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A sensitive HPLC-MS-MS assay for quantitative determination of midazolam in dog plasma

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Abstract

The clinical pharmacokinetics of midazolam have been extensively studied, due to its high clearance by CYP3A4 and sensitivity to drug–drug interactions. In order to investigate the potential to model drug–drug interactions with midazolam in the dog, a selective and sensitive high performance liquid chromatography-tandem mass spectroscopy (HPLC-MS-MS) method has been developed, with sufficient sensitivity to allow analysis of dog plasma samples generated following administration of a clinically relevant dose. The method involves extraction of midazolam and internal standard (flunitrazepam) from dog plasma, using 96-well Oasis® MCX solid phase extraction plates. The assay has been validated over a concentration range of 0.1–10 ng/ml and its specificity, accuracy and precision demonstrated. The relative bias of the assay was within ±15% for all standards with intra- and inter-assay precision (coefficient of variation—%CV) of less than 15%. The assay was applied to the analysis of plasma samples (0.2 ml), generated following intravenous or oral administration of midazolam to male beagle dogs, at a dose level of 0.05 mg/kg, and pharmacokinetic parameters were derived from the resulting data.

Keywords: Pharmacokinetics; Midazolam; Dog; High performance liquid chromatography; Mass spectrometry; Drug interaction

1. Introduction

Midazolam (Fig. 1) is a potent, short acting benzodiazepine [1], which is commonly used clinically as a probe substrate for drug–drug interaction studies involving the CYP3A subfamily [2–4]. These studies have shown that co-administration of the antimycotic agent ketoconazole, a potent CYP3A inhibitor, has a marked effect on the pharmacokinetics of midazolam. Following ketoconazole therapy (200 mg/day, 3 days), AUC increased five-fold after intravenous midazolam administration with an 84% decrease in intrinsic clearance. The utility of the dog as an animal model for this type of interaction study has also been demonstrated following intravenous administration of midazolam at a dose level of 0.5 mg/kg [5]. Ketoconazole administration (200 mg b.i.d. for 30 days), caused a two-fold increase in the AUC of midazolam on day 1 increasing to three- to four-fold on day 30, with a concomitant increase in plasma half life and decrease in clearance. However, at the dose levels used for these studies, side effects such as transient agitation, weakness, ataxia and hyper responsiveness to noise have

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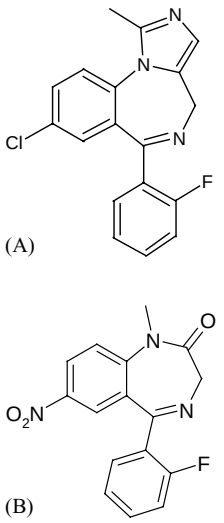

Fig. 1. Chemical structures of midazolam (A) and flunitrazepam (B) (internal standard).

been reported in dogs following i.v. administration of midazolam [6,7]. To minimise these adverse reactions and to provide a greater clinical relevance, a lower dose was selected (0.05 mg/kg) for the current studies. However, this approach required the development of a more sensitive assay in order to measure drug levels in plasma.

Several analytical methods for midazolam have already been developed. The existing HPLC-UV assays [8–10] were considered to be of insufficient sensitivity (LOQ 2.5–15 ng/ml) for the dose level used in these studies. The coupling of HPLC with positive TurboIonSpray leads to a more sensitive and specific technique and inclusion of a second stage of mass analysis (MS-MS) further increases selectivity. There are many examples of analytical methods for benzodiazepines, which incorporate mass spectrometry as an analytical endpoint [11–13], and this approach has been used to quantify levels of midazolam and its major metabolites in monkey plasma, [14] with appropriate sensitivity (0.1 ng/ml). However, sample preparation involved the use of a liquid/liquid extraction step, which reduces the potential for assay automation.

This paper describes a 96-well solid phase extraction method for the isolation of midazolam and internal standard from dog plasma, with HPLC-MS-MS detection. Validation criteria were considered during the development of the analytical procedure and the analytical technique was applied to the analysis of plasma samples from a single dose i.v./oral crossover study in the male Beagle dog at a dose level of 0.05 mg/kg. The limit of quantitation of the method was 0.1 ng/ml for a plasma volume of 0.2 ml.

2. Experimental

2.1. Materials

Midazolam and flunitrazepam (internal standard); Fig. 1 were obtained from Pfizer Global Research and Development, Sandwich, UK (purity >95%). Super purity grade methanol and dichloromethane were obtained from Romil Ltd. (Cambridge, UK). Dichloromethane, isopropanol, ammonium acetate (99%+) and ammonia solution (35%) were from Sigma-Aldrich Co. (Gillingham, Dorset, UK). Orthophosphoric acid and hydrochloric acid were from Fischer Scientific (Loughborough, UK). Purified water was produced from a Milli-Q plus ultrapure water system (Millipore, Watford, UK). 96-well solid phase extraction plates (Oasis, 30 mg MCX—2 ml capacity) were obtained from Waters Chromatography (Elstree, UK).

2.2. Preparation of standard solutions

Primary stock solutions of the analyte were prepared at an initial concentration of 100 μg/ml in methanol and working solutions (0.01 and 0.1 μg/ml) were prepared by serial dilution of the stock with methanol. A stock solution of internal standard (flunitrazepam) at a concentration of 150 μg/ml was prepared in methanol and a working dilution of internal standard was prepared at a final concentration of 0.1 μg/ml. All solutions were stored at 4 °C for up to 14 days.

2.3. Preparation of calibration curves

Control dog plasma was defrosted at room temperature and centrifuged at 3000 rpm for 5 min. 200 μl were transferred to 10 ml glass tubes (Fischer) using a glass Pasteur pipette and appropriate volumes of midazolam (0.01 and 0.1 μg/ml) added, to yield a concentration range of 0.1–10 ng/ml. Samples were vortex mixed prior to solid phase extraction along with blank

plasma samples, which contained neither drug or internal standard. The calibration curve was prepared by weighted linear regression analysis ($1/y^2$) of the peak area ratio (drug/internal standard) against concentration of calibration samples (Analyst v1.2—Applied Biosystems, Foster City, USA). Midazolam concentrations were calculated using the regression line parameters and corrected for any dilutions used. Samples that had concentrations exceeding the highest calibration sample were diluted with control plasma prior to analysis.

2.4. Preparation and analysis of QC samples

The intra-day precision and accuracy of the method over the concentration range was investigated by the replicate analysis of midazolam in dog plasma at three concentrations across the calibration range (0.1, 1 and 10 ng/ml), relative to the calibration standards (n = 8 per concentration). Inter-day assay precision and accuracy was determined by the analysis of duplicate samples at three separate concentrations (0.2, 1 and 9 ng/ml). These QC samples were analysed when freshly prepared, relative to calibration standards prepared from unrelated stock solutions.

2.5. Extraction procedures

Unknown plasma samples were thawed and centrifuged as described previously, and transferred to glass vials. Internal standard (8 μl) was added to all samples except the non-spiked plasma blanks. The samples were then acidified by addition of 20 μl of concentrated orthophosphoric acid and made up to a final volume of 1 ml with purified water. The samples were then vortex mixed prior to solid phase extraction.

Solid phase extraction was carried out using 2 ml capacity Oasis 96-well 30 mg MCX extraction plates conditioned and equilibrated with 1 ml methanol, followed by 1 ml purified water. The acidified plasma samples were applied to the sample wells and passed through the sorbent at a flow rate of approximately 1 ml/min using a vacuum manifold (Porvair Sciences, Kings Lynn, UK). The sorbent was washed with 1 ml 0.1 M hydrochloric acid, followed by 1 ml methanol and dried under full vacuum for 5 min. The analytes were eluted into 96 deep well microplates (Porvair Sciences) using 1 ml dicloromethane:isopropanol:ammonia (78:20:2 v/v/v) and reduced to dryness under a stream of nitrogen heated to 37 °C (Micro DS 96 sample concentrator, Porvair Sciences). The residues were then reconstituted in 250 μl of HPLC eluent and the collection plate centrifuged at 2500 rpm for 5 min prior to injection of 200 μl onto HPLC-MS-MS system.

2.6. HPLC-MS-MS analysis

The HPLC-MS-MS analysis was performed using a Hypersil 100 C18 column (50 mm × 4.6 mm; 5 μm; ThermoHypersil-Keystone, Runcorn, UK) with a mobile phase consisting of a 70:30 (v/v) mixture of 90:10 methanol/water and 10:90 methanol/water (both containing 2 mM ammonium acetate) and 0.027% formic acid at a flow-rate of 1 ml/min. The HPLC system comprised an 1100 binary pump (Agilent Technologies, Waldbronn, Germany) and an HTS Pal autosampler (CTC Analytics, Zwingen, Switzerland). The HPLC flow was split 5:1 using an Accurate™ flow splitter (LC packings, Amsterdam, The Netherlands), such that 200 μl/min was directed to the mass spectrometer. An API 4000 instrument (MDS Sciex, Concord, Ont., Canada) was used in positive TurboIonSpray mode with a source temperature of 400 °C, using nitrogen as the nebuliser, auxiliary, collision and curtain gas. The resolution was set at 0.7 amu width at half-height in both Q1 and Q3. Analytes were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) with a 100 ms dwell time. MRM transitions were optimised by direct infusion of midazolam and flunitrazepam at a concentration of 100 ng/ml using a model 22 syringe pump (Harvard Apparatus, MA, USA) at a flow-rate of 5 μl/min. The optimal transitions were m/z 326 → 291 for midazolam and m/z 314 → 268 for flunitrazepam.

2.7. Dog study

Two male beagle dogs (weight range 14–16 kg; Pfizer colony, Sandwich, UK) received a single dose of 0.05 mg/kg midazolam by intravenous infusion over a period of 15 min. Blood samples (5 ml) were collected at the end of infusion (0.25 h) and at 0.35, 0.5, 1, 1.5, 2, 3, 4, 7, 12 and 24 h post dose for analysis of midazolam concentrations. Plasma was

separated from red blood cells by centrifugation and stored below −15 °C, prior to analysis.

2.8. Pharmacokinetic analysis of data

The results were analysed using linear regression analysis (PKIN, Tessella support services, Abingdon, UK) and pharmacokinetic parameters derived to the last time at which concentrations were above the quantitation limit (4 h). Areas under the plasma midazolam concentration–time curves to infinity (AUC_∞) were calculated using the linear trapezoidal rule. The maximum plasma concentration (C_{max}) and the time to peak (t_{max}) for the oral dose were taken directly from the experimental data. The elimination rate constant (k_{el}) was estimated by least square regression analysis from the data of the last three to four points of each plasma concentration–time curve. The terminal elimination half-life ($t_{1/2}$) was calculated as $\ln(2)/k_{el}$. Bioavailability (F%) was calculated using the AUC_∞ values for the oral and intravenous doses, where

$$F\,(\%) = \frac{\mathrm{AUC}_\infty(\mathrm{Oral})}{\mathrm{AUC}_\infty(\mathrm{Intravenous})} \times 100$$

3. Results and discussion

3.1. Chromatography and specificity

Under the selected LC-MS conditions, typical retention times for midazolam and flunitrazepam were 1.5 and 1.1 min with no interferences from endogenous plasma components at the retention times corresponding to the analytes. A variable broad peak was detected between 0.7 and 1 min with the same m/z transition as midazolam, which was observed in blank samples as well as samples spiked with the analyte or internal standard. Gradient conditions were set up so that this did not interfere with the analyte, however this did cause some deterioration in the peak shape. The total run time of the assay was 3 min. There was no evidence of ion suppression in samples generated from different matrices.

3.2. Linearity

Calibration curves were linear over a concentration range of 0.1–10 ng/ml. A mean coefficient of determination of 0.9975 was derived from three analytical runs, using a $1/y^2$ weighting. The back calculated values for the calibration standards are shown in Table 1, and example chromatograms for blank plasma and calibration standards at 0.1 and 10 ng/ml are shown in Fig. 2.

3.3. Precision and accuracy

The intra-assay precision and accuracy values of the method, assessed using eight spiked plasma samples at concentrations of 0.1, 1 and 10 ng/ml are shown in Table 2. Overall, assay precision and accuracy were similar throughout the range of the calibration with

Table 1
Calibration curve details for the analysis of midazolam in dog plasma

Midazolam measured (ng/ml)	Midazolam added (ng/ml)							
	0.1	0.2	0.4	0.8	1.6	3.0	6.0	10.0
Analysis 1	0.099	0.21	0.39	0.83	1.66	2.80	5.81	10.6
Analysis 2	0.097	0.21	0.42	NR	1.70	2.69	5.81	10.8
Analysis 3	0.096	0.21	NR	0.86	1.78	2.94	5.47	9.72
Mean	0.097	0.21	0.41	0.85	1.66	2.81	5.70	10.4
S.D.	0.002	0.000	0.021	0.021	0.06	0.13	0.20	0.58
Accuracy (relative bias, %[a])	−2.7	5.0	1.3	5.6	7.1	−12.2	−5.1	3.7
Precision (CV[b])	1.6	0.0	5.2	2.5	3.6	4.5	3.4	5.5

NR: No result. Values for coefficient of determination of the calibration in dog plasma ranged from 0.9969 to 0.9986 ($n = 3$).

[a] $\text{Relative bias} = \left(\frac{\text{mean measured value} - \text{prepared value}}{\text{prepared value}}\right) \times 100.$

[b] $\text{CV (coefficient of variation)} = \frac{\text{S.D.} \times 100}{\text{mean}}.$

the mean determined concentrations within 10% of the theoretical values, and coefficients of variation below 15%. The inter-day precision and accuracy were assessed by the analysis of quality control samples (0.2, 1 and 10 ng/ml) in duplicate, on three separate occasions. The results are summarised in Table 3. Precision in this case was within 5% of the theoretical value for each concentration, coefficients of variation were less than 15%.

3.4. Limits of quantitation

The upper limit of quantitation was defined as 10 ng/ml, which gave a 100-fold calibration range for the standard concentrations. The lower limit of quantitation (LLQ) was established at 0.1 ng/ml on the basis of precision and accuracy following replicate analysis. A typical chromatogram of a LLQ sample is given in Fig. 2.

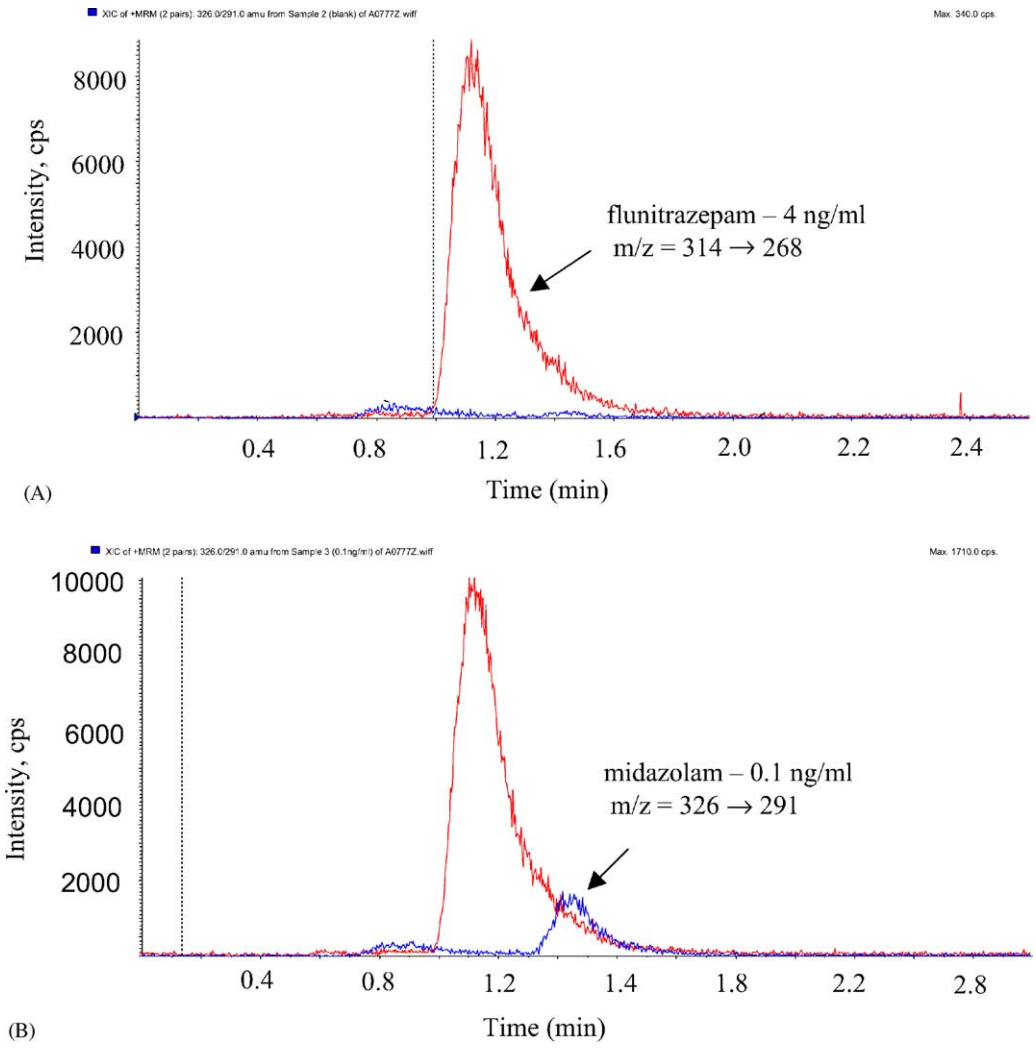

Fig. 2. Representative chromatograms of midazolam (*m*/*z* 326 → 291) and internal standard (flunitrazepam—*m*/*z* 314 → 268) in dog plasma (200 μl). (A) Control plasma blank spiked with 4 ng/ml flunitrazepam. (B) Control plasma spiked with 0.1 ng/ml midazolam and 4 ng/ml flunitrazepam. (C) Control plasma spiked with 10 ng/ml midazolam and 4 ng/ml flunitrazepam. (D) Male dog plasma 1 h after intravenous infusion of midazolam at 0.05 mg/kg.

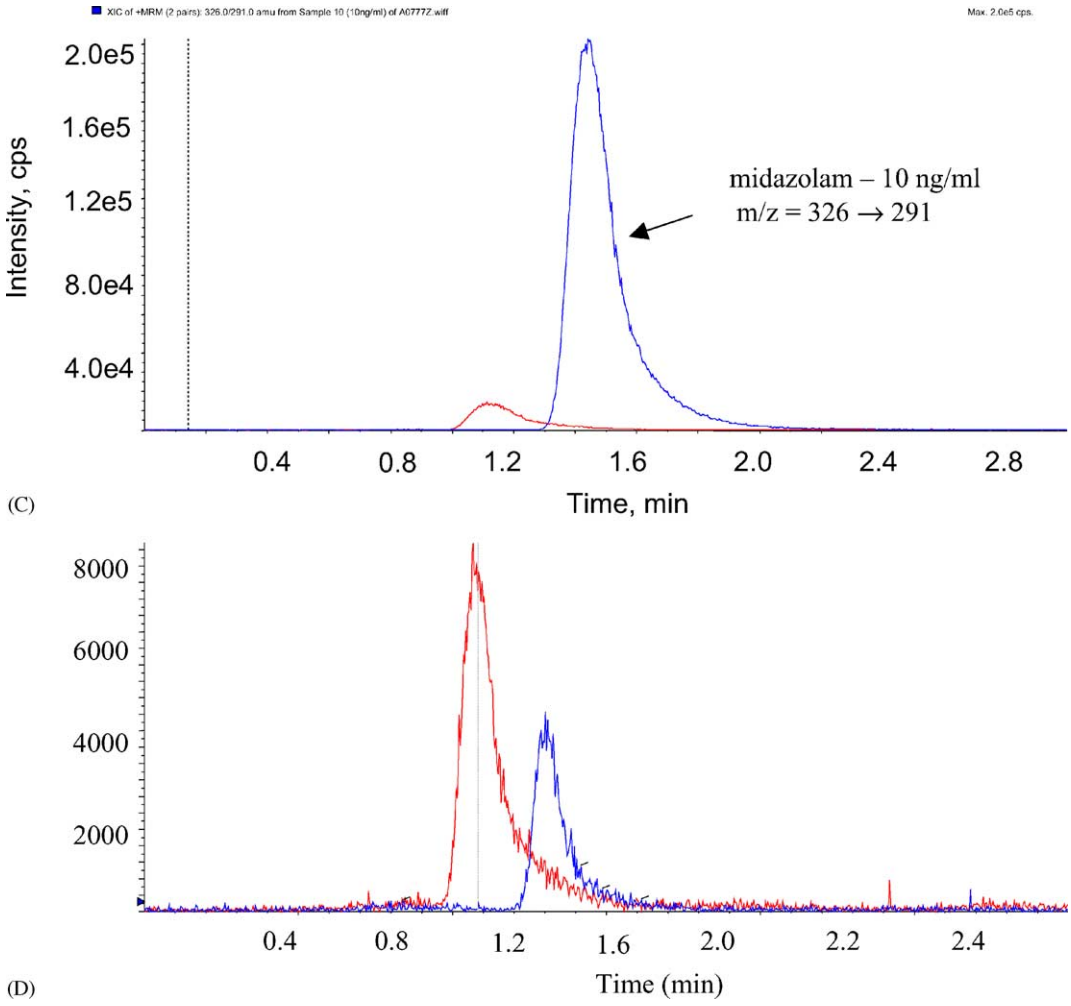

Fig. 2. (*Continued*).

Table 2
Intra-day assay accuracy and precision of the determination of midazolam in dog plasma

Added concentration (ng/ml)	Measured concentration (mean ± S.D., $n = 8$) (ng/ml)	Accuracy (relative bias, %)	Coefficient of variation (%)
0.1	0.095 ± 0.007	−5.2	7.7
1	0.93 ± 0.021	−6.7	2.3
10	9.77 ± 1.014	−2.3	10.4

Table 3
Inter-day assay accuracy and precision of the determination of midazolam in dog plasma

Added concentration (ng/ml)	Measured concentration (mean ± S.D., $n = 6$) (ng/ml)	Accuracy (relative bias, %)	Coefficient of variation (%)
0.2	0.20 ± 0.008	0.1	3.9
1	1.01 ± 0.074	0.7	7.3
9	8.65 ± 0.921	−3.9	10.7

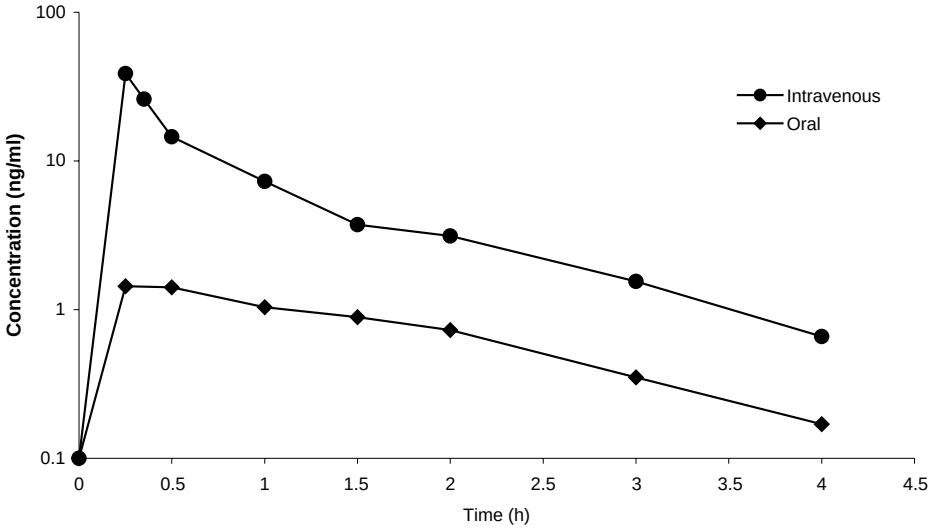

Fig. 3. Mean plasma concentrations of midazolam in male beagle dogs, after single intravenous infusion or oral administration at 0.05 mg/kg.

3.5. Pharmacokinetics in the dog

Pharmacokinetic parameters are shown in Table 4, and plasma profiles following oral or intravenous administration are represented in Fig. 3.

Following both intravenous and oral administration, assay sensitivity was sufficient to measure plasma concentrations up to 4 h post dose. This was considered adequate for the purposes of the study.

3.6. Intravenous phase

Following the end of infusion, plasma concentrations declined with a mean elimination half life of 0.9 h. This value resulted from a mean plasma clearance of 38 ml/min/kg combined with a mean volume of distribution of 2.4 l/kg, AUC_{∞} was 21.9 ng h/ml.

Table 4
Pharmacokinetic parameters in male dogs ($n = 2$) following single intravenous or oral administration of midazolam at a dose level of 0.05 mg/kg

	Intravenous	Oral
$t_{1/2}$ (h)	0.9	–
Clearance (ml/min/kg)	38	–
V_d (l/kg)	2.4	–
AUC_{∞}	21.9	3.0
C_{max} (ng/ml)	–	1.5
T_{max} (h)	–	0.4
F%	–	14

3.7. Oral phase

Following the oral dose, the average C_{max} was 1.5 ng/ml at 0.4 h post dose. Comparison of the AUC_{∞} (3.0 ng h/ml) with the intravenous value yielded a mean oral bioavailability of 14%.

Administration of midazolam by the intravenous and oral routes at a dose level of 0.05 mg/kg to the dog resulted in an oral bioavailability in the dog of 14%. This was comparable to the human bioavailability of midazolam at a similar dose (27 ± 11%) [4]. However, following midazolam administration at 0.5 mg/kg to dogs, oral bioavailability has been reported to be 69% [7], suggesting that the pharmacokinetics of midazolam are non-linear in this species.

4. Conclusions

An HPLC-MS-MS assay for the quantitation of midazolam has been developed and validated in dog plasma. The new assay was rapid, sensitive, specific and reproducible, and should be amenable to automation. The assay has been successfully applied to the analysis of dog plasma samples following both

oral and intravenous administration at a dose level of 0.05 mg/kg.

The use of the sensitive and specific HPLC-MS-MS assay described here has allowed administration of midazolam to the dog at a dose level which is more clinically relevant, and which potentially provides a more relevant model system for studying drug–drug interactions involving CYP3A.

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