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The simultaneous determination of diazepam and its three metabolites in dog plasma by high-performance liquid chromatography with mass spectroscopy detection

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Abstract

A fast, sensitive and specific LC/MS/MS method for the simultaneous determination of diazepam and its three metabolites, oxazepam, temazepam and desmethyldiazepam, in dog plasma is described. The method consists of an automated 96-well solid phase extraction procedure and electrospray LC/MS/MS analysis. D_5-Diazepam is used as the internal standard for all the compounds. Intra-day and inter-day assay coefficients of variations are less than 12.7%. The lower limit of quantitation (LLOQ) is 1 nM for each analyte, based on 0.1 ml aliquots of dog plasma. The analytical run time was 5 min. Linearity is observed over the range of 1–500 nM. This method has been used to support the discovery of pharmacokinetic studies. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: HPLC method; Electrospray ionization; MS/MS detection; Dog plasma; Automated SPE

1. Introduction

Diazepam (7-Chloro-1-methyl-5-phenyl-3H-1, 4-benzodiazepin-2[1H]-one) is used in the treatment of anixiety disorders. Oxazepam, temazepam (3-hydroxydiazepam) and desmethyldiazepam, are three major active metabolites of diazepam (Fig. 1).

A number of methods are in use for the qualitative and quantitative analysis of 1,4-benzodiazepines. Apart from chromatographic methods, such as thin-layer chromatography (TLC) [1], column-switch high-performance liquid chromatography (HPLC) [2,3], and gas chromatography [4–6], immunoassay [7], as well as mass spectrometry are used in the analysis of diazepam and other 1,4-benzodiazopines. Although several

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selective and sensitive CE/MS/MS [8,9] and LC/MS/MS [10] methods were developed, so far, no LC/MS/MS method has been reported for the simultaneous determination of diazepam and its three metabolites, oxazepam, temazepam and desmethyldiazepam, in dog plasma.

A fast, sensitive and specific method to measure diazepam, oxazepam, temazepam and desmethyldiazepam in dog plasma using automated 96-well solid phase extraction and LC/MS/MS analysis is presented in this paper.

2. Experimental

2.1. Chemicals and reagents

Diazepam, oxazepam, temazepam, desmethyldiazepam and internal standard (D_5-Diazepam) were purchased from Sigma (St. Louis, MO). Control dog plasma (EDTA as anticoagulant) was purchased from Bioreclamation Inc. (Hicksville, NY). HPLC grade methanol and

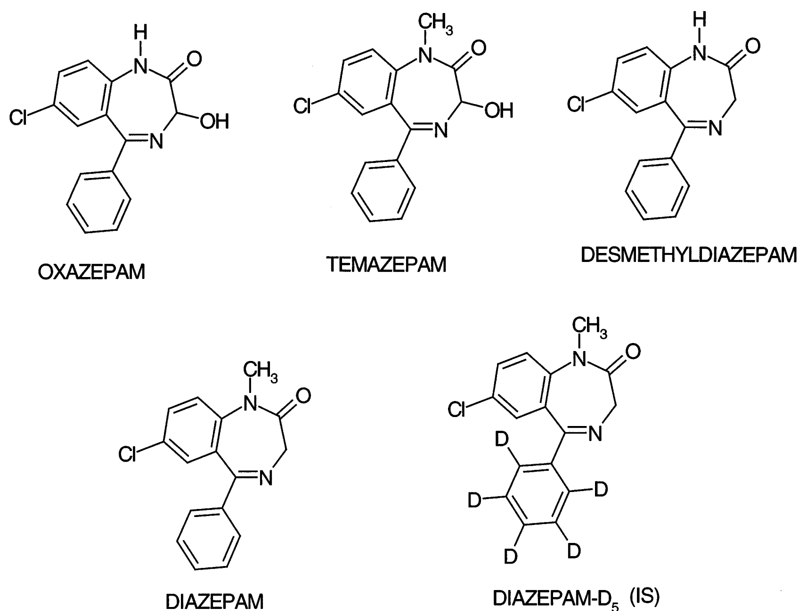

Fig. 1. Structure of oxazepam, temazepam, desmethyldiazepam, diazepam and D_5-diazepam (internal standard).

Table 1
Positive product ion mass spectra of diazepam, its three metabolites and D_5-Diazepam

Compound	$[M+H]^+$	Other significant ions (% relative abundance)			MRM ion combination
		a	*b*	*c*	
Diazepam	285	154 (45%)	193 (45%)	222 (10%)	285 → 154
Oxazepam	287	241 (85%)	269 (15%)		287 → 241
Temazepam	301	255 (90%)	283 (10%)		301 → 255
Desmethyl diazepam	271	140 (60%)	208 (20%)	165 (20%)	271 → 140
D_5-Diazepam (internal standard)	290	154 (50%)	198 (30%)	227 (20%)	290 → 154

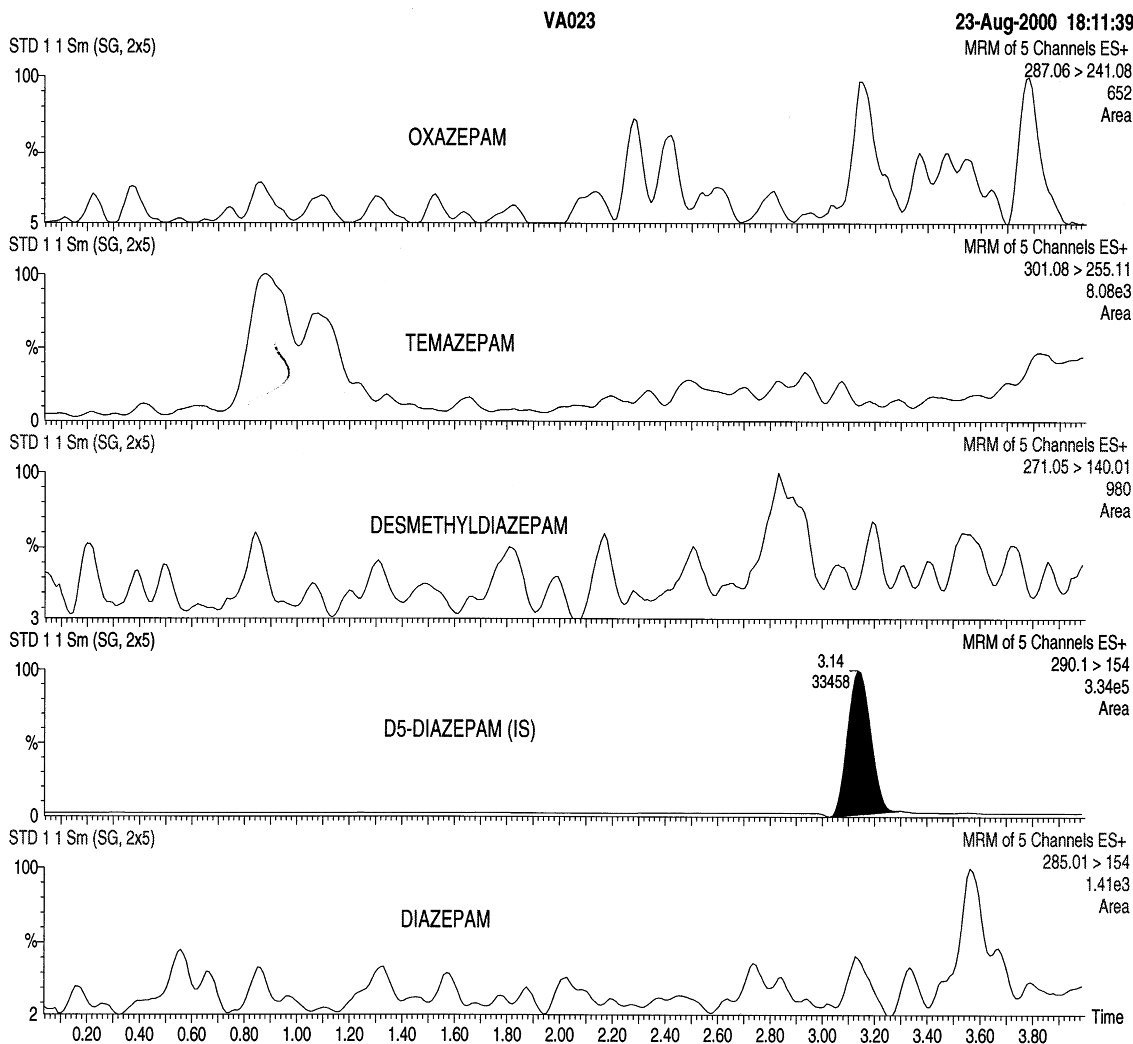

Fig. 2. Representative chromatogram of extracted blank dog plasma.

acetonitrile were purchased from EM Science (Gibbstown, NJ). All other reagents were of analytical grade.

2.2. Equipment

LC/MS/MS was performed using a Micromass (Manchester, UK) Quattro Ultima tandem triple quadrupole mass spectrometer interfaced via an electrospray probe to a liquid chromatograph consisting of a Shimadzu 10A HPLC system (Shimadzu, Japan) and CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC). A Discovery C-18 column (50 × 2.1 mm i.d., 5 μm), supplied by Supelco (Bellefonte, PA), was used at ambient temperature. The solid phase extraction was performed with a Packard Multiprobe II station (Packard, CA). A 3M Empore C-18 High Performance Extraction Disk Plate was used as extraction plate (3M, St. Paul, MN).

2.3. Calibration curve

Stock solutions of four analytes (1 mg/ml) were

individually prepared in methanol. Dilutions of these solutions were made in order to prepare the plasma standards needed to construct the calibration curves. D_5-Diazepam was used as the internal standard. All stock solutions and spiking solutions were stored at −20°C. Duplicate calibration standards were prepared fresh for each batch analysis by adding aliquots of the spiking solutions to give eight appropriate final concentrations in drug-free dog plasma. The concentration of the standards at the respective points on the calibration graphs were 1, 2, 4, 20, 100, 200, 400 and 500 nM in dog plasma based on 0.1 ml of plasma. The quality control (QC) samples, at concentration of 1, 4, 100 and 400 nM in dog plasma for these four analytes, were prepared similarly from a different stock solution. Peak integration, regression and calculation of concentration were computed using Micromass's MassLynx (version 3.3) software. The calibration curve was constructed using a weighted ($1/x^2$) linear regression of the standard dog plasma concentrations to measured peak-area ratios.

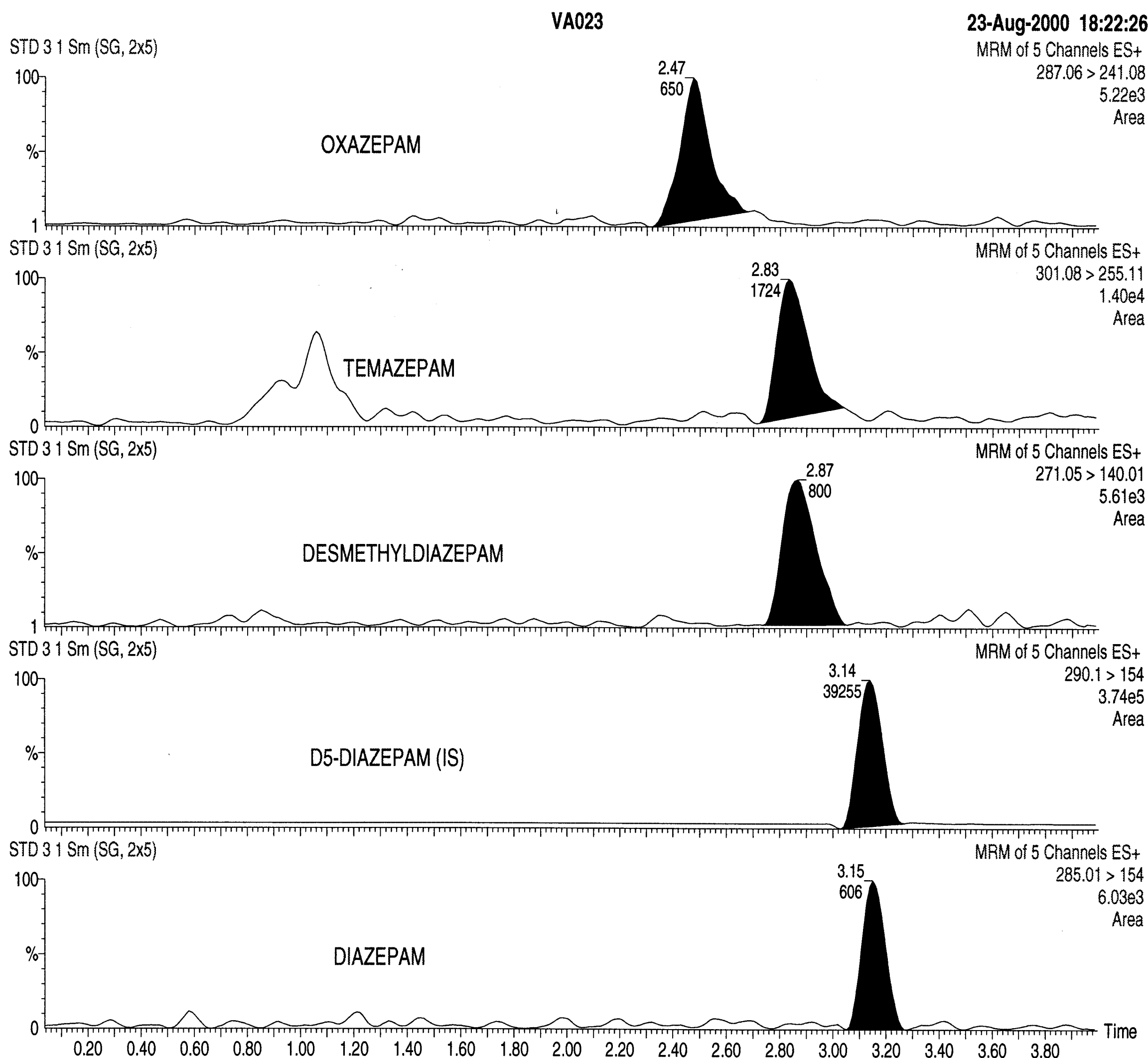

Fig. 3. Representative chromatogram of four analytes from an extracted dog plasma LLOQ (1 nM) sample.

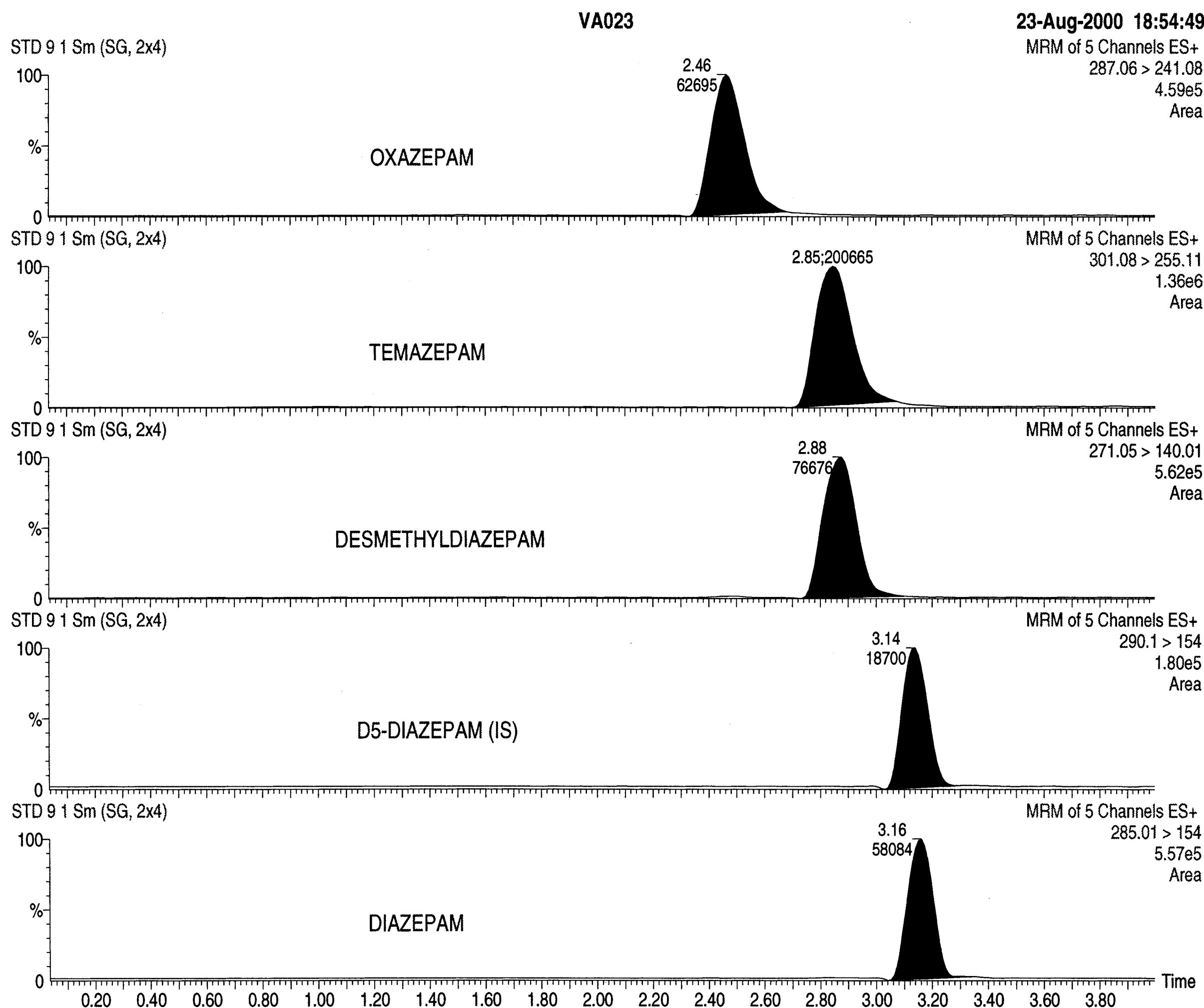

Fig. 4. Representative chromatogram of four analytes from an extracted dog plasma ULOQ (500 nM) sample.

2.4. Extraction procedure

Diazepam, oxazepam, temazepam, desmethyldiazepam and internal standard (D_5-Diazepam) were extracted from dog plasma using a Packard Multiprobe II station with 3M Empore C-18 High Performance Extraction Disk Plates. Disk SPE plates were conditioned by 0.1 ml of methanol followed by 0.1 ml of water. Dog plasma samples pre-mixed with buffer (0.1 ml of plasma with 0.1 ml of 0.1 M ammonium phosphate buffer, pH 6.8) containing internal standard were loaded onto the plates. The plate was washed with 0.5 ml of water followed with 0.5 ml of 15% methanol. After drying the plate for 5 min by centrifuging at 3000 rpm, the final elution was performed with 0.3 ml of methanol. The eluted solutions were dried under nitrogen at 30°C for approximately 30 min and the dried residue was reconstituted in 0.1 ml of 30% acetonitrile. A 0.01 ml aliquot of the reconstituted sample was injected onto the LC/MS/MS system. For each batch, duplicates of

Table 2
Linear regression parameters obtained from the calibration curves of diazepam and its three metabolites in dog plasma

Compound	Slope	Intercept	r^2
Oxazepam	0.0158	−0.0048	0.9927
Temazepam	0.0323	−0.0093	0.9923
Desmethyldiazepam	0.0087	−0.0034	0.9940
Diazepam	0.0095	−0.0048	0.9961

the calibration standard samples, double blank sample, control blank sample and triplicate or six replicates of QC samples were used.

2.5. Chromatographic and detection conditions

The analytes were chromatographically separated using isocratic conditions with a step gradient imposed at the end of the separation. The initial mobile phase consisting of 30% acetonitrile and 70% 0.01 M ammonium acetate (pH 6.8) was delivered to the MS without splitting at a flow rate of 0.25 ml/min. At 3 min, the organic composition was increased to 100%. The total chromatographic run time was 5 min. A Micromass Quattro Ultima system with an electrospray interface was used at a source temperature of 130°C.

The analytes were detected using the multiple reaction monitoring (MRM) mode of the transition m/z 287→241 for oxazepam, 301→255 for temazepam, 271→140 for desmethyldiazepam, 285→154 for diazepam and 290→154 for D_5-Diazepam (internal standard), respectively. The instrument was operated at a desolvation temperature of 350°C, collision gas pressure of $1\times e^{-3}$ mBar, nebulizing gas flow rate of 600 l/h and dwell time of 0.1 s per transition.

3. Results and discussion

3.1. MS/MS optimization

Quantitation was conducted using the multiple reaction monitoring (MRM) mode. The mass spectrometer was programmed to transmit the protonated molecules (precursor) through the first quadrupole (MS1). Following collision-induced dissociation (fragmentation) in the collision cell with argon gas, product ions were transmitted through by the third quadrupole (MS2). Table 1 summarizes the product ion spectra of the four analytes and D_5-Diazepam. The best sensitivities and minimum interferences were achieved by monitoring the transitions stated in Section 2.5.

The fragment ions observed at m/z 241 for oxazepam and m/z 255 for temazepam are be

Table 3
Mean calculated values, accuracy and precision data for the calibration curves

Compound		Concentration (nM)							
		1	2	4	20	100	200	400	500
Oxazepam	Mean calculated value (nM)	1.0	2.0	3.8	19.6	98.4	203.2	410.7	517.0
	Accuracy (%)	2.2	−2.0	−3.8	−2.0	−1.6	1.6	2.7	3.4
	Precision (%)	7.6	9.4	7.5	4.6	5.4	6.1	4.0	3.9
Temazepam	Mean calculated value (nM)	1.0	2.0	4.1	19.6	99.4	199.6	403.9	507.1
	Accuracy (%)	0.5	−2.1	1.7	−1.8	−0.6	−0.2	1.0	1.4
	Precision (%)	6.4	4.6	3.4	8.2	4.6	6.4	4.5	5.6
Desmethyldiazepam	Mean calculated value (nM)	1.0	2.0	4.0	20.2	102.7	188.9	397.3	503.0
	Accuracy (%)	0.7	−2.0	0.0	0.8	2.7	−5.6	−0.7	0.6
	Precision (%)	7.1	11.0	8.1	12.4	2.9	3.1	5.2	3.5
Diazepam	Mean calculated value (nM)	1.0	1.8	3.8	20.2	103.1	204.2	398.7	498.4
	Accuracy (%)	3.7	−10.0	−4.0	1.1	3.1	2.1	−0.3	−0.3
	Precision (%)	7.0	10.6	9.1	4.8	2.5	4.0	4.2	2.9

Table 4
Inter-day and Intra-day accuracy and precision for all four analytes from dog plasma

Compound	Nominal concentration (nM)	Inter-day variation ($n = 12$)			Intra-day variation ($n = 6$)		
		Concentration (nM)	CV (%)	Relative error (%)	Concentration (nM)	CV (%)	Relative error (%)
Oxazepam	1	1.0	7.5	3.6	1.0	8.5	2.2
	4	3.9	7.2	−3.0	4.2	4.1	4.6
	100	97.2	6.4	−2.8	96.4	6.7	−3.6
	400	399.0	6.8	−0.2	387.4	5.0	−3.1
Temazepam	1	1.0	6.9	1.7	1.0	6.7	−0.7
	4	4.1	7.7	3.3	4.0	7.1	−1.1
	100	98.2	4.9	−1.8	97.7	8.7	−2.3
	400	405.2	5.8	1.3	389.7	7.4	−2.6
Desmethyldiazepam	1	1.1	5.7	8.9	1.1	6.8	5.3
	4	4.1	6.1	2.8	4.1	3.8	2.0
	100	101.7	2.9	1.7	100.6	6.1	0.6
	400	391.7	6.3	−2.1	386.8	7.0	−3.3
Diazepam	1	1.0	10.8	4.4	1.0	12.7	4.0
	4	4.1	8.4	3.1	4.3	3.5	8.1
	100	100.8	7.1	0.8	102.4	4.1	2.4
	400	395.0	2.2	−1.3	398.7	4.3	−0.3

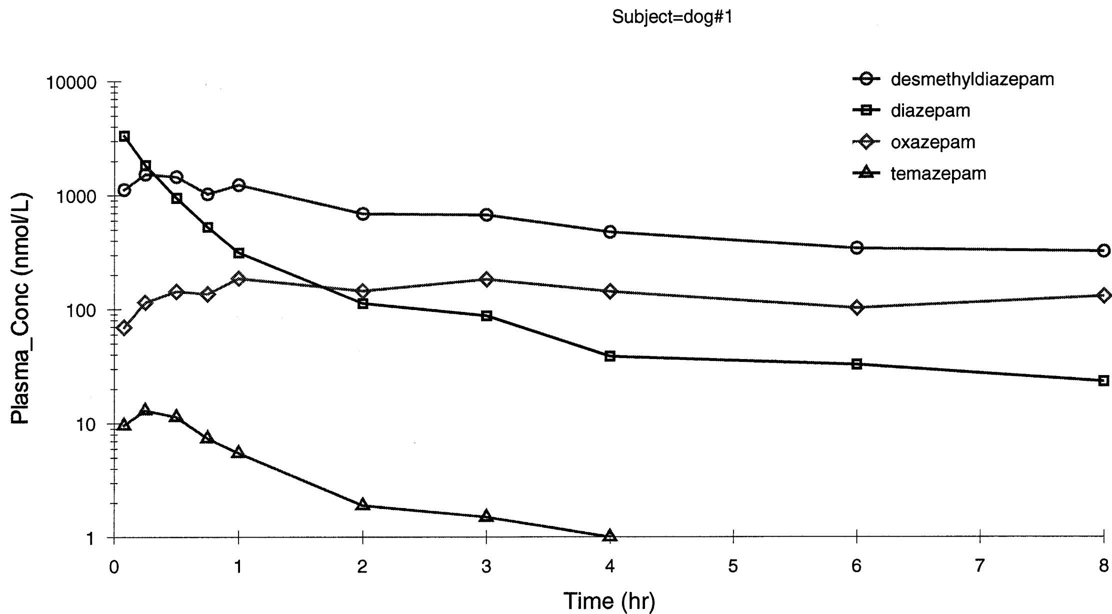

Fig. 5. The plasma concentration-time profiles of diazepam, oxazepam, temazepam and desmethyldiazepam in a dog following a 1 mg/kg i.v. bolus administration of diazepam.

lieved to be derived from loss of CO and H_2O. The fragment ions observed at m/z 140 for desmethyldiazepam and m/z 154 for diazepam and D_5-Diazepam can be attributed to the methyl and dimethyl, chlorophenyl iminium ions (C_7H_7NCl and C_8H_9NCl), respectively.

Collision-induced dissociation (CID) efficiency for the selected product ions was assessed as a function of collision gas energy. CID conditions were adjusted in order to obtain the maximum intensity of the product ions for these four analytes. A collision energy of 30 V was chosen to obtain the highest intensity of the base product ions.

3.2. Chromatography

Various bonded phases including ODS (C18), Octyl (C8), phenyl and CN were evaluated in an attempt to satisfactorily separate all four analytes. Among the various bonded phases, the Supleco Discovery C18 (2.1 × 50 mm, 5 μ) provided the best overall chromatographic separation, with good resolution for these four analytes.

An aqueous solution of 0.01 M ammonium acetate and acetonitrile was used as mobile phase. The addition of buffer instead of water resulted in more reproducible retention times on repeated injections. Peak shape and retention time were optimized at a flow rate of 0.25 ml/min.

3.3. Specificity

The specificity of the method is documented by the absence of interferences from endogenous substances from drug free dog plasma. Six different donors of blank dog plasma were screened and no endogenous dog plasma components were observed at the retention times corresponding to all four analytes and D_5-Diazepam (internal standard). Typical chromatograms of an extracted drug-free dog plasma blank, plasma spiked with all four analytes at 1 nM (LLOQ), and plasma spiked with all four analytes at 500 nM (ULOQ), are illustrated in Figs. 2–4, respectively. The elution order is oxazepam, temazepam, desmethyldiazepam, diazepam and D_5-Diazepam (internal

Table 5
Inter-day and Intra-day accuracy and precision for all four analytes from dog CSF

Compound	Nominal concentration (nM)	Inter-day variation ($n = 12$)			Intra-day variation ($n = 6$)		
		Concentration (nM)	CV (%)	Relative error (%)	Concentration (nM)	CV (%)	Relative error (%)
Oxazepam	1	1.1	10.3	9.4	1.1	9.1	10.0
	4	3.7	5.7	−8.0	4.1	10.8	2.5
	100	98.1	7.9	−1.9	102.0	10.5	2.0
	400	389.2	8.6	−2.7	402.4	9.9	0.6
Temazepam	1	1.0	10.6	−1.2	1.0	8.4	−3.3
	4	3.9	11.5	−1.8	4.2	12.8	5.0
	100	100.6	11.6	0.6	102.4	9.1	2.4
	400	407.1	7.6	1.8	412.5	7.8	3.1
Desmethyldiazepam	1	1.0	11.1	1.9	1.1	14.4	10.0
	4	4.2	7.3	6.0	4.4	7.9	10.0
	100	102.7	7.3	2.7	106.8	7.9	6.8
	400	389.4	11.3	−2.6	396.8	12.5	−0.8
Diazepam	1	1.0	17.7	−0.1	1.0	11.9	−1.7
	4	4.0	11.0	−0.3	4.2	9.3	4.5
	100	99.2	2.8	−0.8	98.0	4.2	−2.0
	400	402.4	5.7	0.6	413.4	3.8	3.4

standard) with retention times of 2.5, 2.8, 2.9, 3.1 and 3.2 min, respectively.

3.4. Linearity

A regression analysis of the peak area ratio versus concentration showed linearity over the 1–500 nM range for dog plasma. The standard curves were fitted to a first-degree polynomial, $y = ax + b$, where (y) is the peak area ratio of analyte to internal standard, (a) is the slope of calibration curve; (b) is constant, and (x) is the analyte concentration (nM). Coefficient of determination (r^2) of 0.9923 or better were obtained in the validation experiments. Table 2 summarizes the slope (sensitivity), intercept and r^2 values for four validation batches from dog plasma. Slightly greater sensitivity was noted for the two hydroxylated metabolites, oxazepam and temazepam, possibly due to their facile fragmentation by loss of H_2O and CO or more efficient formation of the protonated parent. Table 3 summarizes the mean calculated values, accuracy and precision data for the calibration curves.

3.5. Lower limit of quantitation (LLOQ)

The lower limit of quantitation (LLOQ) is defined as the lowest concentration on the calibration graph for which an acceptable accuracy (nominal $\pm$ 20% and precision of 20% CV) were obtained. The current assay has an LLOQ of 1 nM in dog plasma for each analyte. The coefficients of variations of replicate determination at the limit of quantitation were 8.5% for oxazepam, 6.7% for temazepam, 6.8% for desmethyldiazepam and 12.7% for diazepam ($n = 6$), respectively.

3.6. Accuracy and precision

To test the accuracy and precision of this assay, the quality control dog plasma samples (LLOQ QC, Low QC, Medium QC and High QC) were prepared and interpolated against the respective calibration curve. The intra-day assay variations were determined by analyzing six aliquots of spiked dog plasma samples containing 1, 4, 100 and 400 nM of four analytes, respectively. The inter-day assay variations were determined by analyzing three aliquots of spiked dog plasma samples containing 1, 4, 100 and 400 nM of four analytes, respectively, on 4 different days. The inter-day and intra-day accuracy and precision for all four analytes are given in Table 4. In both cases, the coefficient of variation for LLOQ (1 nM) were less than or equal to 8.5% for oxazepam, 6.9% for temazepam, 6.8% for desmethyldiazepam and 12.7% for diazepam, respectively. The coefficients of variation were smaller than 8.7% for all other concentrations investigated (Table 4).

3.7. Recovery

The percent recovery (extraction efficiencies) of four analytes was determined by comparing the peak area ratio of analyte to the internal standard (D_5-Diazepam) in a dog plasma sample that had been spiked with four analytes prior to extraction (pre-extraction spiked QC) with samples to which four analytes had been added post-extraction. The internal standard was added to both sets of samples post-extraction. The overall extraction recoveries from dog plasma were 76.6% for oxazepam, 59.8% for temazepam, 86.6% for desmethyldiazepam and 43.7% for diazepam from three concentration levels (4, 100 and 400 nM).

3.8. Matrix effect

The matrix effect of four analytes was determined by comparing the peak area of analyte in a dog plasma sample that had been spiked with four analytes after extraction (post-extraction added QC) with samples to which four analytes had been added to the initial mobile phase (mobile phased added QC).

There was no significant signal suppression due to matrix effect during the ionization process. The average matrix effect measured was 0.9 for oxazepam, 0.7 for temazepam, 0.8 for desmethyldiazepam and 1.0 for diazepam from three concentration levels (4, 100 and 400 nM).

3.9. Stability

All stability experiments were performed with

two different concentration levels (4 and 400 nM) in triplicate. Diazepam and its three metabolites were found to be stable in dog plasma for at least 4 hours at ambient temperature and in the reconstitution solution at 6°C for at least 24 h. After 4 h at ambient temperature, the concentration decreased 2.1% for oxazepam, 11.1% for temazepam, 8.0% for desmethyldiazepam and 5.6% for diazepam, respectively. After storing at autosamples for 24 h at 6°C, the concentration decreased 5.4% for oxazepam, 10.5% for temazepam, 7.9% for desmethyldiazepam and 10.6% for diazepam, respectively. The stability of diazepam and its three metabolites to repeated freeze-thaw cycles was also examined using the spiked dog plasma samples. After three freeze-thaw cycles, the proportion of diazepam and its three metabolites remaining, relative to the initial analysis, was 91.8% for oxazepam, 89.1% for temazepam, 92.3% for desmethyldiazepam and 94.4% for diazepam, respectively. The analytes in plasma can, therefore, tolerate at least three freeze-thaw cycles without degradation.

3.10. Application

The method was used for the determination of diazepam and its three metabolites (oxazepam, temazepam and desmethyldiazepam) as bioanalytical support for a drug discovery study. Diazepam was administered at 1 mg/kg by bolus i.v. to Mongrel dogs. The plasma was collected at specific time points for the determination of the analytes. The plasma concentration-time plot of diazepam and its three metabolites in dog # 1 is shown in Fig. 5. These results are similar to those reported previously [11] showing that rapid and significant exposure to active metabolites occurs in dogs after diazepam administration. Furthermore, analysis of dog cerebrospinal fluid (CSF) (data not shown) confirms previous observations that CSF penetration of the drug and these metabolites is rapid and limited to the unbound drug fraction [12].

4. Conclusions

A fast, sensitive and specific LC/MS/MS assay for the simultaneous quantitation of diazepam and its three metabolites, oxazepam, temazepam and desmethyldiazepam, in dog plasma at LLOQ of 1 nM has been demonstrated. Validation results support the precision and accuracy of the methods over the range of 1–500 nM. This procedure was also validated and applied to the quantitation of diazepam and its three metabolites in dog CSF (Table 5).

The use of a D_5-Diazepam as internal standard improved the accuracy of the quantitative analysis of diazepam. Because it is closely related in structure and chemical properties with the other three metabolites, D_5-Diazepam also served as an appropriate internal standard for oxazepam, temazepam and desmethyldiazepam. This method has been used to support discovery pharmacokinetic studies in dog, but may be applied to other species or matrices.

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