electrospray ionization source (ESI). Main parameters were as follows: positive ion mode (Ps); ionspray voltage, 5.5 kV; declustering potential, 50 V; mass range m/z 100-800 Da; source temperature, 600°C. HPLC runs were carried out with the same parameters used for HPLC-FL and reported in the previous section.

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). Other compounds tested as internal standards were retigabine and metoclopramide. They were supplied in powder form by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN), methanol (MeOH), dichloromethane (CH_2Cl_2), diethyl ether (Et_2O) 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). Formic acid (HCOOH) was purchased from Sigma-Aldrich (Steinheim, Germany). All other reagents and materials were of analytical grade and supplied from commercial sources. The LC mobile phase were filtered through 0.2 µm cellulose acetate membrane filters (Sartorius Stedim Biotech S.A., Aubagne Cedex, France) with a solvent filtration apparatus.

Standard Solutions

Singular stock solutions of FLU and IS in water were prepared, at a concentration of 500 µg mL^{-1} and 1000 µg/mL respectively, using volumetric flasks. These solutions were successively diluted (water) to reach final concentrations of 1, 10, 20, 50, 100, 200, 500, 1000 and 2500 ng/mL for FLU and 5, 10, 20, 50, 100 and 250 µg/mL for the IS. These were stored at −20°C. Canine drug-free plasma was provided by the blood bank of the Veterinary Hospital of the University of Pisa. It was spiked with the following FLU concentrations 1, 10, 100, 200, 500 and 1000 ng/mL. The calibration curve was obtained using the six known spiked samples above mentioned and was based on analyte/IS peak area ratios. Correlation coefficients for the calibration curves were $r^2>0.98$.

Sample Extraction

The procedure was performed in a 15 mL polypropylene vial. A 500 µL aliquot of plasma sample was added to 100 µL of IS (100 µg mL^{-1}) and vortexed for 60 sec. Four mL of AcOEt:CH_2Cl_2 (7:3v/v) were added, then the sample was vortexed (30 sec), shaken (100 osc/min, 10 min) and centrifuged at 3,000 g for 10 min at 10°C. Three mL of the supernatant were collected in a separate 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 were injected onto the HPLC-FL.

Bioanalytical Method Validation

The described method was validated in terms of linearity, Limit Of Detection (LOD), Limit Of Quantification (LOQ), recovery, specificity, stability, precision and accuracy according to international guidelines on the bioanalytical method validation (Anonymus, 2011).

Within-run and between-run accuracy and precision were assessed on quality control samples (QC samples) and determinated by replicate analyses using 3 determinations of different concentration levels: LOQ (1 ng/mL), low QC (50 and 100 ng/mL), medium QC (500 ng/mL) and high QC (1000 ng/mL).

Stability studies were performed to ensure good reproducibility of the method. Stock solution of the analyte and IS (10 µg/mL) and high and low QC samples were tested for short-term room temperature conditions, long term storage conditions (−20°C) and freeze–thaw stability. Short-term stability determinations were obtained by thawing the QC samples and keeping them at room temperature for 24 h, whereas long-term stability was assessed by storing the samples for a period of 30 days at −20°C.

Animal Treatment and Sampling

Blood samples were obtained from two healthy female adult dogs administered with a single oral dose of FLU (5 mg kg^{-1}) after a 12 h overnight fasting period. Accurate dosing was achieved by partitioning the commercially available hard capsules (Efiret® 100 mg hard capsules, FLU maleate, Meda Pharma S.p.A. Milano, Italy). The blood (2-3 mL) was collected via indwelling catheter previously inserted in the left jugular vein, at assigned times (0, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 24 h). The study protocol was approved by the University of Pisa's ethics committee for animal welfare (CEASA) and transmitted to the Italian Ministry of Health (protocol # 0014896).

Quantification

When unknown samples were assayed, a blank and a fortified blank sample were processed simultaneously for quality control. LOD and LOQ were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively.

Statistical Analysis and Pharmacokinetic Evaluation

The statistical analyses were evaluated using the Student's t-test. The results were presented as mean ± Standard Deviation (SD). In all the experiments, differences were considered significant if the associated probability level (P) was lower than 0.05. The pharmacokinetic calculations were carried out by Win-Nonlin v 5.2.1 (Pharsight Corp, Sunnyvale, CA, USA), using the standard non-compartmental analysis.

Results

Detection Method Development

Derivation of the method was obtained by combining data from early studies (Obermeier *et al*., 1985; Narang *et al*., 1984), with some modifications. Two mobile phases were preliminarily tested (water + formic acid [200:1 v:v]:ACN, 40:60 v/v; $AcONH_4$[20 mM]:ACN, 40:60 v/v). The latter mobile phase with a 1.0 mL/min flow rate was found to optimally provide separation between FLU, IS and matrix interference peaks. A range of buffer pH (4.0, 5.0, 6.0, 6.8) were tried to optimize the chromatographic separation. The retention time of FLU was insensitive to pH showing negligible variations. The IS was influenced by different pH (Fig. 2).

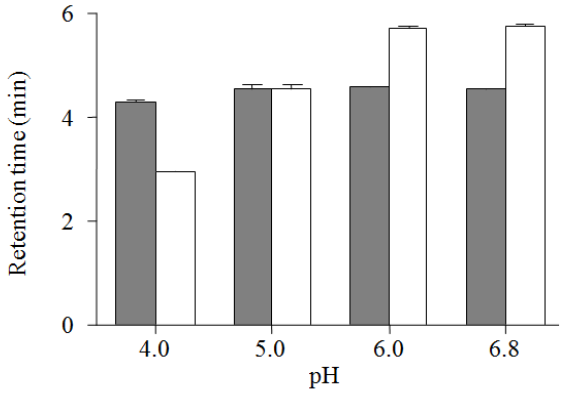

Fig. 2. Effect of the pH on the retention time of FLU (gray bars) and IS (white bars)

At pH 6 the peak was flatter than those found at pH 6.8; at pH 5 an almost complete overlapping of FLU and IS peaks was shown; at pH 4 the IS peak moved to a retention time of 2.9 min, overlapping the matrix peaks. A pH value of 6.8 provided the best separation between the FLU, IS and matrix peaks. These analytical conditions were an excellent compromise in terms of sensitivity, peak separation and backpressure (125 bar).

A full scan of pure FLU and IS by the fluorescence detector showed the values of 323 and 370 nm as the optimal excitation and emission wavelenghts for the two compounds, respectively.

Three compounds (retigabine, metoclopramide and trazodone) with amenable chemical features were evaluated as ISs. Among these, trazodone was found to be the best candidate with an excellent resolution and a suitable retention time, providing the shortest chromatographic course and with peaks well distinct and separated from both the FLU peak and matrix interferences (Fig. 3). On the other hand, retigabine, the most similar to FLU in chemical and physical characteristics, showed partial overlap with FLU, especially at high concentrations. Metoclopramide was not well separated from the interferences due to the matrix (data not shown).

Optimization of the Extraction Method

Solvents such as AcOEt, CH_2Cl_2 and Et_2O were examined. AcOEt and CH_2Cl_2 were selected as the most suitable organic solvents in terms of analyte extraction and minimization of matrix components. Variations in the proportions of the selected extraction solvents (AcOEt:CH_2Cl_2, 7:3, 3:7, 3:2, v/v) were also assessed in terms of recovery and selectivity. AcOEt:CH_2Cl_2, 7:3 v/v showed the best recovery for FLU (range 82-89%) and the IS (77.41±9.82%) (Table 1).

Method Validation

It was necessary to validate each step in the analytical method because to date, methodology for the determination of FLU from canine plasma samples through a fluorimetric detector is yet to be published. The calibration curves were constructed by plotting the ratio of the peak areas versus concentrations in the working range of 1-1000 ng/mL. The calibration equation was Y=9980X-36800 and the correlation coefficient was r^2=0.980. A good linearity was achieved in the investigated range of 1-1000 ng/mL for FLU. According to EMA guidelines, LOD and LOQ were calculated based on signal-to noise approach. The typical signal-to-noise ratios were 10:1 and 3:1 for LOQ and LOD, respectively. Both the trueness and the precision of these values lay within the proposed criteria (RSD%, <20%). The specificity was investigated in regard to the other co-eluting components by comparing the chromatograms of different batches of blank matrices to those from spiked plasma solutions and test samples. It was found that under optimized chromatographic conditions, peaks due to the matrix did not interfere with FLU and IS (Table 2). Typical retention times for FLU and IS was 4.6±0.2 and 5.8±0.2 min, respectively (Fig. 3). The trueness (94.14%) was evaluated by determining the recovery for five replicates of the QC samples (Table 2). The FLU recovery ranged from 89.30% to 82.97%. The LOQ was 1 ng/mL: it has been considered excellent for determining the pharmacokinetic profiles of FLU (Chen *et al*., 2001). The CV% value was 4.47%. Intra-day value consistency (repeatability) was evaluated for three replicates of the QC samples during the same day (3.7-4.1%) (Table 3). Inter-day value consistency (intermediate precision) was evaluated by quantization of FLU in QC samples on three different days (0.6-4.8%) (Table 4). Relative errors for both the intra-day and inter-day trueness were <5%.

Short term stability (room temperature) showed a CV% 3.9%. Data obtained after three freeze-thaw cycles showed that FLU was stable in dog plasma (CV%, <5%). Robustness of the methodology was determined by the reproducibility of results using the analytical method in different laboratories or under different circumstances. The present study evaluated three plasma aliquots from the treated dogs in two different labs (Department of Surgical, Medical, Molecular Pathology and Critical Area, University of Pisa, Italy) and obtained variations of less than 7.2%. The validation parameters were in agreement with the EMA guidelines (Anonymus, 2011).

HPLC-MS Analysis

The selectivity of the method was also confirmed by HPLC-MS analysis of plasma samples collected in treated dogs. No compounds co-eluting with the analyte of interest were detected by full scan acquisitions in positive ion mode. This is particularly demonstrated from the HPLC-MS extracted ion chromatogram (m/z 305 and 372 Da for FLU and IS, respectively) that exhibited a good correspondence with the HPLC–FL chromatogram. Retention times of both FLU and IS were not substantially altered (4.70±0.2 and 5.85±0.3 min for FLU and IS respectively) (Fig. 4).

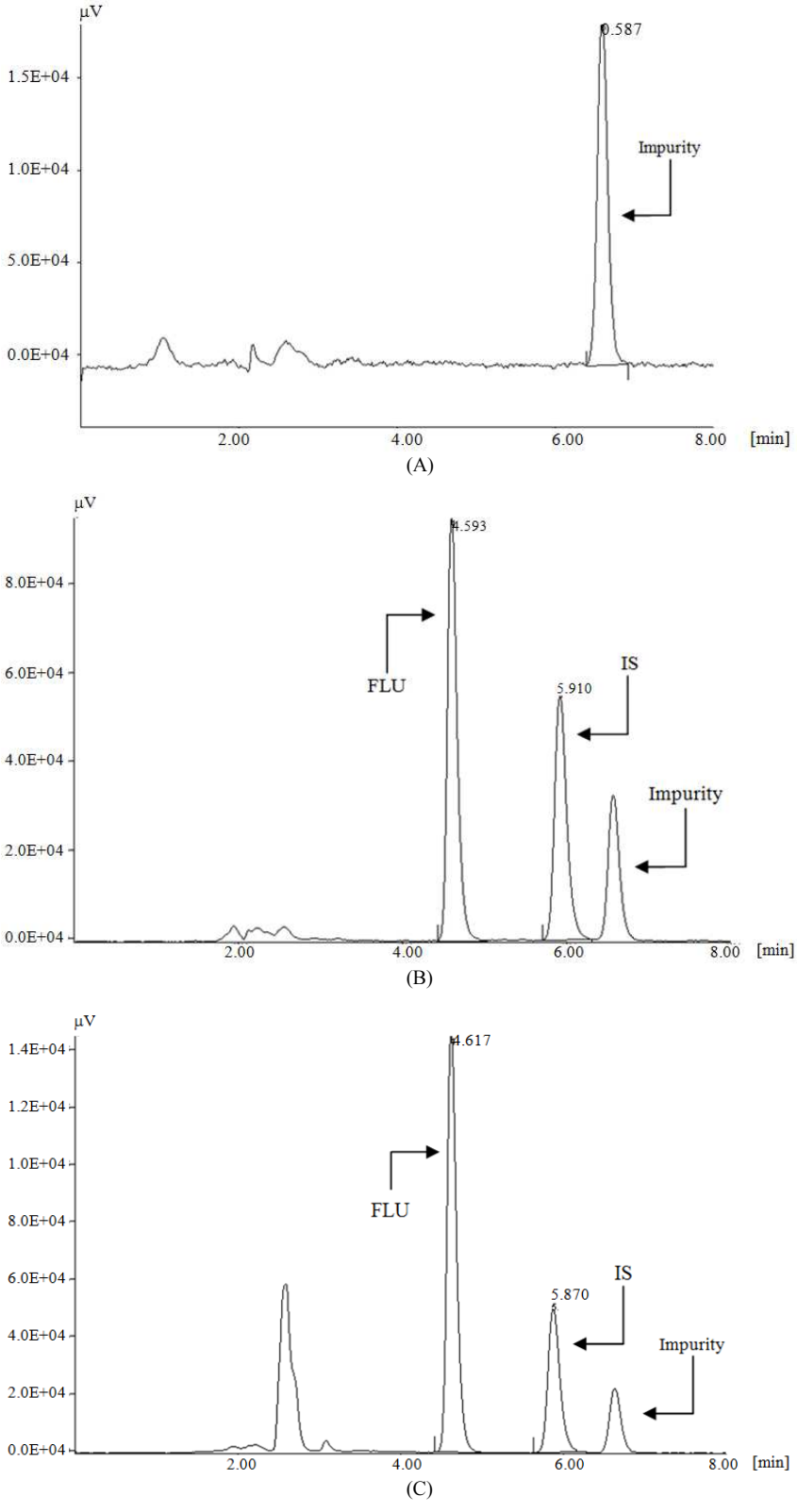

Fig. 3. Chromatographic curve by HPLC-FL. (A) Chromatographic curve from canine control plasma. (B) Chromatographic curve from fortified sample (FLU 50 ng/mL; IS 10 µg/mL). (C) Chromatographic curve from plasma sample collected in a dog (6h) orally administered with FLU (5 mg kg^{-1})

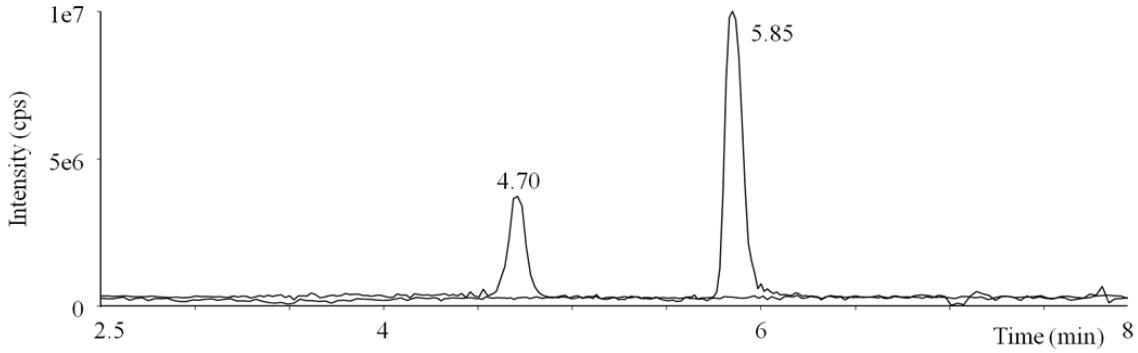

Fig. 4. HPLC-MS extracted ion chromatogram. It was acquired from a real sample and is relative to the ions at m/z 305 and 372 Da. The peaks from FLU and IS, clearly shown at 4.70 min and 5.85 min, respectively, confirm a good correspondence between HPLC

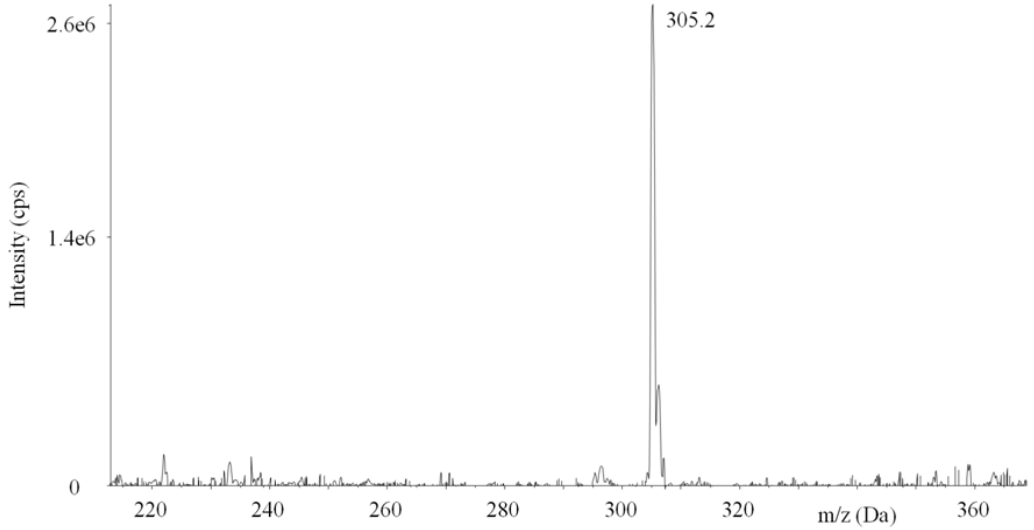

Fig. 5. Mass spectrum in positive ion mode. Spectrum obtained from the analyte peak at 4.70 min. A real sample [5 mg kg^{-1} treated dog (1 h)] acquired in positive ion mode

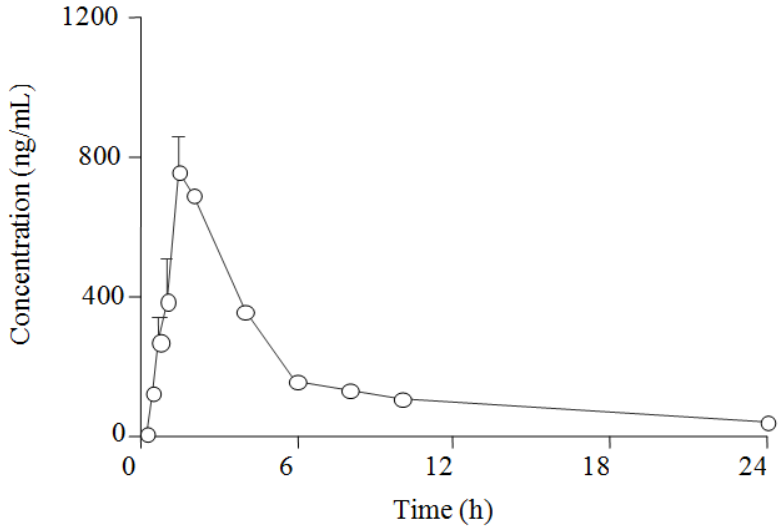

Fig. 6. Mean concentration-time curve of FLU after a single oral dose (5 mg kg^{-1}) of Efiret® in 2 adult dogs

Table 1. Extraction recovery % (±SD) of FLU and IS spiked at 10, 50, 100 ng/mL of FLU and 10 μg/mL of IS

	Recovery %				
			FLU		IS
Organic solvents	v/v ratio	10ng/mL	50ng/mL	100ng/mL	10μg/mL
AcOEt:CH_2Cl_2	7:3	89.30±13.76	85.70±4.54	82.97±11.39	77.41±9.82
AcOEt:CH_2Cl_2	3:7	47.06±12.12	42.07±4.60	51.27±3.28	40.11±7.22
AcOEt:CH_2Cl_2	3:2	71.97±1.69	78.69±9.36	65.84±4.27	62.80±11.97

Table 2. Summary of validation data for FLU

Property	Units	Flupirtine
Linear range	ng/mL	1-1000
Calibration equation		Y=9980X-36800
Correlation coefficient	r^2	0.980±0.0594
LOQ	ng/mL	1
LOD	ng/mL	0.3
Trueness	%	94.14
Precision	%	
Inter-day		0.6-4.8
Intra-day		3.7-4.1
Specificity		Specific

Table 3. Intra-day values of FLU

Concentration level (%)	Spiked concentration (ng/mL)	Concentration obtained (ng/mL)	Mean repeatability (%)
	1	0.95	
1	1	0.98	95.9
	1	0.94	
	50	50.7	
50	50	51.6	100.8
	50	49.0	
	100	101.2	
100	100	102.6	101.3
	100	103.0	
	500	487.5	
500	500	501.0	99.9
	500	510.0	
	1000	1029	
1000	1000	1040	103.7
	1000	1042	

Table 4. Inter-day values of FLU

Concentration level (%)	Spiked concentration (ng/mL)	Concentration obtained (ng/mL)[§]	Mean repeatability (%)
	1 (day 1)	0.99, 1.0, 0.98	
1	1 (day 2)	0.98, 1.0, 0.99	99.4
	1 (day 3)	1.0, 0.97, 1.03	
	50 (day 1)	50.6, 49.8, 50.9	
50	50 (day 2)	47.8, 49.4, 49.6	100.8
	50 (day 3)	52.1, 51.6, 50.6	
	100 (day 1)	108.9, 109.5, 108.2	
100	100 (day 2)	103.6, 104.2, 103.0	104.8
	100 (day 3)	100.9, 101.6, 100.2	
	500 (day 1)	527.0, 520.1, 533.9	
500	500 (day 2)	499.5, 500.9, 4.98.1	102.9
	500 (day 3)	517.0, 515.9, 518.1	
	1000 (day 1)	989.0, 991.5, 986.5	
1000	1000 (day 2)	1041.0, 1039.0, 1037.0	101.7
	1000 (day 3)	1025.5, 1020.5, 1023.0	

[§]Each row reports three concentration values obtained in the same day

Table 5. Main pharmacokinetic parameters of FLU after a single oral dose (5 mg/kg) of Efiret® in 2 adult dogs

		Oral 5 mg/kg (n=2)	
Parameter	Units	Mean	±SD
R^2		0.99	0.01
λ_Z	1/h	0.09	0.03
$T_{1/2}\ \lambda_Z$	h	7.48	2.10
T_{max}	h	1.75	0.35
C_{max}	ug/mL	0.85	0.22
$AUC_{0-\infty}$	h*ug/mL	4.10	0.29
Vz/F	mL/kg	13066.55	2767.52
CL/F	mL/h/kg	1222.83	86.89
MRT	h	8.50	2.62

R^2, correlation between observed/predicted points; λz, the rate constant associated with the terminal elimination phase;$T_{1/2}\lambda_Z$, half-life of the elimination phase; T_{max}, time of peak; C_{max}, peak plasma concentration; $AUC_{0-\infty}$, area under the plasma concentration–time curve extrapolated to infinity; CL/F, apparent clearance; Vz/F, apparent volume of distribution; MRT, mean resident time

Moreover, mass spectra in positive ion mode showed only one significant ion at m/z 305.2 Da, confirming that FLU is the only compound responsible for the peaks at 4.70 min (Fig. 5). This ion, as confirmed by further MS–MS experiments, is the pseudo molecular ions ($[M^+H]^+$) of FLU.

The peak at 2.5 min reported in Figure 3c has not any fragmentation similar to FLU (data not shown). Hence, it has been concluded that it is an artefact due to the individual dog matrix.

Application of the Method

The applicability of this method has been verified by determining FLU in canine plasma after single oral treatment with 5 mg kg^{-1} of Efiret®. HPLC analysis of the plasma confirmed the presence of FLU in time related amounts (Fig. 6). The average FLU concentration in plasma ranged between 4.3 and 760 ng/mL. The described method allowed monitoring of the concentration versus time curves of the analyte and the calculation of the basic pharmacokinetic parameters (Table 5).

Discussion

A number of analytical methods have been employed for the detection of FLU, mostly in biological matrices. Obermeier *et al.* (1985) used the HPLC-UV to assess the pharmacokinetic profile of FLU. Unfortunately in that study, the lack of both method validation and determination of key parameters (i.e., LOQ, recovery, accuracy, etc) prevents a comparison with the present method. The two HPLC-FL methods present in the literature (Niebch *et al*., 1992; Narang *et al*., 1984) are partially validated. They reported only some of the key parameters requested by the EMA guidelines (Anonymus, 2011) and showed longer chromatographic curves then the present study. The LOQ detected in the present study was lower than those reported in the previous HPLC-FL methods (Niebch *et al*., 1992; Narang *et al*., 1984). In contrast, methods carried out with expensive devices such as LC MS-MS with Atmospheric Pressure Chemical Ionization (APCI) (Chen *et al*., 2001), Electrospray Ionization (ESI) (Zhong *et al*., 2001; Kandasamy *et al*., 2011) and Ultra-Performance Liquid Chromatography (UPLC) (Satheesh *et al*., 2012) showed comparable values of selectivity, accuracy, precision, stability and specificity to those found in this study. The findings concerning short and long term stability indicated that the room temperature and storage of analyte in plasma samples at −20° C is adequate and no stability-related problems would be expected during routine analyses for pharmacokinetic study. The results of the validation parameters demonstrated that the method enables accurate quantification of FLU. Narang *et al*. (1984) simultaneously detected FLU and its acetylate metabolite in human plasma. However, the reported LOQ for this metabolite was 200 ng/mL, too high to determine its pharmacokinetic profile after oral administration. According to our preliminary LC-MS-MS no other significant amount of compounds structurally related to FLU has been detected in dog-treated plasma samples (data not shown). It is likely that the metabolic patter between human and dog may differ quantitatively and/or qualitatively (Lin and Lu, 1997). This discrepancy might be due to the earlier proven metabolic interspecies differences (Martignoni *et al*., 2006). However, further *ad hoc* studies need to clarify this issue and pharmacokinetic data have to be considered with the caveat that they are based on measurements from two dogs only and should be integrated with further studies in this animal species.

Conclusion

The analytical method described in this work is fully validated in canine plasma according to the EMA guidelines (Anonymous, 2011). It provides selective and accurate analysis of FLU without the need for expensive clean-up steps, solvent consuming flows or expensive devices. The low LOQ shows that the method could be useful for drug measurement even when administered in

sub-clinical doses. As FLU is a drug recently considered for the veterinary medicine application, this method is the most suitable to be used for pharmacokinetic investigations in canines. In summary, this is the first time that a HPLC-FL technique is validated for determination and quantification of FLU in canine plasma.

Conflicts of Interest and Source of Funding

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

Funding Information

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Author's Contributions

V. De Vito: Participated in all experiments, performed the *in vivo* study, the data-analysis and contributed to the writing of the manuscript.

A. Saba: Coordinated/performed the LC-MS/MS work, and contributed to the writing of the manuscript.

H. Owen: Coordinated the data analysis, contributed to the writing of the manuscript.

M. Giorgi: Contributed to the HPLC study, coordinated the whole study, contributed to the writing of the manuscript.

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