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Determination of constituents of Telazol®—tiletamine and zolazepam by a gas chromatography/mass spectrometry-based method

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Abstract

Tiletamine and zolazepam injection (Telazol®) is used in veterinary surgical practice to induce short-term anesthesia and also to immobilize wild animals. The present work describes a sensitive method to measure tiletamine and zolazepam concentrations in plasma by means of GC/EI–MS on a 5% phenyl/95% methylpolysiloxane column. A simple liquid extraction procedure with ethyl acetate was used to isolate the two compounds and the same were separated and analyzed by GC/MS without derivatization. A formal validation of the assay demonstrated good accuracy and precision for both tiletamine (98–100.8%; C.V.$_{total}$ < 6.7%) and zolazepam (98.3–103.4; C.V.$_{total}$ < 13.2%). With 500 μl of plasma, the limits of quantification for both tiletamine and zolazepam were found to be 10 ng/ml. Both compounds were stable after three freeze-thaw cycles. The assay was used to analyze plasma samples collected from a pig after intramuscular administration of 10 mg/kg of Telazol®. The plasma concentration–time profile of tiletamine and zolazepam from this representative pig is also provided.

Keywords: GC/MS; Pharmacokinetics; Pig; Telazol®; Tiletamine; Zolazepam

1. Introduction

An equal weight (1:1 ratio) combination of tiletamine hydrochloride and zolazepam hydrochloride (Fig. 1) is marketed as Telazol® (Fort Dodge Animal Health, Fort Dodge, IA, USA). Tiletamine and zolazepam possess dissociative anesthetic and tranquilizing properties, respectively [1]. Tiletamine does not affect cranial nerve and spinal reflexes and its effect is devoid of muscle relaxation; the addition of zolazepam achieves muscle relaxation. This combination has the approved indication of use in cats and dogs for short surgical procedures [1]. Telazol® is also used intramuscularly to induce short-term anesthesia for surgical purposes and for immobilization and restraining in various other animal species [1–7]. There is scarcity of information on the pharmacokinetics of the components of Telazol®. In our personal experience with pigs, we have seen that similar doses of Telazol® produce anesthesia of varying intensity and duration. This may be due to variation in the levels of tiletamine and zolazepam achieved in different animal subjects. Therefore, elucidation of pharmacokinetics of tiletamine and zolazepam is important to better understand the variability in the effects of Telazol®. Previously reported assay methods for tiletamine and zolazepam include high-performance liquid chromatography coupled with UV detection [8,9] and GC/MS [10,11]. These methods have been published as part of either pharmacokinetic or toxicological investigations without formal validation of procedures. The present work focuses on development and validation of a sensitive GC/MS assay for determination of tiletamine and zolazepam in plasma samples.

2. Experimental

2.1. Materials and chemicals

The materials and chemicals used included: acetic acid (HPLC grade—Fisher Chemical, Fair Lawn, NJ, USA); diazepam and diethyl ether with and without 1M HCl (ACS

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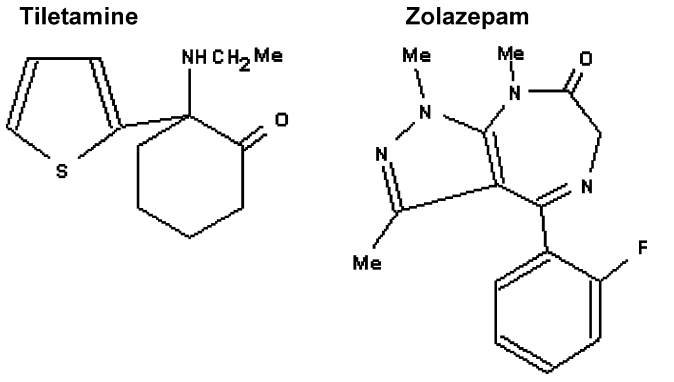

Fig. 1. Chemical structures of tiletamine and zolazepam.

grade) obtained from Sigma–Aldrich, St. Louis, MO; ethyl acetate (HPLC grade—Burdick Jackson, Muskegon, MI, USA); ketamine HCl (1 mg/ml analytical standard in methanol—Cerilliant, Round Rock, TX, USA); methanol (HPLC grade); methylene chloride (ACS grade); sodium acetate (ACS grade); sodium hydroxide (ACS grade) obtained from Fisher Chemical, Fair Lawn, NJ, USA; and water (HPLC grade—EMD Chemicals, Gibbstown, NJ, USA).

Tiletamine and zolazepam are not available commercially as pure standards and therefore were extracted from a Telazol® vial (lot no. A71285) obtained from a local pharmacy. A vial of Telazol® contains 250 mg each of tiletamine and zolazepam (as pure base). The extraction strategy employed was as following. The contents of one Telazol® vial were dissolved into 5 ml of water and the resultant solution (pH 2.8) was raised to pH 6 with 0.1N NaOH. This solution was extracted three times with equal volumes of ethyl acetate in a separatory funnel. Drying of the ethyl acetate fraction in a Rotavapor® flask produced crystalline zolazepam. The residual aqueous fraction was pH adjusted to 11 with 0.1N NaOH and was extracted three times with equal volumes of ethyl acetate. Vacuum evaporation of ethyl acetate in a Rotavapor® flask produced tiletamine with semisolid consistency. The zolazepam crystalline mass (from above) was dissolved in 10 ml of a boiling mixture of diethyl ether and methanol (in 1:1 ratio). The solution was evaporated overnight at atmospheric pressure leading to crystallization of zolazepam as well-defined rod-shaped crystals. The crystals were washed with cold diethyl ether in a Buchner funnel and were kept aside for drying. The semisolid tiletamine (from above) was purified by a second extraction. The tiletamine mass was dissolved in 5 ml of methylene chloride and the resultant solution was back-extracted with 10 ml of 100 mM pH 5 acetate buffer in a separatory funnel. The pH of the aqueous fraction was raised to 11 with 1N NaOH and this solution was extracted with 10 ml of methylene chloride three times. The methylene chloride fraction was transferred to a Rotavapor® flask and dried under vacuum. To the resultant tiletamine mass (semisolid appearance), 4 ml of ether with 1 M HCl was added leading to formation of a hydrochloride salt of tiletamine. The ether was evaporated in the Rotavapor® flask leaving behind a brownish white powder of tiletamine HCl.

Purity of tiletamine and zolazepam was ascertained by analyzing the two components by GC/MS in the scanning mode (50–500 amu) under the chromatographic conditions described later in this paper. A small amount of tiletamine HCl and zolazepam were dissolved in methanol to produce two working solutions. Injection of each of these working solutions into the GC/MS produced only one peak without a second interfering peak.

2.2. GC apparatus and operating conditions

The GC/MS system consisted of an Agilent 6890 series II Gas Chromatograph coupled with an Agilent 5973 series Mass Selective detector and an Agilent 7673 Autosampler. A 30 m long DB-5 capillary column (J&W scientific, Folsom, CA, USA) with internal diameter of 0.32 mm and film thickness of 0.25 μm was used for analytical separation. The carrier gas was ultra pure (5.0 grade) helium flowing at a rate of 1 ml/min. The injection size was 2 μl and a split ratio of 10:1 was utilized. In each analytical run the column temperature was initially maintained at 120 °C for 1 min, raised to 260 °C in 14 min and kept at 260 °C for 5 min (total run time 20 min). The injection port, electron impact source, and MS analyzer were set at temperatures of 280, 230, and 150 °C, respectively. The retention times for tiletamine, ketamine, zolazepam, and diazepam were 8.4, 10.2, 14.6, and 15.8 min, respectively and the dominant ions for these four analytes had *m/z* ratios of 166, 180, 285, and 256, respectively. Therefore, for subsequent quantitative analysis, the MS was operated in the selected ion monitoring (SIM) mode at *m/z* values of 166 (from 3 to 10 min); 180 (from 10.1 to 11 min); 285 (from 11 to 15 min); and 256 (from 15.1 to 20 min) with a dwell time of 50 ms.

2.3. Spiking of plasma for calibration curve

A stock solution of tiletamine HCl in water (equivalent to 1 mg/ml of free base) was prepared and further diluted with water to prepare eight working solutions (250, 500 ng/ml and 1, 5, 25, 50, 75, 100 μg/ml). Zolazepam was dissolved in methanol to obtain a 1 mg/ml solution. This stock solution was diluted with methanol to yield eight working solutions (250, 500 ng/ml and 5, 25, 100, 200, 300, 400 μg/ml). Heparinized plasma from a pig not exposed to Telazol® was used as the blank matrix. Eight pig plasma samples (500 μl each) were spiked with 10 μl of each of these working solutions to yield effective plasma concentrations of 5, 10, 20, 100, 500 ng/ml and 1, 1.5 and 2 μg/ml for tiletamine (free base) and 5, 10, 100, 500 ng/ml and 2, 4, 6, 8 μg/ml for zolazepam.

The 1 mg/ml methanolic stocks of diazepam and ketamine HCl (as free base) were diluted with methanol to prepare 10 μg/ml solutions of both diazepam and ketamine. Ten microliters of each of these internal standard solutions was added to each plasma sample.

2.4. Spiking of plasma for quality control standards

The concentrations chosen as quality controls (QCs) were 40 ng/ml (low QC), 200 ng/ml (medium QC) and 800 ng/ml (high QC) for tiletamine and 200 ng/ml (low QC), 1000 ng/ml

(medium QC) and 5000 ng/ml (high QC) for zolazepam. Validation of lower limit of quantification (LOQ) was focused on the concentration of 10 ng/ml for both tiletamine and zolazepam. Separate 1 mg/ml stocks of both tiletamine (free base; in water) and zolazepam (in methanol) were prepared. The tiletamine HCl stock was further diluted (with water) to prepare four working solutions of 500 ng/ml and 2, 10, 40 μg/ml. The zolazepam stock was further diluted (with methanol) to prepare four working solutions of 500 ng/ml and 10, 50, 250 μg/ml. Addition of 10 μl of each of these working solutions to 500 μl of plasma yielded effective plasma concentrations of 10 ng/ml (LOQ), 40 ng/ml (low QC), 200 ng/ml (medium QC) and 800 ng/ml (high QC) for tiletamine and 10 ng/ml (LOQ), 200 ng/ml (low QC), 1000 ng/ml (medium QC) and 5000 ng/ml (high QC) for zolazepam. The quality control samples were extracted after addition of 10 μl of each of the two internal standard solutions as mentioned above.

2.5. Extraction from plasma samples

One milliliter of 0.5N NaOH was added to each plasma sample (500 μl) in a 13 mm × 100 mm test tube and vortexed for 10 s. Two milliliters of ethyl acetate were added to each tube and the mixture was vortexed for 20 s. The ethyl acetate layer was removed to another test tube. The aqueous layer was extracted two more times with 2 ml of ethyl acetate. The three ethyl acetate fractions from each sample were pooled together and dried under a gentle stream of nitrogen gas at 30 °C in a TurboVap® evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted with 200 μl of ethyl acetate and 2 μl of this extract was injected into GC.

2.6. Study design for method validation

Plasma samples for standard curve and validation were spiked and processed in the manner described above. An eight-point standard curve along with three sets (low, medium, and high QC; $n = 5$ for each set) of validation samples was run on five separate days to determine intra- and inter-day accuracy and precision. On three of these days, one set ($n = 5$) of LOQ samples were also run. The stability of tiletamine and zolazepam after three freeze-thaw cycles was determined at the levels of low, medium and high QC ($n = 5$ at each level) for both compounds. For this purpose, separate sets of plasma samples (500 μl each) were spiked with tiletamine and zolazepam standards to produce necessary concentrations and were extracted and analyzed with other validation samples. Responses from one set of validation run were compared with unextracted standards containing the same amount (low, medium, and high QC; $n = 5$ for each set) of analytes to determine extraction recovery.

2.7. Analysis of plasma samples obtained from pig

Blood samples were collected at periodic intervals from one pig undergoing a surgical procedure after administration of 10 mg/kg of Telazol® via deep intramuscular injection. These samples were processed in the manner described above and analyzed with standard and QC samples of a validation run.

2.8. Data analysis

The standard curves were obtained by plotting the peak area ratio of tiletamine to ketamine and zolazepam to diazepam against the corresponding analyte concentrations using a $1/x$ weighting function. The peak area ratios of analytes to internal standards in the validation samples were used to back-calculate the analyte concentrations from the standard curve. For each concentration data point, the calculated values were subjected to a one-way analysis of variance and the within-day component of variation (S^2_{wd}), the between-day component of variation (S^2_b), and total variance (S^2_t) were calculated [12,13] as follows:

$$S^2_{wd} = [MS_{wd}]$$

$$S^2_b = \left[\frac{MS_b - MS_{wd}}{r}\right]$$

$$S^2_t = S^2_b + MS_{wd}$$

where MS_{wd} and MS_b are the within- and between-day mean sum of squares, respectively from the ANOVA output table and r is the number of replicates.

The within-day and between-day components of variation and the total coefficient of variation ($C.V._{wd}$, $C.V._b$, and $C.V._t$) for each concentration were determined by dividing the S_{wd}, S_b, and S_t, respectively by the mean of the calculated concentration values and multiplying it by 100 [12]. Accuracy (%) was determined by dividing the mean of calculated concentration by the true mean and multiplying it by 100.

3. Results and discussion

Fig. 2 shows the characteristic mass spectrums of tiletamine and zolazepam with predominant ion fragments being 166 and 285, respectively. A representative mass chromatogram with retention times is shown in Fig. 3. The standard curves obtained were linear ($r^2 > 0.995$) for both tiletamine and zolazepam over the tested range. The intercept and slope (mean ± S.D., $n = 5$) of the standard curves (peak area ratio of analyte and internal standard versus concentration [ng/ml]) for tiletamine were -0.00344 ± 0.00079 and 0.00314 ± 0.00028, respectively. These parameters for zolazepam were 0.00305 ± 0.00596 and 0.00270 ± 0.00075, respectively. The extraction recovery was almost complete for tiletamine and was about 60% for zolazepam (Table 1). The efficiency of the extraction procedure was less at higher analyte concentrations for both tiletamine and zolazepam. As the analyte concentrations were expected to be in the μg/ml range for zolazepam, no effort was made to further improve the extraction recovery.

The assay was sensitive for both tiletamine and zolazepam with a LOQ of 10 ng/ml and good accuracy and precision around this concentration (Tables 2 and 3). While there does not appear

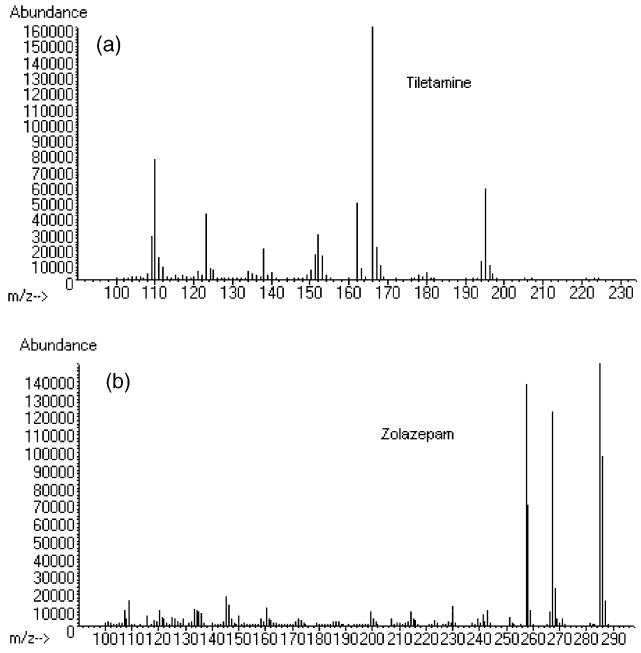

Fig. 2. (a) Mass spectrum of tiletamine. (b) Mass spectrum of zolazepam.

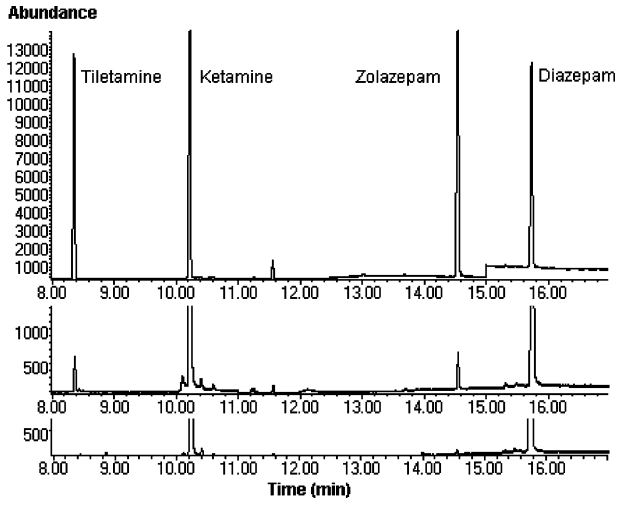

Fig. 3. Chromatograms of tiletamine and zolazepam in extracted plasma samples spiked with internal standards (ketamine and diazepam). Bottom panel: blank plasma sample; middle panel-plasma sample with 10 ng/ml (LOQ level) of tiletamine and zolazepam; top panel: plasma sample from a pig after intramuscular administration of Telazol®.

Table 1
Extraction recoveries of tiletamine and zolazepam from pig plasma

Level tested	% Average recovery (% C.V.) ($n = 5$)
Tiletamine	
Low QC (40 ng/ml)	100.6 (6.14)
Medium QC (200 ng/ml)	95.9 (8.76)
High QC (800 ng/ml)	88.5 (3.64)
Zolazepam	
Low QC (200 ng/ml)	60.0 (3.85)
Medium QC (1000 ng/ml)	59.0 (4.58)
High QC (5000 ng/ml)	54.5 (7.35)

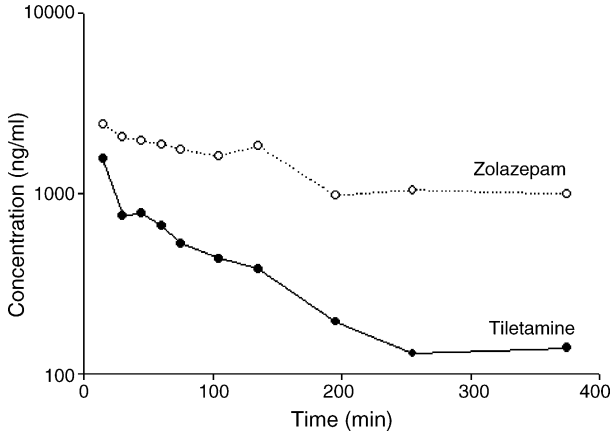

Fig. 4. Plasma concentration–time profile of tiletamine and zolazepam in a pig after intramuscular administration of a 10 mg/kg dose of Telazol®.

to be a likelihood of obtaining such low concentrations in animal samples, the LOQ value can further be reduced by simply extracting a larger amount of plasma and by reconstituting the extracted analytes in a smaller amount of solvent. Both tiletamine and zolazepam had good stability after three freeze-thaw cycles at the three tested levels (Table 4).

The plasma concentration–time profile after intramuscular administration of a 10 mg/kg dose of Telazol® (5 mg/kg each of tiletamine and zolazepam) to one pig is shown in Fig. 4. Concentrations of both tiletamine and zolazepam were highest at the first observation point (15 min) and declined afterwards. By 6 h post-administration, tiletamine concentration had declined to about 10% of the peak while the zolazepam concentration was still about 50% of the peak.

Tiletamine and zolazepam have been assayed previously by HPLC and GC/MS methods. Semple et al. [9] have inves-

Table 2
Intra- and inter-assay precision and variability for tiletamine (shown as % accuracy and % C.V.)

Concentration (ng/ml)	n	Mean measured concentration (ng/ml)	Accuracy (%)	S_{wd} (ng/ml)	C.V.$_{wd}$ (%)	S_b (ng/ml)	C.V.$_b$ (%)	S_t (ng/ml)	C.V.$_t$ (%)
10 (LOQ)	15	10.0	100.1	0.43	4.3	0	0	0.37	3.7
40 (low QC)	25	39.2	98.0	1.5	3.9	2.1	5.4	2.6	6.7
200 (medium QC)	25	199.2	99.6	7.3	3.7	8.6	4.3	11.3	5.7
800 (high QC)	25	806.1	100.8	38.3	4.8	28.7	3.6	47.9	5.9

S_{wd}, S_b, and S_t are within-day, between-day, and total standard deviation, respectively. C.V.$_{wd}$, C.V.$_b$ and C.V.$_t$ are within-day, between-day, and total coefficient of variation.

Table 3
Intra- and inter-assay precision and variability for zolazepam (shown as % accuracy and % C.V.)

Concentration (ng/ml)	n	Mean measured concentration (ng/ml)	Accuracy (%)	S_{wd} (ng/ml)	C.V.$_{wd}$ (%)	S_b (ng/ml)	C.V.$_b$ (%)	S_t (ng/ml)	C.V.$_t$ (%)
10 (LOQ)	15	10.07	100.7	0.75	7.4	0	0	0.74	7.4
200 (low QC)	25	206.8	103.4	9.7	4.7	23.6	11.4	25.5	12.3
1000 (medium QC)	25	995.1	99.5	70.87	7.1	110.3	11.1	131.1	13.2
5000 (high QC)	25	4912.5	98.3	158.9	3.2	605.8	12.3	626.3	12.7

S_{wd}, S_b, and S_t are within-day, between-day, and total standard deviation, respectively. C.V.$_{wd}$, C.V.$_b$ and C.V.$_t$ are within-day, between-day, and total coefficient of variation.

Table 4
Freeze-thaw stability (shown as % accuracy and % C.V.)

	Tiletamine			Zolazepam		
	Low QC (40 ng/ml)	Medium QC (200 ng/ml)	High QC (800 ng/ml)	Low QC (200 ng/ml)	Medium QC (1000 ng/ml)	High QC (5000 ng/ml)
Cycle 1 ($n=5$)	100.3 (2.1)	101.3 (1.3)	87.9 (1.8)	89.1 (1.2)	92.2 (10.6)	93.6 (9.1)
Cycle 2 ($n=5$)	101.8 (1.1)	101 (1.2)	89.7 (3.1)	91.1 (1.5)	107.1 (2.9)	97.2 (9.3)
Cycle 3 ($n=5$)	101.6 (2.4)	101.2 (2.8)	92.3 (9.8)	92.3 (1.4)	111.2 (3.9)	99.1 (10.1)

tigated the pharmacokinetics of tiletamine and zolazepam in polar bears. The authors used a two-stage liquid extraction with ethyl acetate/hydrochloric acid/ethyl acetate for isolating these two compounds from serum followed by their separation using HPLC with UV detection [8]. The authors reported extraction recoveries of 88% for both tiletamine and zolazepam. However, the details of extraction methodology and validation parameters were unclear from their report. Two methods employing GC/MS instrumentation have also been reported [10,11]. Chung et al. [10] extracted tiletamine and zolazepam from 1 ml of alkalinized blood with 5 ml of ethyl acetate three times, back-extracted the pooled ethyl acetate into acid, and re-extracted the acidic solution twice with 5 ml of ethyl acetate. This publication was a case report on a death related to abuse of Telazol® and the authors did not perform a formal validation of the assay procedure. A second GC/MS based method was used to determine postmortem levels of tiletamine and zolazepam in blood and tissue samples [11]. The authors used a solid phase extraction procedure to isolate tiletamine and zolazepam from a 3 ml whole-blood sample. This report also did not provide detailed information regarding performance of assay methodology.

4. Conclusion

A sensitive GC/MS based method was developed to assay tiletamine and zolazepam concentrations in plasma. To our knowledge, the current report is the first validated assay for determination of Telazol® components in plasma. The methodology described by us employs a simple one-step liquid–liquid extraction and offers estimation of tiletamine and zolazepam with good sensitivity and precision. We believe that this assay will be helpful to other investigators interested in studying pharmacokinetics of tiletamine and zolazepam.

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