Method Article

A quick and simple method for the determination of ivermectin in dog plasma by LC–MS/MS

Eva Morbidelli[a], Julie Rambaldi[a], Laura Ricci Bitti[b], Anna Zaghini[a], Andrea Barbarossa[a,*]

[a] Alma Mater Studiorum – University of Bologna, Department of Veterinary Medical Sciences, Via Tolara di Sopra 50, 40064, Ozzano dell'Emilia, BO, Italy
[b] FATRO S.p.A., Via Emilia 285, 40064, Ozzano dell'Emilia, BO, Italy

A B S T R A C T

Ivermectin is an endectocide belonging to the macrocyclic lactone class, commonly used in dogs as a heartworm preventative and for the treatment of several external and internal parasite infections. Among the analytical methods for ivermectin determination in plasma available in literature, many require a laborious clean-up step on SPE cartridge, and use fluorescence detection instead of the more reliable mass spectrometry. In the context of a project aimed at its pharmacokinetic evaluation in this species, a liquid chromatography-tandem mass spectrometry method for the determination of ivermectin in dog plasma was developed and validated, using blank plasma provided by a local canine blood transfusion service. Samples underwent a quick liquid-liquid extraction before analysis in the LC–MS/MS system, operating in selected reaction monitoring (SRM) mode. The method provided satisfactory linearity (R^2 >0.99), accuracy (bias <3%) and precision (CV <10%) over the 0.5–20.0 ng/mL range.

- This is to our knowledge the first validated LC–MS/MS method for ivermectin determination in dog plasma.
- Sample preparation is simple, rapid and inexpensive, without compromising sensitivity.
- The modest amount of plasma required makes the proposed technique suitable for pharmacokinetic studies also in small dogs.

* Corresponding author.
  E-mail address: andrea.barbarossa@unibo.it (A. Barbarossa).

Specifications Table

Subject area	Veterinary Science and Veterinary Medicine
More specific subject area	Veterinary Pharmacology
Method name	Ivermectin in dog plasma by LC–MS/MS
Name and reference of original method	N/A
Resource availability	N/A

Method details

A method was validated for the determination of ivermectin (IVM) in dog plasma, using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Blank plasma samples were provided by a local canine blood transfusion service. The extraction procedure is rapid and simple, without requiring a SPE purification step, and allows the determination of IVM at levels as low as 0.5 ng/mL. The low volume of sample needed makes this approach suitable for pharmacokinetic studies also in small size dogs. The proposed technique has already been successfully employed to study IVM pharmacokinetics within a drug approval process, in which measured concentrations reached a maximum of 4.66 ng/mL and fell between the LLOQ and the LOD only at the final timepoints at the end of the elimination phase.

Materials

Ivermectin chemical reference substance (CRS) was supplied by the European Directorate for the Quality of Medicines and HealthCare (EDQM).

Sample Preparation

1 After thawing at room temperature, transfer 0.5 mL aliquots of each dog plasma sample into a 10 mL conical-bottomed polypropylene test tube.
2 Add 4 mL of acetonitrile (HPLC grade).
3 Add 2 mL of n-hexane (HPLC grade).
4 Agitate on vortex mixer for 10 min.
5 Centrifuge for 10 min at 1500 $\times g$ at 5 °C.
6 Transfer 4 mL of the lower phase into a new 10 mL conical-bottomed polypropylene test tube.
7 Evaporate under gentle nitrogen stream at 70 °C.
8 Dissolve the dry residue with 0.2 mL of dilution phase, consisting of a methanol:water (50:50, v/v) solution containing 5 mM ammonium formate and 0.1% formic acid.
9 Agitate on vortex mixer for 5 min.
10 Place into an ultrasonic bath for 6 min.
11 Agitate on vortex mixer for 5 min.
12 Centrifuge for 5 min at 1500 $\times g$.
13 Filter through a 0.22 μm DURAPORE® PVDF syringe filter into a LC vial.

LC–MS/MS conditions

The LC system was an Agilent 1290 pump, equipped with autosampler and thermostated column compartment. Chromatographic separation was achieved on an Agilent Zorbax RRHD Eclipse Plus C18 column (1.8 μm, 2.1 × 50 mm) combined with a guard column (1.8 μm, 2.1 × 5 mm) of the same phase.

The column temperature was maintained at 40 °C and the flow rate was 0.4 mL/min. The mobile phase consisted of a water (A) and methanol (B) mixture with 5 mM ammonium formate and 0.1% formic acid. The following gradient program (time, %A–%B) was applied: 0 min, 50-50; 4 min, 5–95;
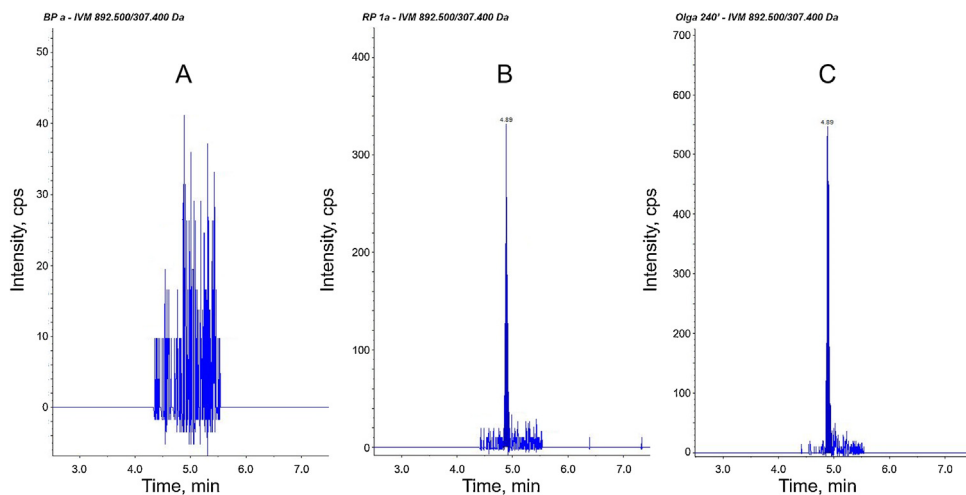
Fig. 1. Chromatograms of a blank sample (A), a blank sample spiked at the LLOQ (0.5 ng/mL) (B), and an actual dog sample collected 240 min after ivermectin oral administration (C), obtained by LC–MS/MS analysis. Early-eluting matrix contaminants and the last part of the LC run were diverted to waste through a Valco® divert valve.

10 min, 5–95; 10.5 min, 50–50; 15 min, 50–50. The injection volume was 20 μL and the retention time for IVM was 4.79 min. An example of the obtained chromatograms is shown in Fig. 1.

An AB Sciex API 3200 triple quadrupole mass spectrometer equipped with TurboIonSpray® interface was used for analyte detection. The instrument operated in positive electrospray ionization (ESI+), with capillary voltage set at 5 kV and source temperature at 350 °C. Acquisition was performed in selected reaction monitoring (SRM) mode, observing the 892.5 > 307.4 m/z [H$_2$B1a-NH$_3$]$^+$ transition with a dwell time of 50 ms. The specific MS/MS parameters were optimized through direct infusion of IVM standard solution into the instrument: cone voltage and collision energy were 40 V and 35 V, respectively. Data analysis was performed using AB Sciex Analyst® 1.5.2 software.

Method validation

The proposed method was validated in accordance with the European guidelines [1]. The specificity was confirmed by the absence of interfering compounds at the specific retention time of the analyte in the chromatograms obtained from blank plasma samples.

Matrix-matched calibration curves (six levels: 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/mL) were freshly prepared during different days to evaluate the linearity of the method. Peak areas were plotted against their concentrations applying a "no weighting" linear regression model, obtaining satisfactory coefficients of correlation (R^2 >0.99). An example of the typical calibration curve is shown in Fig. 2. The homogeneity of variance (homoscedasticity) and the regression model significance over the calibration range were assessed by using the Cochran test and the analyses of variance Lack of Fit test, respectively. The lower limit of quantification (LLOQ) of the method, corresponding to the lowest concentration with signal to noise (S/N) ratio >10 and acceptable precision and accuracy, was 0.5 ng/mL. The limit of detection (LOD), corresponding to the lowest concentration with signal to noise (S/N) ratio >3, was 0.2 ng/mL.

Accuracy and precision (intra- and inter-day) were tested by spiking blank plasma samples at three different concentrations (0.5, 1.0 and 20 ng/mL) in sextuplicate, and in three different days. The analysis of these QC samples proved the good accuracy (percentage difference always lower than ±3%) and precision (intra- and inter-day CV% always below ±10%) of the method (Table 1). The recovery was tested at two different concentrations within the validation range (0.5 and 5 ng/mL), resulting always >80%.
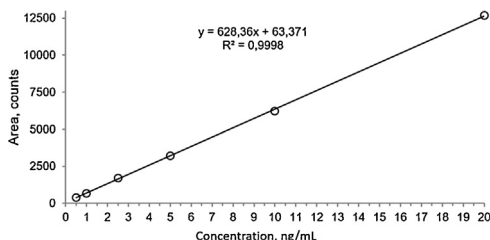
Fig. 2. Example of one of the matrix-matched calibration curves, prepared fortifying blank dog plasma at six different concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/mL) and plotting peak areas against their concentrations.

Table 1
Intraday and interday accuracy (D% = percentage difference between the experimental and theoretical values) and precision (CV %) for IVM (ivermectin), calculated on data obtained analyzing QC (quality control) samples prepared in three different days (total n = 54).

	IVM at 0.5 ng/mL			IVM at 1.0 ng/mL			IVM at 20.0 ng/mL		
	Day 1 (n = 6)	Day 2 (n = 6)	Day 3 (n = 6)	Day 1 (n = 6)	Day 2 (n = 6)	Day 3 (n = 6)	Day 1 (n = 6)	Day 2 (n = 6)	Day 3 (n = 6)
Intraday D%	−1.7	−1.7	−6.0	−2.7	−1.5	−3.2	−1.9	−4.7	−5.0
Intraday CV%	8.5	9.7	6.0	6.9	6.8	4.5	2.5	2.5	3.9
Interday D%		−1.7			−2.4			−3.9	
Interday CV%	8.1			5.8			3.2		

Analyte stability was tested in solvent, fortified samples and sample extracts. Ivermectin was stable in solvent (methanol) at 2–8 °C after 31 days, as confirmed by areas variation below ±10% compared to day 0. Stability of IVM in fortified samples was evaluated in triplicate at the lowest and highest levels of the validation range, resulting stable (percentage difference always below ±15%) within 6 h at room temperature, 24 h at 2–8 °C, 49 days at −15 and −70 °C, and following three freeze/thaw cycles at −15 and −70 °C. Sample extracts (fortified at 0.5 and 20.0 ng/mL, in triplicate) were stable within 24 h after preparation when stored in amber vials at room temperature in the autosampler.

To evaluate the robustness of the method (defined as a measure of its capacity to remain unaffected by small but deliberate variations) different parameters have been observed and modified individually. Results demonstrated that variations of the volume of the extraction solvent (±2.5%), of the composition of the mobile phase (±2%) and of the collision energy (±2%) do not have effects on the response of the analyte.

Additional information

Background

Ivermectin, an endectocide belonging to the macrocyclic lactone class, is commonly used in dogs as a heartworm preventative, and for the treatment of certain types of external and internal parasite infections. Ivermectin is a mixture of homologues comprising not less than 80% of 22,23-dihydroavermectin B1a and not more than 20% of 22,23-dihydroavermectin B1b. Effective dosages of ivermectin in dogs are very low, and therefore also its plasma concentrations. In order to study the pharmacokinetics of IVM, a sensitive and reliable method for its quantification in canine plasma is therefore necessary. Most of the existing methods in literature require a purification step on solid phase extraction (SPE) cartridge [2–4], which is laborious, time consuming and expensive if compared to the quick liquid-liquid extraction here described. For these reasons, the proposed protocol might result preferable in PK studies, where large sets of samples must be processed. In addition, while liquid chromatography-fluorescence detection was used for ivermectin determination in previous studies on

dog plasma [5–7], no other methods using LC–MS/MS are available to date. The use of a triple quadrupole instrument operating in selected reaction monitoring mode (SRM) grants unmatched specificity, and at the same time allows to perform only little sample preparation without affecting the high sensitivity provided.

Acknowledgements

References

[1] EMEA/CHMP/EWP/192217/2009. Guideline on bioanalytical method validation, 1–23. www.ema.europa.eu/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf (Accessed October 18, 2018).
[2] K. Kojima, K. Yamamoto, Y. Nakanishi, H.J. Katae, Determination of 22,23-dihydroavermectin B1a in dog plasma using solid-phase extraction and high-performance liquid chromatography, Chromatography 413 (1987) 326–331.
[3] S.T. Dunn, L. Hedges, K.E. Sampson, Y. Lai, S. Mahabir, L. Balogh, C.W. Locuson, Pharmacokinetic interaction of the antiparasitic agents ivermectin and spinosad in dogs, Drug Metab. Dispos. 39 (2011) 789–795.
[4] M. Boterman, M.J. Dröge, F. Oostebring, M.A.H. Boezeman, E. Oosting, A sensitive automated online SPE-LC-MS/MS method for the quantitative determination of ivermectin B1a in bovine plasma, 11th European Association for Veterinary Pharmacology and Toxicology (2009).
[5] V.S. Magalhães, Y.P. Cid, T.P. Ferreira, D.M.V. Medeiros, L.C. de S O Batista, T.R. Correia, A.L.M. Albert, F.B. Scott, Evaluation of pharmacokinetics and efficacy of ivermectin following oral administration in dogs against experimental infection of Ctenocephalides felis felis and Rhipicephalus sanguineus, Vet. Parasitol. 228 (2016) 167–171.
[6] M. Genchi, A. Geneteau, P. Forget, R. Delcombel, C. Genchi, Pharmacokinetics and efficacy of an ivermectin implant for long-term prevention of Dirofilaria immitis infection in dogs, Parasitol. Res. 116 (2017) 1723–1728.
[7] G. Eraslan, M. Kanbur, B.C. Liman, Y. Cam, M. Karabacak, S. Altinordulu, Comparative pharmacokinetics of some injectable preparations containing ivermectin in dogs, Food Chem. Toxicol. 48 (2010) 2181–2185.