Validation of an HPLC-FL Method for the Determination of Tepoxalin and its Major Metabolite in Horse Plasma

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Abstract

A sensitive, simple, and accurate high-performance liquid chromatographic (HPLC) method has been developed for the determination of tepoxalin, its major metabolite RWJ-20142 and the internal standard RWJ-20294 (Schering-Plough Co.) in horse plasma. The plasma samples (0.5 mL) were directly extracted with acetonitrile 0.9 mL; after high speed centrifugation to precipitate proteins 50 µL were injected into a HPLC. Separation was performed on a Gemini C18 column (250 mm × 4.6 mm i.d., 5 µm particles) with mobile phase A consisting of 0.01 M 1-octane-sulfonic acid in 0.01 M acetic acid aqueous solution and mobile phase B consisting of tetrahydrofuran, using an isocratic elution at 50% and 50% at 1.0 mL/min flow rate. Detection and quantitation were performed by fluorimetric detection at λex = 290 nm and λem = 440 nm. Detection and quantitation limits for tepoxalin and RWJ-20142 were 30 and 50 ng/mL, and 10 and 30 ng/mL, respectively. Recovery values for tepoxalin and RWJ-20142 were near 100%. The linear concentration range for tepoxalin and RWJ 20142 were 50–5000 and 30–5000 ng/mL, respectively. The validated reversed-phase HPLC-fluorescence detection method was used for determination of preliminary pharmacokinetic data of the drug in horse plasma.

Introduction

Tepoxalin [5(4-chlorophenyl)-N-hydroxy-(4-methoxyphenyl)-N-methyl-1H-pyrazole-3-propanamide] (Figure 1) is an orally active dual cyclooxygenase/lipoxygenase inhibitor, with a favourable gastrointestinal profile with respect to gastric mucosal injury (1). Tepoxalin is indicated for the control of pain and inflammation associated with osteoarthritis in dogs. After oral administration in dogs and humans, tepoxalin is rapidly converted to its active, carboxylic acid metabolite RWJ-20142, which inhibits cyclooxygenase but not lipoxygenase (2,3). Its administration could be useful in equine clinical practice despite it has not formulated yet for this animal species. Potentially, a dual inhibitors as tepoxalin has broader spectrum of anti-inflammatory activity than that of pure COX inhibitors, representing a hypothetical alternative to classical non steroidal anti-inflammatory drug therapies in horses. Recently its pharmacokinetic ability has been reported in rabbits (4) and poultry (5), but no validated method has been used for these matrices. The objective of the present study was to validate the analytical method for the quantization of tepoxalin and RWJ-20142 in horse plasma, in order to use such method for pharmacokinetic evaluations in prospective studies.

Experimental

Materials and methods

Tepoxalin, RWJ-20142, and RWJ-20294 standard powders were gifted from Schering-Plough Co. (Summit, NJ). High-performance liquid chromatography (HPLC)-grade water from Baker Analyzed Reagent (J.T. Baker, Deventer, Holland) was used for solvent A preparation. Methanol, tetrahydrofuran, and acetonitrile used were of HPLC grade from Carlo Erba (Milan, Italy). Acetic acid, and 1-octane-sulfonic acid were of analytical-reagent grade from Sigma (St. Louis, MI).

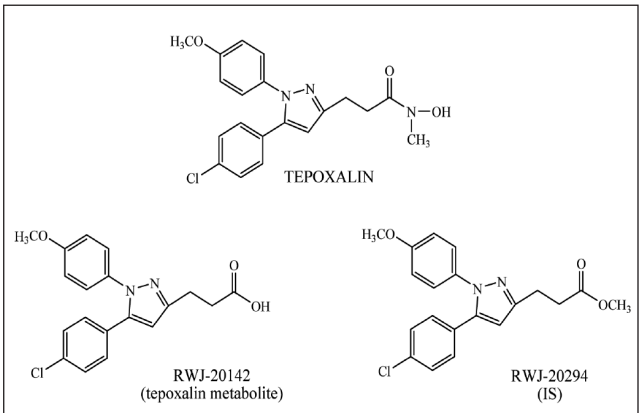

Figure 1. Molecular structure of tepoxalin, RWJ-20142 (metabolite) and RWJ-20294 (internal standard).

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Standards drug solution

Accurately weighed tepoxalin standard (99.9% pure, 100 mg) was transferred to a 100-mL volumetric flask. The drug was dissolved in methanol and the solution was then diluted to volume with the same solvent. This gave a standard stock solution of concentration 1000 µg/mL. The stock solution was appropriately diluted to prepare 10 µg/mL working standard. The same procedure was repeated for RWJ-20142 and RWJ-20294.

Extraction procedure

The drug was extracted from horse plasma by the following procedure. The plasma sample (0.5 mL) was spiked with 10 µg/mL RWJ-20294 (100 µL) as internal standard in a 2 mL plastic Eppendorf. The mixture was vortex mixed for 30 s and left rest for 5 min. Acetonitrile (0.9 mL) was added to the mixture to allow the protein precipitation. This mixture was vortex mixed, shaken for 10 min at 150 oscillation/min on a shaker, and then centrifuged at 14000 *g* in a refrigerated (4°C) centrifuge for 15 min. Four hundred microliters of the upper layer was transferred in a clean Eppendorf tube and centrifuged (15 min at 14000 *g*) again. Two hundred microliters were sundered in a 500 µL Eppendorf. Sample extracts were filtered through a 0.45-µm syringe filter (Spartman, Whatman, Maidstone, UK) before being injected into the HPLC system.

Chromatographic conditions

HPLC was performed with a Jasco PU 980 (Easton, MD) isocratic pump attached to a Jasco AS-1550 auto-sampler and Jasco 821-FP, fluorimetric detector. Integration was performed with Borwin chromatographic software, version 1.21. The compounds were separated on a Gemini C18 column (250 mm × 4.6 mm i.d., 5 µm particles) with a solvent A, consisted of 0.01 M 1-octane-sulfonic acid in 0.01 M acetic acid in water, and a solvent B consisted of tetrahydrofuran, mixed at 50:50 (v/v). The flow rate of the mobile phase was maintained at 1.0 mL/min. Injection volume was 50 µL and the detector wavelengths were set at 290 and 440 nm as excitation and emission wavelengths, respectively.

Validation procedure

The method was validated in accordance with its intended use. The specificity of the method was checked by extraction and analysis of blank plasma (from six different horses). Specificity was confirmed by the absence of any peaks at the expected retention times of tepoxalin, RWJ-20142 and RWJ-20294. The linearity experiment was performed in triplicate with spiked samples in the range from 50 to 5000 ng/mL and 30 to 5000 ng/mL for tepoxalin and RWJ-20142, respectively. The samples for the linearity test were prepared by adding different amounts of tepoxalin or RWJ-20142 and a fixed amount (100 µL) solution of internal standard solution (10 µg/mL) to plasma to obtain the required concentration range. These samples were extracted by use of the extraction procedure described previously and injected into the chromatographic system. The drug/IS and metabolite/IS peak area ratio was plotted against the concentration of the drug and metabolite, respectively, and the correlation coefficients, slopes, and intercepts of the plots were evaluated for both analytes. RSD (%) values of slopes and intercepts for all three calibration plots were < 2% and values of the correlation coefficient for all three plots were > 0.999. LOD (limit of detection) and LLOQ (lower limit of quantitation) were determined by calculating the ratio of the response to the analyte (peak-height signal) to the baseline response (noise). The signal was defined as the distance between the drug peak baseline and the peak top; noise was the distance between the average lower and upper limits of the noise in an uncontaminated (peak-free) region of the chromatogram. LOD and LLOQ were calculated as the concentration corresponding to a signal-to-noise ratio of > 3:1 and > 10:1, respectively. When solutions at the LOD and LLOQ levels were prepared and injected six times the RSD (%) of the response was < 2. Accuracy and precision were evaluated using quality control (QC) samples (low 100 ng/mL, medium 250 ng/mL, and high 1000 ng/mL). Five replicate QC samples at each concentration were analyzed in a single sequence to evaluate within-day variation. For evaluation of between-day variation four replicate QC samples at each concentration were analyzed with standards on three different days. The accuracy of the method was determined by statistically comparing with the nominal concentrations and expressed as a percentage of the actual concentrations in the QC samples. The precision of the method was evaluated as RSD (%) of the area ratios for the QC samples. Nominal concentrations were between 85% and 115% and RSD were lower than 15%. Tepoxalin and RWJ-20142 were checked for bench-top stability, auto-sampler stability, and freeze–thaw stability by use of QC samples. In the bench-top stability study, plasma samples (QC levels) spiked with the drug or metabolite were kept at room temperature for 4 h, then extracted and analyzed with freshly extracted samples spiked at the QC levels. Auto-sampler stability was evaluated by keeping QC samples in an auto-sampler for 6 h and 12 h and then analyzing these samples with freshly extracted QC levels. Evaluation of freeze-thaw stability was performed for three cycles of freezing and thawing. Spiked plasma was kept in the deep freeze at –20°C for 12 h (1st cycle) and then left to thaw at room temperature. The samples were then extracted and analyzed with the freshly extracted QC samples. The remaining samples were kept for the 2nd and 3rd cycles and then analyzed. The long-term stability of tepoxalin and RWJ-20142 was evaluated for 14 days. It was checked by storing QC samples at –20°C, then extracting and analyzing with freshly extracted QC levels. The stability was evaluated by comparing the difference (%) between area ratio values of stability-test samples and fresh QC samples. The difference was less than 15%. The system-suitability test was performed by six-fold analysis of a mixed solution of drug or metabolite and internal standard in the linear region of the calibration plot and measuring the RSD (%) of the capacity factor, tailing factor, response, and resolution. The system-suitability test was performed before analysis of every batch of samples. This helped to ensure the performance and reproducible out-put of the chromatographic system during analysis.

Animal treatment

The study protocol was approved by the ethics committee of the University of Pisa authorization no. 22275 and transmitted to Italian Ministry of Health. The mare was previously determined to be clinically healthy based on physical examination and full chemistry hematologic analyses. The animal was fasted for 12 h

before administration of tepoxalin (10 mg/kg body weight; Zubrin 200 mg/tablet, Schering-Plough Co.) by nasogastric tube: the nasogastric tube was finally rinsed with 500 mL water to ensure complete delivery of the drug into the stomach. Blood samples were collected at 0, 5, 15, 30, and 45 min and 1, 1.5, 2, 4, 6, 8, 24, 34, 48, 58, and 72 h by a 14-gauge catheter previously placed in the right jugular vein. The blood was then placed into collection tubes containing lithium heparin. The samples were centrifuged within 30 min after collection and the harvested plasma was frozen at –20°C until analysis.

Pharmacokinetic evaluation

The pharmacokinetic calculations were carried out using WinNonLin v 5.2.1 (Pharsight Corp, Cary, NC). C_{max}, the highest observed plasma concentration, and T_{max}, the time required to reach C_{max}, were obtained from the individual plasma concentration/time curves. Half-life of the terminal phase ($T_{1/2}\lambda z$) was calculated from the slope of the logarithm of concentration versus time profile. Area under the concentration/time curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by the linear trapezoidal rule.

Results and Discussion

During development of the method, different solvents—dichloromethane, *n*-hexane, ethylacetate, and *t*-methylbutane—were investigated for extraction of tepoxalin and metabolite. Recoveries were sometimes low (< 50% with ethylacetate), or highly variable (RSD% > 20% with dichloromethane and *n*-hexane). However, in all the instances large interferences from plasma peaks were also observed. Finally, the extraction with acetonitrile was selected as the best among those tested. It provided cleaner extracts and better precision. Similar extractive procedure was reported in a previous study (5). Nominal values (%) for recovery of tepoxalin and RWJ-20142 from QC samples in tests of intraday and interday accuracy ranged within 99.8–100.2% and 100.2–100.7%, and between 99.8–100.5% and 99.2–100.4%, respectively.

The mobile phase was optimized to provide sufficient selectivity in a short separation time. After a series of screening experiments, it was concluded that 1-octane-sulfonic solution gave better peak shapes than the cheaper acetate and phosphate buffers, resulting also in higher sensitivity and selectivity. With methanol as organic component of the mobile phase, the three peaks showed less theoretical plates and higher retention times compared to tetrahydrofuran. Tetrahydrofuran as organic component resulted in better sensitivity and selectivity but variation of the amount of tetrahydrofuran in the mobile phase effected resolution and runtime. Variation of the pH of the mobile phase resulted in bad peaks' shape, increased interferences from the plasma and in the shift of the analytes at the beginning of the chromatogram: for this purpose a standard amount of acetic acid was added to regularize the pH. Finally, the optimized mobile phase was, 0.01 M 1-octane-sulfonic acid in 0.01 M acetic acid in water and tetrahydrofuran, mixed at 50:50 (v/v).

Injection volume was optimized to 50 µL, because use of

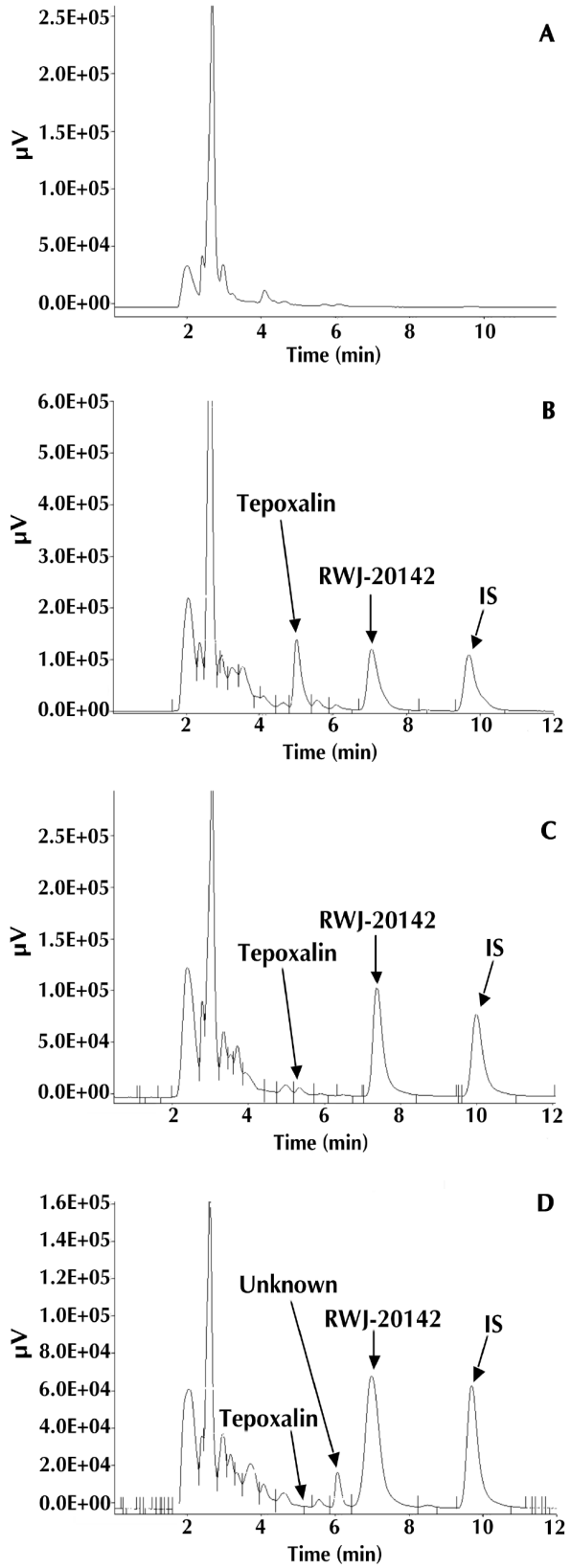

Figure 2. Chromatographic curve from blanks of horse plasma (A); chromatographic curve from fortified sample (tepoxalin, RWJ-20142 and IS each at spiking level of 666 ng/mL) of horse plasma (B); chromatographic curve from incurred samples of horse plasma (1 h) (C); and chromatographic curve from incurred samples of horse plasma (4 h) (D).

greater volumes resulted in loss of peak symmetry. The column temperature was not controlled and was approximately 25°C (ambient). Small changes in column temperature (± 2°C), detection wavelength (± 2 nm), and mobile phase concentration (± 2%), and changing the batch of the column, did not affect the sensitivity and selectivity of the method.

RWJ-20294, which has physicochemical properties similar to those of the analytes and a similar chemical structure (Figure 1), was chosen as the internal standard (IS). It was well separated from tepoxalin, RWJ-20142 and endogenous interferences with recoveries near to 100% at the concentration employed (666 ng/mL). This molecule has just been used as IS in previous studies concerning the tepoxalin plasma detection (2,5). Typical chromatograms obtained from blank plasma, from blank plasma spiked with tepoxalin, RWJ-20142 and internal standard, and from a plasma sample from the horse after administration of Zubrin tablets are shown in Figure 2A–2D. The tepoxalin, RWJ-20142 and IS eluted at 5.1, 7.1, and 9.7 min, respectively. An unknown time-dependent peak has been observed in plasma samples following drug administration, and has been speculated to be a new metabolite (Figure 2D) (5).

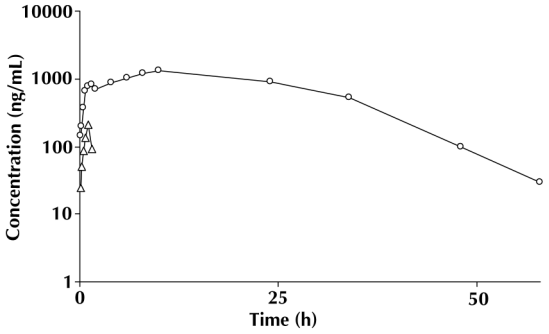

Figure 3. Tepoxalin (Δ) and RWJ-20142 (o) semi-logarithmic concentration vs time curves (ng/mL) in plasma of horse administered with a single oral dose of 10 mg/kg by nasogastric tube.

The acceptances of the system-suitability test criteria were in accordance with those proposed by Vander Heyden et al. (6).

Detector response was found to be linear in the range 50–5000 ng/mL and 30–5000 ng/mL, with $r^2 = 0.999$ and $r^2 = 1$ for tepoxalin and RWJ-20142, respectively. The lowest limits of detection were 30 and 10 ng/mL for tepoxalin and RWJ-20142, respectively. The limits of quantification were 50 and 30 ng/mL, for tepoxalin and RWJ-20142, respectively, indicative of the high sensitivity of the method. Coefficient of variance (RSD %) for tepoxalin and RWJ-20142 QC samples ranged from 1.5% to 2.6% and 2.2% to 4.6% for intra-day precision and from 1.5% to 3.6% and 2.9% to 5.1% for inter-day precision, respectively, indicating that the method is precise. Results from determination of intra-day and inter-day accuracy, and precision are listed in Table I. The absence of a peak at the retention times of tepoxalin, RWJ-20142 and the internal standard indicates the specificity of the method.

Low values of percentage differences (< 15%) between area ratios for stability-test samples and fresh QC samples confirmed the stability of drug on the bench top for 4 h, in an auto-sampler for 12 h, and inside the freezer for 14 days. Results from stability studies are listed in Table II.

The features of this (specific, rapid, sensitive, low-cost, and validated) method HPLC makes it appropriate for pharmacokinetic studies, despite other methods reported higher sensitivity and specificity (7).

The plasma concentration vs time curve (Figure 3) of tepoxalin showed both little maximal concentration of the drug (Cmax 210.7 ng/mL) and its rapid elimination ($T_{1/2}\lambda z$ 0.36 h), though its major metabolite RWJ-20142 showed both higher Cmax (1303.5 ng/mL) and $T_{1/2}\lambda z$ (5.78 h) (Table III). Such pharmacokinetic profile is similar to those previously reported in dogs (2), rabbits (4), chickens (5), and humans (3).

Table I. Results from Studies of Intra- and Inter-day Accuracy and Precision for Tepoxalin and RWJ-20142*

	Tepoxalin			RWJ-20142		
	LQC	MQC	HQC	LQC	MQC	HQC
Intra-day						
Mean ± SD	100.1 ± 2.6	249.5 ± 3.8	1001.5 ± 26.2	100.3 ± 4.6	250.4 ± 7.8	1006.7 ± 22.0
CV(%)	2.6	1.5	2.6	4.6	3.1	2.2
Nominal (%)	100.1	99.8	100.2	100.3	100.2	100.7
Inter-days						
Mean ± SD	100.5 ± 3.6	249 ± 3.8	1005.4 ± 26.2	100.9 ± 5.1	248 ± 9.8	1003.9 ± 29.1
CV(%)	3.6	1.5	2.3	5.1	3.9	2.9
Nominal (%)	100.5	99.8	100.5	100.9	99.2	100.4

*LQC: 100 ng/mL; MQC: 250 ng/mL; HQC: 1000 ng/mL.

Table II. Results from Study of Stability for Tepoxalin and RWJ-20142*

Statistical properties	Auto–sampler stability				Freeze–thaw stability				Bench–top stability				Long–term			
	LQC		HQC		LQC		HQC		LQC		HQC		LQC		HQC	
	Tep	RWJ	Tep	RWJ	Tep	RWJ	Tep	RWJ	Tep	RWJ	Tep	RWJ	Tep	RWJ	Tep	RWJ
Difference (%)	–4.3	–2.5	–39.1	–52.8	–3.7	–1.5	11.1	–29.9	–3.6	–2.9	–46.1	49.4	5.2	3.8	–39.4	–18.8
S.D.	0.25	0.32	17.3	32.2	0.1	0.7	7.8	21.2	0.9	1.4	24.5	35.1	2.3	1.5	28.4	25.4
CV (%)	0.25	0.32	1.7	3.2	0.1	0.7	0.8	2.1	0.9	1.4	2.5	3.5	2.3	1.5	2.8	2.5
Nominal (%)	95.7	97.5	96.1	94.7	96.3	98.5	101.1	103	96.4	97.1	95.4	104.9	105.2	103.8	96.1	98.1

*LQC = 100 ng/mL; HQC = 1000 ng/mL

Conclusion

The present study has developed a specific, rapid, sensitive, and low-cost HPLC method for the determination of tepoxalin and its major metabolite in horse plasma. The method involves a simple extraction procedure then separation on a reversed-phase column, with an internal standard, and fluorimetric detection. The accuracy, precision, recovery, quantization limit, and detection limit enable the use of the method in pharmacokinetic and clinical studies of tepoxalin. The run time and stability are suitable for processing many samples daily. To date this is the first time that a HPLC method for the quantization of tepoxalin and its main metabolite in plasma is validated. This could be useful to perform certified quantization of the drug in plasma matrices (i.e., pharmacokinetics). The pharmacokinetic data obtained are in agreement with reference values reported in other animal species, indicating the utility of method for estimating tepoxalin and RWJ-20142 concentrations in horse plasma.

Table III. Main Pharmacokinetic Parameters of Tepoxalin and RWJ-20142 After Oral Administration of 10 mg/kg Tepoxalin in a Healthy Horse

Parameter*	Tepoxalin	RWJ-20142
C_{max} (ng/mL)	210.7	1303.5
T_{max} (h)	1	10
$AUC_{0-\infty}$ (h ng/mL)	201.2	37244
$T_{1/2}\lambda z$ (h)	0.36	5.78

*$AUC_{0-\infty}$ = area under the plasma concentration – time curve extrapolated to infinity;
C_{max} = peak plasma concentration;
T_{max} = time of peak;
$T_{1/2}\lambda z$ = terminal half-life.

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References

1. D.C. Argentieri, D.M. Ritchie, M.P. Ferro, T. Kirchner, M.P. Wachter, D.W. Anderson, M.E. Rosenthale, and R.J. Capetola. Tepoxalin: a dual cyclooxygenase/5-lipoxygenase inhibitor of arachidonic acid metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. *J. Pharmacol. Exp. Ther.* **271:** 1399–1408 (1994).
2. L.M. Homer, C.R. Clarke, and A.J. Weingarten. Effect of dietary fat on oral bioavailability of tepoxalin in dogs. *J. Vet. Pharmacol. Ther.* **28:** 287–291 (2005).
3. S.A. Waldman, C. Vitow, B. Osborne, L. Gillen, D.C. Argentieri, F.A. Wong, I.L. Smith, A.T. Chow, J. Misiti, and T.D. Bjornsson. Pharmacokinetics and pharmacodynamics of tepoxalin after single oral dose administration to healthy volunteers. *Clin. Pharmacol.* **36:** 462–468 (1996).
4. C.G. Pollock, J.W. Carpenter, D.E. Koch, and R.P. Hunter. Single and multiple-dose pharmacokinetics of tepoxalin and its active metabolite after oral administration to rabbits (Oryctolagus cuniculus). *J. Vet. Pharmacol. Ther.* **31:** 171–174 (2008).
5. S. De Boever, E. Neirinckx, K. Baert, P. De Backer, and S. Croubels. Pharmacokinetics of tepoxalin and its active metabolite in broiler chickens. *J. Vet. Pharmacol. Ther.* **32:** 97–100 (2009).
6. Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M Vandeginste, and D.L. Massart. Guidance for robustness: ruggedness tests in method validation. *J. Pharm. Biomed. Anal.* **24:** 723–753 (2001).
7. D.J. Burinsky, B.L. Armstrong, A.R. Oyler, and R. Dunphy. Characterization of tepoxalin and its related compounds by high-performance liquid chromatography/mass spectrometry. *Pharm. Sci.* **85:** 159–164 (1996).

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