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Article

An LC–MS Method for Determination of Milbemycin Oxime in Dog Plasma

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An HPLC-MS method has been developed and validated for the quantitative determination of milbemycin oxime (MBO) in dog plasma. The developed method has been then applied in *in vivo* clinical studies to obtain pharmacokinetics of MBO in blood after its oral administration. Samples were extracted using solid-phase extraction (SPE). Sample proteins were precipitated with acetonitrile (ACN) and sodium chloride (NaCl) and then diluted with methanol and water. Calibration standards were prepared by using plasma matrix and following the same SPE procedure. Chromatographic separation was performed on a Waters C_{18} packed column (3.5 μm particles diameter; 3 × 100 mm) with a C_{18} guard column (3.5 μm particles diameter; 3 × 20 mm). The mobile phase was an 85:15 (v/v) mixed solution of ACN and 5 mM ammonium acetate. The calibration curve was linear over a concentration range of 2.0–500 ng/mL with a limit of quantitation of 2.0 ng/mL. The oral administration of a pellet of 2.5 mg MBO produced blood concentrations ranging from 6.10 ± 0.92 to 78.81 ± 4.38 ng/mL within 6 h, with a terminal half-time of 11.66 ± 0.93 h. This study determined the suitability of the herein proposed method to investigate the pharmacokinetics of MBO after oral administration.

Introduction

Milbemycin oxime (MBO) (Figure 1) belongs to the group of macrocyclic lactones (MLs), isolated from the fermentation of *Streptomyces hygroscopicus* var. *aureolaccrimosus*, which consists of milbemycin A_4 oxime and milbemycin A_3 oxime at an 80:20 ratio. MBO was discovered by a Sankyo group in 1967, and the closely related chemical structure to avermectins leds to its insecticidal activities against important pests, as has been found with the avermectins abamectin and emamectin, and launched as a parasiticide for the control of *Dirofilaria immitis* (1) and other nematodes and arthropods. MBO, like avermectins and other milbemycins, increases nematode and insect membrane permeability to chloride ions via glutamate-gated chloride ion channels (related to vertebrate GABA and glycine receptors). This leads to hyperpolarization of the neuromuscular membrane and flaccid paralysis and death of the parasite (2). And now, MBO is used as a feline and canine anthelminitic, its efficacy in cats against heartworms (3) has been recently investigated and several studies have confirmed its suitability for the control of *Dirofilaria immitis* (4), *Toxocara canis*, *Ancylostoma caninum* (5) and *Trichuris vulpis* (6, 7) and some ectoparasites (8–10) in dogs. In recent years, MBO has been used with other anthelmintics, such as lufenuron (11, 12), praziquantel (13), spinosad (14) to treat with *Toxocara canis*, *Baylisascaris procyonis*, *Toxocara cati* and other canine intestinal nematode parasites.

Even though some collies were reported sensitive to this drug at higher dosages (15, 16), MBO appears to be safer and associated with fewer side effects. Furthermore, the closely similar structure of MBO to that of avermectins has provided MBO with excellent parasiticide activities as well as cross resistance. The wide usage in animal area has made the determination method of MBO in animal matrix emergency.

The determination of avermectins, such as abamectin, ivermectin (17), eprinomectin (18), doramectin and selamectin (19) and milbemycins (20) such as moxidectin (21) in animal blood and tissues has been reported based on microbiology and immunoassay methods, HPLC and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (22–24). The authors have developed an HPLC-fluorescnece method for the detection of milbemycin in dog plasma with a derivatization, which is not related to this work. And the determination of MBO in animal blood was reported by ELISA in the 20 century (1); however, the sensitivity was low. HPLC-MS as a new method for the detection of drugs has been widely used and, to some extent, added specificity for the detection of MBO compared with other methods; however, to the best of our knowledge, the method has not been used in the plasma samples of MBO.

The objective of the present work was to develop an HPLC-MS method, considering the SPE for sample preparation and MS detection, for the HPLC analysis and pharmacokinetics of MBO in dog plasma.

Experimental

Chemicals and reagents

Milbemycin A_4 oxime (98%) was purchased from Wako Chemicals (Richmond, VA, USA). MBO pellets (2.5 mg/tablet) were obtained from Zhejiang Hisun Pharmaceutical Co. Ltd. HPLC-grade ACN and methanol were from Merk (Darmstadt, Germany). Deionized water for LC–MS was prepared using a Milli Q50 (Millipore, Bedford, MA, USA) water purification system. Prepacked cartridges (Supelclean LC_{18}, 100 mg, 1 mL) for SPE were supplied by Sigma-Aldrich (St. Louis, MO, USA). For the preparation of in-house quality control and calibration samples, dog plasma was collected from normal dogs. All other chemicals used in this study were of analytical grade.

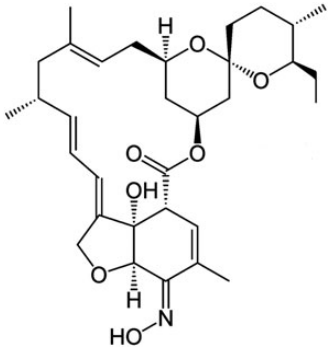

Figure 1. Structure of milbemycin A4 oxime.

Instruments and analytical conditions

A Finnigan Spectrasystem (Thermo Fisher Scientific Hemel Hempstead, UK) comprising a Finnigan Spectrasystem P2000 Binary Gradient Pump and a Thermo Separation Products TCP Spectrasystem AS3000 autosampler was used. The HPLC system was interfaced with a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Thermo Fisher Scientific Hemel Hempstead, UK) operated under the Xcalibur software (version 1.4, Thermo Electron Corporation, Hemel Hempstead, UK), a 2000-40M air compressor (JUN-AIR International A/S, Denmark) and a nitrogen generator system 75-72 (Parker Hannifin Corp, Kent, UK). Analyses were carried out using a Waters (Milford, MA, USA) Xterra C_{18} (3.5 μm particles diameter; 3 × 100 mm) with C_{18} guard column (3.5 μm particles diameter; 3 × 20 mm).

The mobile phase was a mixture of ACN at 85% and 5 mM ammonium acetate at 15%. The mobile phase was performed at a flow rate of 0.25 mL/min. Mass spectral analysis for MBO was carried out using electrospray ionization (ESI) in positive ion mode (4.8 kV) at a capillary temperature of 300°C, capillary voltage of 29 V and a tube lens of 105 V. For single ion monitoring analysis, the first quadruple was set to scan for m/s 558.85 and 570.85, which represented $[MH]^+$ and $[MNa]^+$, respectively.

In vivo MBO administration

The concentrations of MBO after its oral administration to beagles 10 ± 1 kg were measured. Blood samples were collected from each dog before oral administration of the drug. No drug was detected in the plasma samples collected from any dogs.

For the oral MBO administration, the animals were slightly anesthetized and received an oral administration of MBO. Blood samples 1 mL/sample were then collected at the end of administration and after 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 h. No medications were given to dogs other than MBO. All procedures were in accordance with the National Institute of Health's guidelines on animal care.

Sample preparation

The blood samples were centrifuged at 2620*g* for 10 min after their collection, then 0.8 mL of ACN and 60 mg NaCl were added to 200 μL of supernatant and vortex mixed for 1 min. After vortex mixing, the mixture was centrifuged at 2620*g* for 5 min and the supernatant was used. The supernatant ≤5 mL was evaporated by drying in nitrogen flow. Then a 3 mL of methanol−5 mmol/L of ammonium acetate (1:9) was added into the tube, vortex mixed and cleaned up by a C_{18} solid-phase column extraction. Automatic sample preparation was performed as reference (20). Three milliliters of methanol was applied to elute the cartridge and the elute was collected and evaporated by drying in nitrogen flow. Two hundred microlitres of mobile phase was added and after filtered with a 0.22-μm nylon membrane, 5 μL was injected for the HPLC−MS analysis.

Method validation

The validation of analytical assay of MBO from plasma extracts was performed according to current guidelines (25).

Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from six dogs with those of corresponding standard plasma sample spiked with MBO and plasma sample after an oral dose. All the six blank plasma samples were extracted to ensure the absence of interfering peaks.

Calibration curve

Stock 5000 ng/mL and substock 100 ng/mL solutions of milbemycin A_4 oxime were prepared in ACN. A total of eight MBO concentrations (2, 5, 10, 50, 100, 150, 200, 500 ng/mL) in drug-free plasma were used as calibrators and three in-house quality control standards (QCs) containing 5, 150 and 400 ng/mL of MBO were used to estimate the accuracy and precision of the assay. All the stock and diluted stock solutions, calibrators and QC standards were stored at −20°C until being used.

Precision and accuracy

Quality control samples were prepared by spiking of substock solution in the blank perfusion fluid. Accuracy was determined at three different concentrations of QC samples (5, 150, 400 ng/mL) each in five replicates. Similarly, precision was measured using five determinations per concentration for all QC samples. Intra- and interassay precision were measured by determinations on a particular day and also on three consecutive days.

Recovery

The analytical recovery of MBO was assessed by comparing the peak area ratio of QCs extracted from plasma with the peak area ratio of reference standards prepared in the same way at the stock solution.

Stability

Freeze and thaw stability. The stability of MBO after freeze and thaw cycles was determined at all the three QC concentrations in triplicate. The QC samples were frozen at −20°C for 24 h and then thawed unassisted for the next 24 h, and this cycle was repeated three times before analysis.

Bench-top stability. Bench-top stability of MBO in the blank matrix was determined by holding separate QC samples at above three concentrations and in triplicate at room temperature for 24 h. Samples were analyzed thereafter with the same method and comparing the accuracy against freshly prepared stock solution.

Stock solution stability. The stability of the stock solution stored at −20°C for 7 days and subsequently for 6 h at room temperature was also determined. Stock solution was spiked in blank perfusion fluid as mentioned above to produce three QC concentrations in triplicate and analyzed against the QC samples prepared from fresh stock solution.

Application to pharmacokinetics of MBO

Tablets of MBO (2.5 mg) were used for oral administration in six healthy dogs with no clinical sighs of either acute or chronic illness. The trial was started after an overnight fast which continued for another 48 h after dosage. Plasma samples were taken by jugular vein puncture after administration of a single dose of a 2.5 mg pellet. Blood specimens were stored at −20°C until analysis. After reconstitution, the samples were treated in the same procedure as for the plasma calibration line. The dose–time curve was analyzed by the pharmacokinetic software 3P97, and then fitted by nonlinear regression and algebra processing. The pharmacokinetic parameters of each animal were analyzed by 3P97 according to compartment model, and demonstrated using $\bar{x} + SD$.

Results

Method validation

The employment of SPE is pivotal to achieving a fast separation combined with a reliable quantitative determination of MBO in biological matrix. The peak shape of target compound was characterized by high symmetry. No interfering compounds or loss of chromatographic efficiency was observed for MBO peaks with the developed program. Under optimal LC–MS conditions, the positive ion signals were chosen for larger than the negative ion signals, which was coincidence with results of David *et al.* (26). Scanning mass spectra of milbemycin A_4 oxime was shown in Figure 2 for milbemycin A_4 oxime, the m/z 558.85 and 570.85 was chosen as monitoring ion.

Separation and specificity

Protein precipitation could be used for the determination of many chemical materials; however, it can induce interfering peaks and general baseline noise and contamination of the LC–MS system. To provide additional sample cleanup, an SPE method was developed. Chromatograms obtained from the preparation of control plasma when using a generic SPE method are shown in Figure 3a–c, in which the typical chromatograms of blank dog plasma, plasma fortified with 5 ng/mL of MBO and a plasma sample collected from a dog 0.5 h after oral administration of MBO (0.25 mg/kg) are shown.

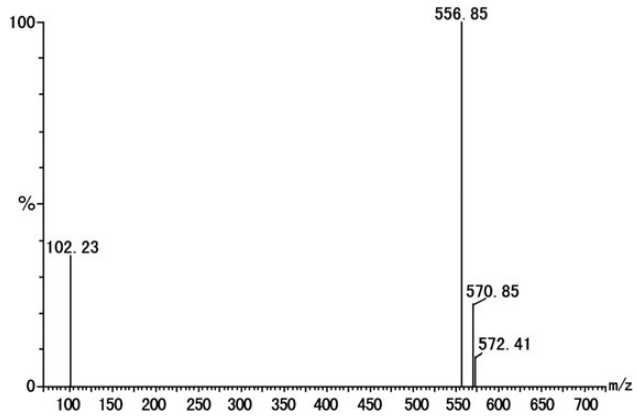

Figure 2. Scanning mass spectra of milbemycin A_4 oxime.

Linearity

The standard curves showed good linearity over the concentration range of 2–500 ng/mL (2, 5, 10, 50, 100, 150, 200, 500 ng/mL) for MBO in dog plasma. The concentration ranges were selected on the basis of drug concentration anticipated while analyzing the samples in the above conditions, the concentration–peak area relationships were described by simple regression analysis and analytical procedure was in a given range to obtain the test results which were directly proportional to the concentration (amount) of analyte in the defined range for the samples. A $1/\chi$ weighted least-square linear regression equation of $y = 94.2379x - 60.4477$ was observed with a correlation coefficient of $r = 0.999551$ for the plot of concentration versus response (peak area).

Accuracy and precision

Precision was expressed as a coefficient of variance (%CV: [standard deviation/mean concentration] × 100) of the quality control samples. Accuracy was determined by calculating the percentage bias of the quality control samples (%bias = [measured concentration − mean nominal concentration] × 100/measured concentration). Interassay variability was expressed as the accuracy and precision of the mean QC concentrations of six separate assays. Intra-assay variability was determined as the accuracy and precision of the eight individual QC concentrations within 1 day, which was within the general assay acceptability criteria for quality control samples (27). The inter- and intra-assay accuracy and precision was within 10% for the three concentrations (Table I).

Limits of detection and quantitation

The LOD was calculated by equation $3.3\sigma/S$, while for the LOQ equation $10\sigma/S$ was used, where σ is SD of response from blank and S is the slope of standard curve (28). LOQ was further confirmed by analyzing the blank perfusate spiked with the compound at the same concentration as described in specificity and selectivity procedure. The lower and upper limits of quantification (LQC and (HQC) were arbitrarily set as the bottom (2.0 ng/mL) and top (500 ng/mL). The LOD was 0.8 ng/mL for this assay.

Recovery

MBO recovery for plasma fortified 5, 150 and 400 ng/mL of MBO was 89% ± 2%, 91% ± 3% and 92% ± 4%, respectively ($n = 6$).

Stability

Determination of MBO stability following showed that for all QC samples, freeze–thaw, storage at room temperature of samples and stock solution had no significant impact on the MBO concentrations measured (Table II).

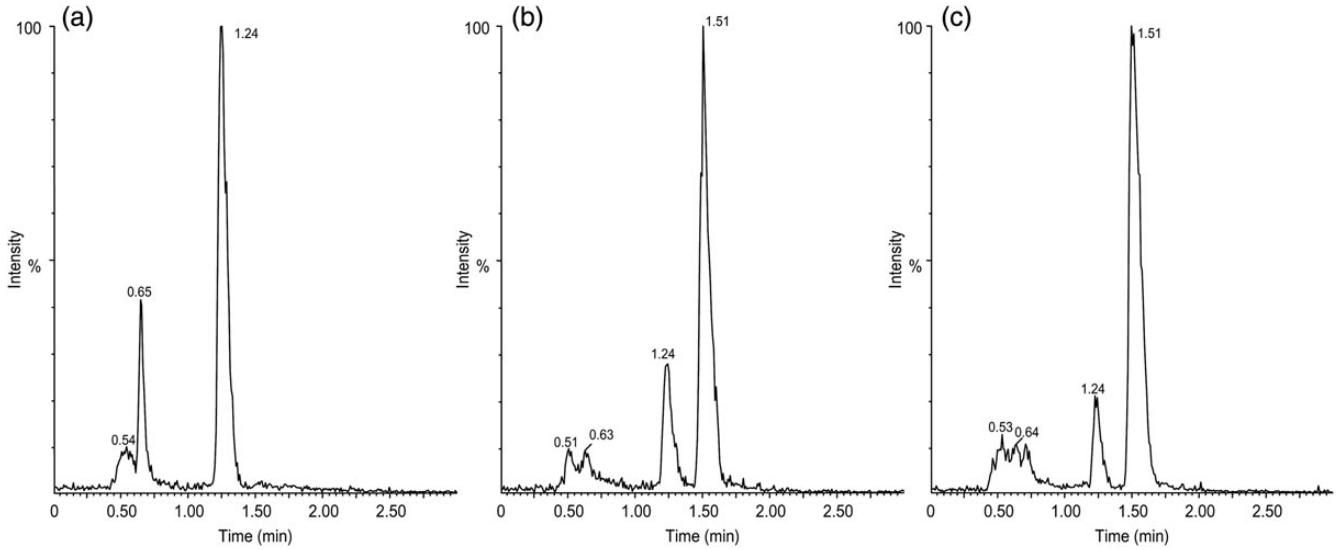

Figure 3. (a) Typical chromatograms of blank dog plasma. (b) Plasma sample fortified with 5 ng/mL of MBO. (c) Plasma sample collected from a dog 0.5 h after oral administration of MBO (0.25 mg/kg).

Table I

Inter- and Intra-Assay Precision and Accuracy for the Measurement of MBO in Dog Plasma as Determined by HPLC-MS

Nominal concentration (ng/mL)	Accuracy (%biasa; mean ± SD)		Precision (%CVb; mean measured concentration)	
	Intra-assay	Interassay	Intra-assay	Interassay
5.0	4.2 ± 2.1	4.0 ± 1.9	5.1 ± 0.3 (5.9)	5.0 ± 0.2 (4.0)
150.0	3.5 ± 2.3	3.1 ± 2.0	148.7 ± 7.6 (5.1)	147.4 ± 5.6 (3.8)
400.0	3.2 ± 2.7	2.8 ± 1.9	407.3 ± 17.9 (4.4)	403.9 ± 14.5 (3.6)

Table II

MBO Concentrations (ng/mL) Were Determined in Freshly Spiked Dog Plasma, for 6 Months at Freeze, for 12 h at Ambient Temperature and three Freeze–Thaw Cycles (−80°C Cycles/Room Temperature)

Treatment	Low concentration	*P*-value	Medium concentration	*P*-value	High concentration	*P*-value
Untreated	4.93 ± 0.15	–	147.2 ± 6.0	–	404.1 ± 14.4	–
6 months at freeze	4.97 ± 0.21	0.519	148.8 ± 5.9	0.956	403.5 ± 15.1	0.884
12 h at ambient temperature	5.00 ± 0.26	0.275	152.2 ± 7.5	0.522	408.6 ± 12.8	0.918
3× freeze/thaw	4.83 ± 0.06	0.184	149.2 ± 10.5	0.465	403.3 ± 28.2	0.297

Quantification of dog samples

Figure 4 reports the plasma MBO concentrations detected in dog following oral administration of a 0.25 mg/kg dose of the drug. No drug was detected in the plasma samples collected before MBO administration or collected from dogs that did not receive the drug. The peak concentration obtained at the end of the infusion process was 79.33 ± 5.08 ng/mL, and then the values decreased with a terminal half-life of 11.09 ± 0.54 h, as calculated by the terminal portion of the semilogaritmic plot reported in inset of Figure 4.

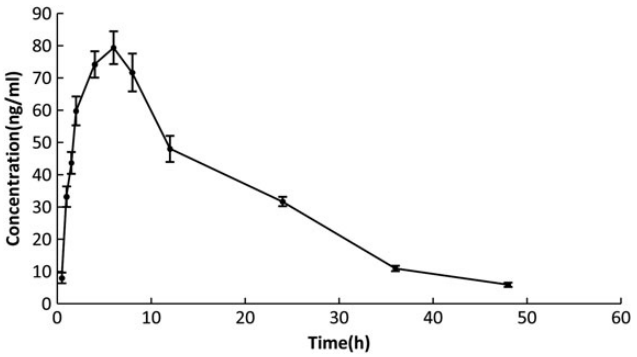

Figure 4. Plasma MBO pharmacokinetic profiles in six dogs receiving MBO (0.25 mg/kg PO).

Discussion

There was little method reported about MBO detection in animal plasma (1), and here a validated, accurate, precise and sensitive LC–MS method has been developed for the detection of MBO in dog plasma. HPLC–MS allows added specificity for the detection of MBO compared with that obtained with HPLC. None of the MBO ions was detected at any time in extracted drug-free plasma samples.

The final composition of the mobile phase was a mixed solution of ACN and 5 mM ammonium acetate (85:15, v/v). Increasing the percentage of the ammonium acetate in the mobile phase enhanced peak symmetry. Ammonium acetate with pH in the range of 3–6 was tested. Peak symmetry increased with decreasing pH of buffer, while simultaneously the retention times of analyte were shortened. Eventually, 5 mm ammonium acetate was chosen as a compromise between the discussed parameters. Separation of the analyte acquired a run time of 3 min.

Early methods for the seperation of MLs from plasma usually involved extensive cleanup based on different combinations of liquid–liquid extraction (LLE), liquid–liquid partitioning (LLP) and sorbent chromatography. We extracted MBO using acetonitrile/water before purifying on a C_{18} SPE catridges. The C_{18} column was selected for the assay because it exhibited excellent peak shape and had sufficient response for MBO. The rapid and easy sample treatment by precipitation as well as the short runtime of the assay 3 min allows rapid sample processing and analysis.

Conclusions

An HPLC-MS method for the quantification of MBO in samples obtained by SPE extraction from 200 μL dog plasma has been successfully applied to determine MBO in plasma extracts and its pharmacokinetics was evaluated after oral administration to dogs. The herein propose method takes the advantage of lower LOQ without negatively affecting its use for clinical pharmacokinetic studies. Satisfactory sensitivity and specificity of the assay are addressed, together with a fully reliable HPLC-MS method of analysis characterized by good linearity, accuracy and precision.

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References

1. Ide, J., Okazaki, T., Ono, A., Saito, A., Nakagawa, K., Naito, S., *et al.*; Milbemycin: discovery and development; *Annual Report of Sankyo Research*, (1993); 45: 1–98.
2. Lee, H.H., Terada, M.; *In vitro* effects of milbemycin oxime: mechanism of action against *Angiostrongylus cantonensis* and *Dirofilaria immitis*; *Parasitology Research*, (1992); 78: 349–353.
3. Genchi, C., Cody, R., Pengo, G., Büscher, G., Cavalleri, D., Bucci, V., *et al.*; Efficacy of a single milbemycin oxime administration in combination with praziquantel against experimentally induced heartworm (*Dirofilaria immitis*) infection in cats; *Veterinary Parasitology*, (2004); 122: 287–292.
4. Blagburn, B.L., Dillon, A.R., Arther, R.G., Butler, J.M., Newton, J.C.; Comparative efficacy of four commercially available heartworm preventive products against the MP3 laboratory strain of Dirofilaria immitis; *Veterinary Parasitology*, (2011); 176: 189–194.
5. Humbert-Droz, E., Büscher, G., Cavalleri, D.; Efficacy of milbemycin oxime against fourth-stage larvae and adults of *Ancylostoma tubaeforme* in experimentally infected cats; *The Veterinary Record*, (2004); 154: 140–143.
6. Kok, D.J., Schenker, R., Archer, N.J., Horak, I.G., Swart, P.; The efficacy of milbemycin oxime against pre-adult *Spirocerca lupi* in experimentally infected dogs; *Veterinary Parasitology*, (2011); 177: 111–118.
7. Kok, D.J., Williams, E.J., Schenker, R., Archer, N.J., Horak, I.G.; The use of milbemycin oxime in a prophylactic anthelmintic programme to protect puppies, raised in an endemic area, against infection with *Spirocerca lupi*; *Veterinary Parasitology*, (2010); 174: 277–284.
8. Holm, B.R.; Efficacy of milbemycin oxime in the treatment of canine generalized demodicosis: a retrospective study of 99 dogs (1995–2000); *Veterinary Dermatology*, (2003); 14: 189–195. 11.
9. Gunnarsson, L.K., Möller, L.C., Einarsson, A.M., Zakrisson, G., Hagman, B.G., Christensson, D.A., *et al.*; Clinical efficacy of milbemycin oxime in the treatment of nasal mite infection in dogs; *Journal of the American Animal Hospital Association*, (1999); 35: 81–84.
10. White, S.D., Rosychuk, R.A., Fieseler, K.V.; Clinicopathologic findings, sensitivity to house dust mites and efficacy of milbemycin oxime treatment of dogs with *Cheyletiella* sp. Infestation; *Veterinary Dermatology*, (2001); 12: 13–18.
11. Bowman, D.D., Legg, W., Stansfield, D.G.; Efficacy of moxidectin 6-month injectable and milbemycin oxime/lufenuron tablets against naturally acquired *trichuris vulpis* infections in dogs; *Veterinary Therapeutics*, (2002); 3: 286–289.
12. Bowman, D.D., Ulrich, M.A., Gregory, D.E., Neumann, N.R., Legg, W., Stansfield, D.; Treatment of *Baylisascaris procyonis* infections in dogs with milbemycin oxime; *Veterinary Parasitology*, (2005); 129: 285–290.
13. Schenker, R., Bowman, D., Epe, C.; Efficacy of a milbemycin oxime-praziquantel combination product against adult and immature stages of *Toxocara cati* in cats and kittens after induced infection; *Veterinary Parasitology*, (2007); 145: 90–93.
14. Schnitzler, B., Hayes, B., Wiseman, S., Snyder, D.E.; Confirmation of the efficacy of a combination tablet of spinosad and milbemycin oxime against naturally acquired infections of canine intestinal nematode parasites; *Veterinary Parasitology*, (2012); 184: 279–283.
15. Rudayna, G.; Parasitic miticial therapy; *Clinical Theniques in Small Animal Practice*, (2006); 21: 135–144.
16. Tranquilli, W.J., Paul, A.J., Todd, K.S.; Assessment of toxicosis induced by high-dose administration of milbemycin oxime in collies; *American Journal of Veterinary Research*, (1991); 52: 1170–1172.
17. Tolan, J.W., Eskola, P., Fink, D.W., Mrozik, H., Zimmerman, L.A.; Determination of avermectins in plasma at nanogram levels using high-performance liquid chromatography with fluorescence detection; *Journal of Chromatography A*, (1980); 190: 367–376.
18. Pollmeier, M., Maier, S., Moriarty, K., DeMontigny, P.; High-performance liquid chromatographic assay for the determination of a semisynthetic avermectin analog (eprinomectin) in bovine milk at parts per billion levels – method development and validation; *Journal of Chromatography B*, (2002); 772: 99–105.
19. O'Keeffe, M., Kennedy, O., Farrell, F.; Research Report No. 46: Food Residue Database Studies, Teagasc, The National Food Centre, Dunsinea, Castleknock, Dublin 15, Ireland, (2001), 7 pp.
20. Xu, Q.Q., Xiang, W.S., Li, J.C., Liu, Y., Yu, X.L., Zhang, Y.T., *et al.*; Development and validation of an HPLC-FLD method for milbemectin quantification in dog plasma; *Journal of Chromatography B*, (2010); 878: 2013–7.
21. Alvinerie, M., Sutra, J.F., Badri, M.; Determination of moxidectin in plasma by high-performance liquid chromatography with automated solid-phase extraction and fluorescence detection; *Journal of Chromatography B*, (1995); 674: 119–124.
22. Malaníková, M., Malaník, V.; Use of thin-layer chromatography for the testing of avermectins produced by *Streptomyces avermitilis* strains; *Journal of Chromatography A*, (1990); 513: 401–404.
23. Reuvers, T., Diaz, R., Gomez, J.; Proceedings of EuroResidue II Conference on Residues of Veterinary Drugs in Food, Veldhoven, The Netherlands, University of Utrecht, Faculty of Veterinary Medicine, University of Utrecht, Utrecht, The Netherlands, (1993), pp. 567.
24. Ali, M.S., Sun, T., McLeroy, G.E., Phillippo, E.T.; Simultaneous determination of eprinomectin, moxidectin, abamectin, doramectin, and

ivermectin in beef liver by LC with fluorescence detection; *Journal of AOAC International*, (2000); 83: 39–52.
25. Rambla-Alegre, M., Esteve-Romero, J., Carda-Broch, S.; Is it really necessary to validate an analytical method or not? That is the question; *Journal of Chromatography A*, (2012); 1232: 101–109.
26. Durden, D.A.; Positive and negative electrospray LC–MS–MS methods for quantitation of the antiparasitic endectocide drugs, abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin and selamectin in milk; *Journal of Chromatography B*, (2007); 850: 134–146.
27. FDA.; Guidance for industry. Bioanalytical method validation, (2001). http://www.fda.gov/cder/guidance/4252fnl.htm (Accessed October 20, 2007).
28. ICK Guideline; Analytical method validation (Q2A, Q2B), (2007): www.fda.gov (Accessed April 05, 2005).