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Quantification of Zolpidem in Canine Plasma

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Abstract: Problem statement: Zolpidem is a non-benzodiazepine hypnotic agent currently used in human medicine. In contrast to benzodiazepines, zolpidem preferentially binds with the GABAA complex ϖı receptors while poorly interacting with the other ϖ receptor complexes. Recent studies have suggested that ZP may be used to initiate sedation and diminish severe anxiety responses in dogs. The aim of the present study is to develop and validate a new HPLC-FL based method to quantify zolpidem in canine plasma. **Approach:** Several parameters both in the extraction and in the detection method were evaluated. The applicability of the method was determined by administering zolpidem to one dog. **Results:** The final mobile phase was acetonitrile: KH_2PO_4 (15 mM; pH 6.0) 40:60 v/v, with a flow rate of 1 mL min^{-1} and excitation and emission wave lengths of 254 and 400 nm, respectively. The best extraction solvent was CH_2Cl_2:Et_2O (3:7 v/v), this gave recoveries ranging from 83-95%. The limit of quantification was 1 ng mL^{-1}. The chromatographic runs were specific with no interfering peaks at the retention times of the analyte. The other validation parameters were in agreement with the EMEA. **Conclusion/Recommendations:** This method (extraction, separation and applied techniques) is simple and effective. This technique may have applications for pharmacokinetic or toxicological studies.

Key words: Z-drugs, plasma, dog, fluorescence, hypnotic

INTRODUCTION

Z-drugs are a group of nonbenzodiazepine drugs with effects similar to those of benzodiazepines, they are used in the treatment of insomnia (NIH, 2004). Names of drugs within this group mostly start with the letter "Z". Zolpidem (ZP) (2-(4-methylphenyl)-N, N, 6-trimethylimidazo [1, 2-a] pyridine-3-acetamide) belongs to this class and it is used primarily as a hypnotic agent (Fig. 1). While ZP is not a benzodiazepine, it does interact with the GABAA complex ϖ receptors. ZP preferentially binds with the GABAA ϖ1 receptor complex and has very little interaction with the other ϖ receptor complexes. Since the GABAA ϖ1 receptor complex is found primarily in the portions of the brain that affect sleep, ZP behaves as a sleep inducer without the muscle relaxant and anticonvulsant effects of the benzodiazepines. ZP has a rapid action and a short half-life and is very effective and safe; for these reasons, ZP is one of the most frequently prescribed hypnotic drugs in the United States and Europe (Morlock *et al*., 2006). Some paradoxical effects have been reported among users however (Haas *et al*., 2010).

Very few reports of paradoxical effects are present in the veterinary literature. Cases of intoxication following ingestion of large doses of ZP have been reported in cats (Czopowicz *et al*., 2010) and dogs (Richardson *et al*., 2002) and paradoxical excitation reactions have also been recorded in dogs (Riviere and Papich, 2009). These anecdotal reports suggest that ZP may be used to initiate sedation and diminish severe anxiety responses in dogs. It could be of particular use for acute-onset and severe phobic states, such as thunderstorm phobia and separation anxiety where there is a need for safe and rapid reduction in responsiveness to environmental stimuli and initiation of sleep, with relatively short duration of action and rapid recovery. Several analytical methods for detecting ZP in plasma are present in the literature. Studies utilizing HPLC (Durol and Greenblatt, 1997; Ring and Bostick, 2000; Nirogi *et al*., 2006), GC (Gaillard *et al*., 1993; Stanke *et*

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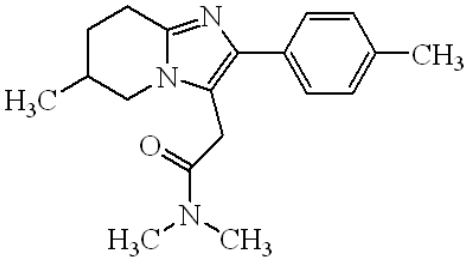
al., 1996) and LC/MS (Bhatt *et al*., 2006) have been reported, however none of these are validated for canine plasma. The aim of the present work is to validate a rapid and sensitive HPLC-FL analytical method to evaluate the plasma concentration of ZP in dogs, such a method could be used in cases of toxicity as well as in pharmacokinetic studies in canine species.

MATERIALS AND METHODS

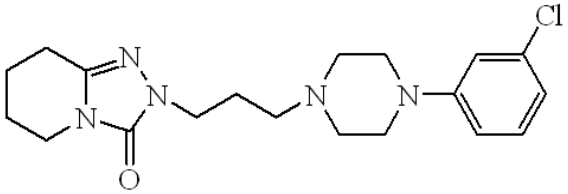
Zolpidem was obtained from Research Biochemicals International (Natick, MA, USA) and the Internal Standard (IS), trazodone (Fig. 1), was obtained from Sigma Chemical Company (St. Louis, MO, USA). All organic solvents used were HPLC grade and purchased from Carlo Erba (Milan, Italy): methanol (CH_3OH), dichloromethane (CH_2Cl_2), ethyl-ether (Et_2O), ethyl acetate (AcOEt), normal hexane (C_6H_{14}) and cycle-hexane (C_6H_{12}). Deionised water was purified by a Milli-Q system (Millipore). Potassium hydroxide pellets (analytical reagent grade) was purchased from Avantor (Modena, Italy) Control dog plasma was collected into lithium heparin anticoagulant from healthy dogs in the veterinary hospital of Pisa.

Instrumentation and chromatographic conditions: The HPLC system was an LC Workstation Prostar (Varian, Inc., Walnut Creek, CA, USA) consisting of a high pressure mixer pump (ProStar, model 230), CTO-10Avp column oven, spectrofluorometric detector (ProStar, model 363) and a loop of 20 μL. Data was processed by a Star LC Workstation (Varian, Inc.). The chromatographic separation assay was performed using a Haisil 100 C_{18} analytical column (250×4.6 mm inner diameter, 5 μm particle size, Higgins Analytical Inc., Mtn. View, CA, USA) maintained at room temperature. The analytical method was derived from a previously described protocol (Ring and Bostick, 2000) with slight modifications. The mobile phase consisted of acetonitrile:buffer (15 mM KH_2PO_4, adjusted to pH 6.0 with NaOH) (40:60 v/v) at a flow rate of 1.0 mL min^{-1}. Excitation and emission wavelengths were set at 254 and 400 nm, respectively.

Animal treatment and sampling: Blood samples were obtained from one healthy male Setter dog orally administered with tablets of ZP (0.5 mg kg^{-1}) (Zolpidem, EG®, Milan, Italy). Blood (2-3 mL) was collected via a catheter, previously inserted in the left jugular vein, at assigned times (0, 5, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10 and 24 h). The blood was then placed into collection tubes containing lithium heparin.



Zolpidem



Trazodone (IS)

Fig. 1: Molecular structures of zolpidem and trazodone (internal standards)

The samples were centrifuged at 3,000 rpm within 10 min of collection and the harvested plasma was frozen immediately and stored at -20°C. Immediately prior to the analysis, the samples were thawed at room temperature. The study protocol was approved by ethics committee of the University of Pisa and transmitted to the Italian Ministry of Health.

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 (5 μg mL^{-1}) and vortexed for 60 sec. Four mL of Et_2O:CH_2Cl_2 (7:3 v/v%) were added, then the sample was vortexed (30 sec), shaken (100 osc min^{-1}, 10 min) and centrifuged at 3,000 rpm for 10 min at 10°C. Three ml of the supernatant was collected in a separate glass vial. The organic phase was evaporated under a gentle stream of nitrogen at room temperature and reconstituted with 500 μL of the mobile phase. 20 μL of this latter solution was 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 (CBL, 2010).

Calibration curves were obtained by spiking the blank matrix with a known concentration of each analyte and IS to provide concentrations of 0.5, 1, 10,

20, 50, 75, 100, 200 and 250 ng mL^{-1}. The calibration curve of peak area *versus* concentration (ng mL^{-1}) of ZP was plotted. Least squares regression parameters for the calibration curve were calculated and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula Y = mX + b, where Y = peak area, X = concentration of the standard in ng mL^{-1}, m = the slope of the curve and b = the intercept with Y axis. Correlation coefficients for each of the calibration curves were > 0.99.

Within-run and between-run accuracy and precision were assessed on quality control samples (QC samples) and determinated by replicate analyses using 5 determinations of different concentration levels: 1, 20, 50, 100, 250 ng mL^{-1}.

Theory and calculation:

Quantification: When unknown samples were assayed, a control 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.

Pharmacokinetic evaluation: The pharmacokinetic calculations were carried out using WinNonLin v 5.2.1 (Pharsight Corp, Sunnyvale, CA, USA). Maximum concentration (Cmax) of ZP in plasma and the time required to reach Cmax (Tmax), were predicted from the data. The area under the concentration *vs* time curve ($AUC_{0-\square}$) was calculated using the linear trapezoidal rule. Akaike's information criterion (Yamaoka *et al*., 1978) was used to select the best-fitting model. Changes in plasma concentrations of ZP was evaluated using the standard compartmental analysis and the relative pharmacokinetic parameters were determined using standard mono-compartmental equations (Gibaldi and Perrier, 1982).

RESULTS AND DISCUSSION

Detection method development: Derivation of the mobile phase was achieved using a previously published method (Ring and Bostick, 2000) with slight modifications. Another method (Nirogi *et al*., 2006) reported a mobile phase constituted by ACN: 20 mM $NH_4H_2PO_4$ (27:63 v/v), however this method was dismissed due to inferior peak resolution. A range of buffer pH (5.0, 6.0, 7.0) were assayed to optimise the chromatographic separation and analytes absorbance. It was shown that varying pH made no difference to absorbance value, while the best separation was achieved at pH 6. For this reason, pH=6 was chosen as optimal.

Different flow rates between 0.5-1.5 mL min^{-1} were tested using the Haisil 100 C_{18} analytical column, 250×4.6 mm inner diameter, 5 μm as stationary phase. The final mobile phase resulted in ACN: KH_2PO_4 (15 mM) pH 6.0 (40:60 v/v) with a 1 mL min^{-1} flow rate. This gave excellent sensitivity and peak separation.

Excitation and emission wavelengths were tested within the ranges 254-410 and 320-400 nm, respectively. These ranges were chosen according to the wavelength intervals used in early studies (Durol and Greenblatt, 1997; Ring and Bostick, 2000; Nirogi *et al*., 2006). Wavelengths of 254 and 400 nm resulted in optimal excitation and emission, respectively.

Optimization of the extraction method: The influence of both solvent type (significant determinant of selectivity of the method) and number of extraction cycles, were studied in order to find the optimal extraction protocol for ZP. Solvents such as CH_2Cl_2, AcOEt, Et_2O and hexanes (C_6H_{12} or C_6H_{14}) were examined. CH_2Cl_2 and Et_2O were selected as the most suitable organic solvents in terms of analyte extraction and minimization of matrix components (interferents). Variations in the proportions of the selected extraction solvents (CH_2Cl_2:Et_2O 3:7, 3:2, 7:3 v/v) were also assessed in terms of recovery and selectivity. The CH_2Cl_2:Et_2O, (3:7 v/v) showed the best recovery of both analytes and IS (Table 1).

Finally, the influence of the number of extraction cycles on the extraction efficiency was also evaluated. The extraction time was set to 10 min and the number of extraction cycles was varied from one to three. No significant improvement in the recovery was shown with multiple extractions. A protocol using one extraction cycle was selected to ensure an efficient extraction of ZP and IS.

Method validation: It was necessary to validate each step in the analytical method so that this protocol can be utilised in future pharmacokinetic and toxicological studies (Salem and Abdallah, 2007; Carrillo-Lopez *et al*., 2010).

The calibration curves were constructed by plotting the ratio of the peak areas *versus* concentrations in the working range. Good linearity was achieved in the investigated range for ZP, the linear regression equations are reported in Table 2.

According to EMEA guidelines, LOD and LOQ were calculated based on signal-to-noise approach. These calculations were performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples, in this way, the minimum concentration at which the analyte can be reliably quantified (LOQ) or detected (LOD) was determined.

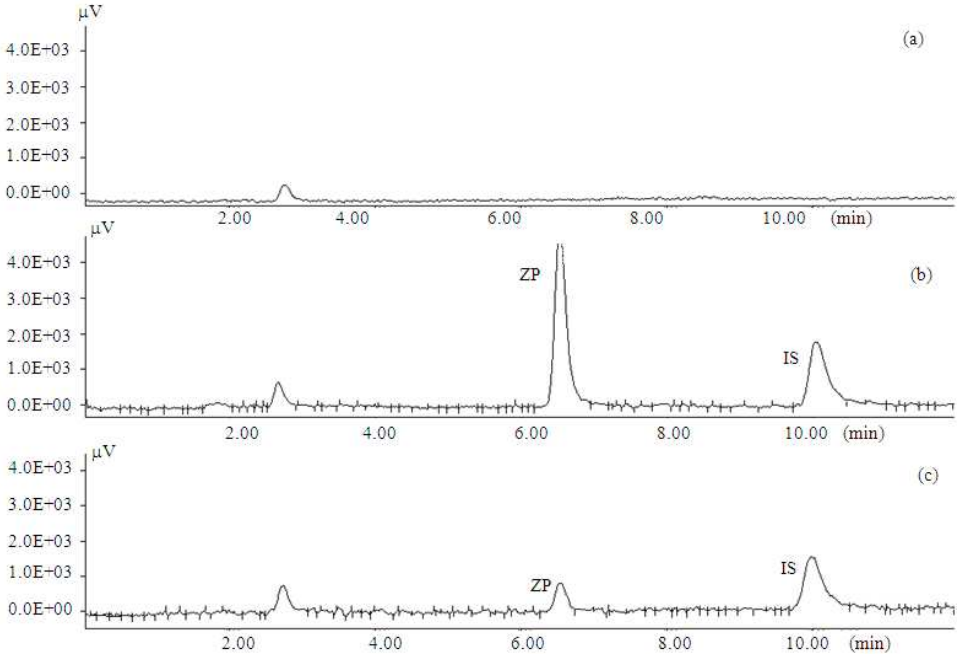


Fig. 2: (a) Chromatographic curve from blanks of canine plasma; (b) Chromatographic curve from fortified sample (ZP 100 ng mL^{-1}; IS 1000 ng mL^{-1}) of canine plasma; (c) Chromatographic curve from plasma sample collected at 15 min in the treated dog

Table 1: Single extraction recovery % (±SD) of ZP and IS spiked at 100 ng mL^{-1} with varied mixtures (n = 3)

		Recovery (%)	
Organic solvents	v/v ratio	ZP	IS
CH_2CI_2: Et_2O	3:7	89±6	93±6
CH_2CI_2: Et_2O	3:2	77±5	94±7
CH_2CI_2: Et_2O	7:3	72±6	85±10

Table 2: Summary of validation data for ZP

Property	ZP
Linear range (ng mL^{-1})	1-250
Calibration equation	y = 730x+430
Correlation coefficient (R^2)	0.993±0.022
LOQ (ng mL^{-1})	1
LOD (ng mL^{-1})	0.3
Accuracy	102.1±4.3
Precision (%)	
Interday	0.5-4.1
Intraday	3.1-4.3
Robustness (%)	<5.2
Specificity	Specific

The typical signal-to-noise ratios were 10:1 and 3:1 for LOQ and LOD, respectively (Table 2). Both the accuracy 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 ZP and IS. Typical retention times for ZP and IS where 6.5±0.1 and 10.1±0.1 min, respectively (Fig. 2b and c).

The accuracy was evaluated by determining the recovery for five replicates of each of the QC samples. The recovery of ZP ranged from 83.1-94.2%. The respective CV (%) values varied from 0.5-6.2%. Intra-day value consistency (repeatability) was evaluated for five replicates of each QC sample during the same day. Inter-day value consistency (intermediate precision) was evaluated by quantitation of ZP in QC samples on five different days. Relative errors for both the intraday and inter-day accuracy were <4.3%.

Stability studies were performed to ensure good reproducibility of the method. Stock solution of the analytes and IS (1000 ng mL^{-1}) and high (250 ng mL^{-1}) and low (20 ng mL^{-1}) 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. Data obtained after three freeze-thaw cycles showed that the analytes were stable in dog plasma (CV% < 5%).

Table 3: Predicted pharmacokinetic parameters for zolpidem

Parameters	ZP
R^2	0.99
$AUC_{0-\infty}$ ((min ng mL^{-1})	3679.12
T_{max} (min)	29.99
C_{max} (ng mL^{-1})	51.21
K_{01} HL (min)	35.94
Cl (mL kg min^{-1})	154.91

R^2, correlation between observed/predicted points; $ACU_{0-\infty}$, area under the plasma concentration-time curve of peak; C_{max}, peak plasma concentration; K_{01} HL, half life of the elimination phase; Cl, total body clearance

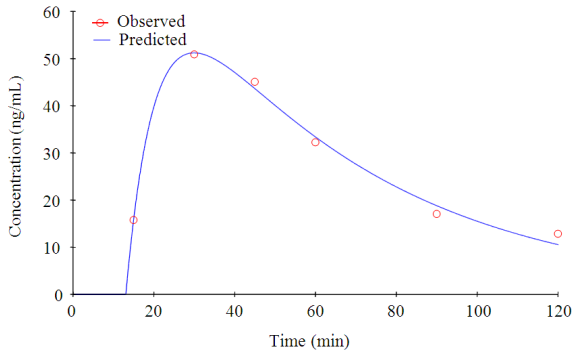


Fig. 3: Observed (O) and predicted (–) values of plasma concentrations of ZP following a single oral dose (0.5 mg kg^{-1}) in one adult Setter dog

These findings indicated that the storage of analytes in plasma samples at -20°C is adequate and no stability-related problems would be expected during routine analyses for pharmacokinetic studies.

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 3 plasma aliquots from the treated dog in two different labs (Veterinary Pharmacology and Pharmaceutical Science, both at the University of Pisa) and obtained variations of less than 5.2%.

These results demonstrate that the method enables accurate quantification of ZP. The validation parameters were in agreement with the EMEA guidelines (CBL, 2010).

Application of the method: The applicability of this method has been verified by determining ZP in canine plasma after oral treatment with 0.5 mg kg^{-1} of ZP. HPLC analysis of the plasma confirmed the presence of ZP in time-related amounts (Fig. 3). The amount of ZP in plasma ranged between 12.85 and 50.89 ng mL^{-1}. The described method allowed monitoring of the concentration *versus* time curves of the active ingredient and the calculation of the basic pharmacokinetic parameters (Table 3). The pharmacokinetic of active ingredients is one of the most important parameter for its evaluation (Lewis *et al*., 2007).

CONCLUSION

The analytical method described in this work provides selective and accurate analysis of ZP without the need for expensive clean up steps, solvent consuming flows or expensive devices. The low LOQs show that the method could be useful for drug measurement even when it is administered in sub-clinical doses. These features make the described method suitable for pharmacokinetic investigations including drug-drug interaction and investigating intoxication cases and demonstrate that it has potential future applications in veterinary medicine such as providing guidance for dose adjustment (Giorgi *et al*., 2012).

In summary, this method (extraction, separation and applied techniques) is simple and efficacious for the determination of ZP in canine plasma.

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