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Liquid chromatographic assay of moxidectin in human plasma for application to pharmacokinetic studies

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Abstract

A sensitive and selective high-performance liquid chromatography (HPLC) method is presented for the analysis of moxidectin in human plasma. Solid phase extraction using Oasis® HLB cartridges is used for sample preparation. The fluorescent derivative is obtained by a dehydrative reaction with trifluoroacetic anhydride and *N*-methylimidazole. Separation is achieved on a Bondapak C_{18} reversed-phase column with a mobile phase composed of tetrahydrafuran–acetonitrile–water (40:40:20, v/v/v). Detection is by fluorescence, with excitation at 365 nm and emission at 475 nm. The retention times of moxidectin and internal standard, ivermectin are approximately 10.7 and 18.6 min, respectively. The assay is linear over the concentration range 0.2–1000 ng/ml for moxidectin in human plasma ($r = 0.9999$, weighted by 1/concentration). Recoveries at concentrations 0.2, 400, 1000 ng/ml are 94, 75, and 71%, respectively. The analysis of quality control (QC) samples for moxidectin (0.2, 400, and 1000 ng/ml) demonstrates excellent precision with relative standard deviations of 11.9, 5.7, and 2.7%, respectively ($n = 6$). The method is accurate with all intra- ($n = 6$) and inter-day ($n = 18$) mean concentrations within (5.0%) from nominal at all QC sample concentrations. Moxidectin was found to be stable after three free-thaw cycles, and with storage at -20 and -80 °C for 12 weeks. The method is suitable for pharmacokinetic studies of moxidectin after oral administration to humans.

Keywords: Moxidectin; Antifilarial; Antiparasitic; Liquid chromatography

1. Introduction

Moxidectin is an antiparasitic drug that is widely used in veterinary medicine and has promising potential use as a human antifilarial agent. Tagboto and Townson have reported superior in vitro and in vivo microfilaricidal activity of moxidectin compared to ivermectin against the human pathogen *Onchocerca volvulus* [1]. Trees et al. reported that prophylactic treatment with moxidectin prevented the development of the adult worm infection of *Onchocerca ochengi* in cattle [2]. *O. ochengi* in cattle has a similar host–parasite relationship with the human pathogen *O. volvulus*. Unpublished efficacy studies in dogs infected with *Brugia pahangi* suggest moxidectin has promising macrofilaricidal activity.

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Onchoceriasis (river blindness), a parasitic infection of *O. volvulus*, is a major public health problem. The disease is found in Africa, in Central and South America and in Yemen. Ivermectin is given orally, usually once a year, which will reduce the microfilarial load to a very low level and prevent blindness that is a major consequence of onchoceriasis in humans. However, there is the need for a macrofilaricial drug, which will kill the adult worm, and alleviate the need for ongoing treatment.

Moxidectin is a semisynthetic derivative of nemadectin [3,4], a macrocyclic lactone produced by cultures of *Streptomyces cyanogriseus*. Moxidectin is chemically related to the milbemycins [5] and avermectins [6], which have activity against a broad spectrum of nematode and arthropod parasites of animals [7] (Fig. 1). The molecular structure includes a fused cyclohexene–tetrahydrofuran ring, a bicyclic 6,6-membered spiroketal and a cyclohexene ring fused to the 16-membered macrocyclic ring. Moxidectin is the 23-(*o*-methyloxime) derivative of nemadectin and differs from ivermectin in having no sugar moiety at the C-13 position and having an unsaturated side-chain at the C-25 position.

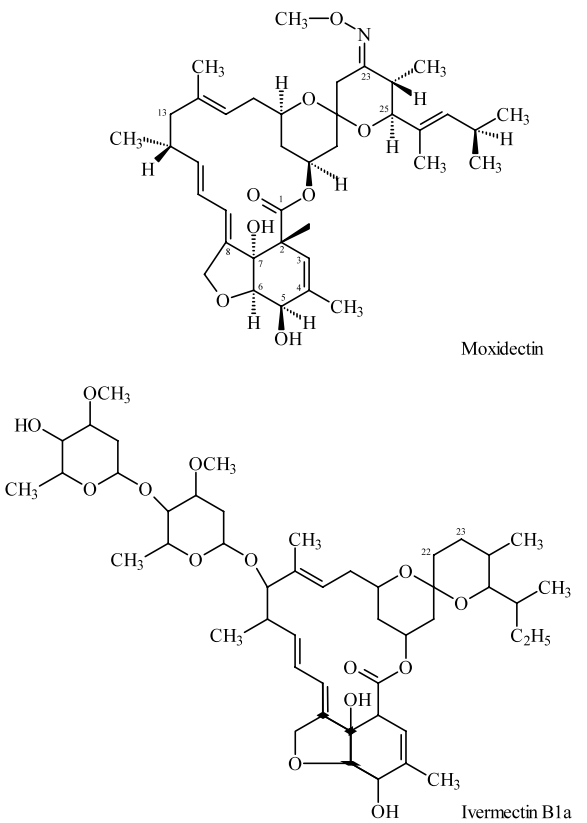

Fig. 1. Chemical structures of moxidectin (top) and internal standard, ivermectin (bottom).

A number of methods for the analysis of moxidectin with high-performance liquid chromatography (HPLC) have been reported [8–13]. Most of these methods were used for the determination of avermectins in various tissues for the quantitation of residues in food. Liquid chromatographic methods have been reported for the determination of moxidectin residues in cattle tissues using liquid extraction [8], C_{18} solid phase extraction (SPE) [9,10] and an alumina column clean-up [11,12]. All of these methods are based upon the formation of a fluorescent derivative obtained by a dehydrative reaction with trifluoroacetic anhydride and *N*-methylimidazole and fluorescence detection. Only one report for the determination of moxidectin in cattle plasma with HPLC was found. This method was validated and used to support cattle pharmacokinetic studies [13]. It is an adaptation of the fluorescent derivatization procedure described above and utilizes SPE with fluorescence detection. This method could not be utilized for a clinical pharmacokinetic study because an internal standard was not used and the analyte could not be separated from interferences.

A specific and sensitive method with an internal standard for determining moxidectin, a candidate antifilarial agent, in human plasma for anticipated clinical trials is described in this report.

2. Experimental

2.1. Solvents and chemicals

Moxidectin (Lot # AC10731-88C) was provided by Analytical Standards Distribution,

America Cyanamid (Princeton, NJ, USA). Ivermectin was purchased from Sigma Chemical Company (St. Louis, MO, USA). Trifluoroacetic anhydride (99%) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). *N*-Methylimidazole (99%) was obtained from Acros Co. (New Jersey, USA). HPLC grade acetonitrile, tetrahydrofuran (THF) and propan-2-*ol* were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultra-pure analytical grade Type I water for HPLC was produced by a Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA).

2.2. Standard stock solutions

Moxidectin and ivermectin were weighed on a Mettler Toledo AG 104 analytical balance (Mettler Toledo, Inc., Hightstown, NJ, USA). The appropriate amounts of drug were dissolved using methanol in volumetric flasks to make a 1 mg/ml stock solution of each. Working standards for each concentration point on the standard curve were prepared as solutions in methanol at concentrations of 0.2, 0.6, 5, 50, 200, 400, 600, 1000 ng/ml by serial dilutions of the stock solution. A 1 μg/ml working solution for the ivermectin was prepared in methanol. For all quantitated samples, 10 μl of working internal standard solution was added. All standard solutions were stored at 4 °C. Alvinerie et al. reported the stock solution and the serial dilution standards were stable for at least 3 months at 4 °C [9].

2.3. Instrumentation

Chromatographic separations were performed on a Waters model 501 solvent delivery system (Waters, Milford, MA, USA) equipped with a Shimadzu fluorescence detector RF-551 (Shimadzu, Kyoto, Japan) and model 717 plus automatic injector (Waters, Milford, MA, USA). Integration results from this automated system were processed by Shimadzu integrator CR-501 (Shimadzu, Kyoto, Japan). A μBondaPak™ C_{18} column, 10 μm particle size, 300 mm length × 3.9 mm internal diameter (Waters, Milford, MA, USA) was used for separation.

2.4. Sample preparation

This procedure was validated using 200 μl of spiked human plasma. Outdated human plasma was obtained from the Blood Bank at the University of Iowa Hospitals and Clinics and stored frozen in aliquots at −20 °C. Extraction of the moxidectin and internal standard was carried out by SPE. Waters Oasis, HLB® cartridge, 30 mg with a 1 ml reservoir (Waters, Milford, MA, USA), and Alltech Vacuum Manifolds-24 ports, (Alltech, Deerfield, IL, USA) were used for SPE.

A human plasma aliquot (200 μl) was spiked with 10 ng (10 μl) of ivermectin in a 100 × 13 mm glass test tube. Prior to extraction, 200 μl of acetonitrile and 40 μl of water were added to all samples, and vortex mixed for 10 min and followed by centrifugation at 2000 × *g* for 10 min. For each sample, a SPE cartridges was activated by aspirating 1 ml of methanol, followed by 1 ml of HPLC grade water. The samples were then loaded onto the cartridges, and aspirated at a reduced vacuum (3 mm Hg). After aspiration, each cartridge was washed with 2 ml of HPLC grade water, and 2 ml of 25% methanol at reduced vacuum (5 mm Hg). Two milliliter of isopropanol was used to elute the samples under a reduced vacuum (3 mm Hg). After elution, the eluant was evaporated using a N-EVAP® (Berlin, MA, USA), under a gentle stream of nitrogen in a water bath set at 50 °C. The residues were dissolved in 100 μl of *N*-methylimidazole solution in acetonitrile (1:1, v/v). To initiate the derivatization, 150 μl of the trifluoroacetic anhydride solution in acetonitrile (1:2, v/v) was added. The reaction completed within 30 s. Aliquot (20 or 200 μl) of this solution was injected directly onto the chromatographic system. For unextracted specimens, appropriate amounts of moxidectin and ivermectin in methanol are added to a 100 × 13 mm glass test tube, then evaporated to dryness under nitrogen at 50 °C in a water bath. The residue is dissolved in 100 μl of *N*-methylimidazole

solution in acetonitrile (1:1, v/v) and carried through the derivatization procedure using trifluoroacetic anhydride outlined above.

2.5. Chromatographic conditions

The mobile phase consisted of acetonitrile–tetrahydrofuran–water (40:40:20,v/v/v). The mobile phase was pumped at a flow-rate of 1.0 ml/min. The injection volume was 200 μl for samples containing moxidectin < 10 ng/ml; otherwise, 20 μl was applied with an automatic injection system onto the μBondaPak™ C_{18} column (10 μm, 300 × 3.9 mm I.D.). The fluorescence detector settings were: excitation 365 nm and emission 475 nm, and sensitivity high.

2.6. Data regression

Chromatographic data were collected and integrated by a Shimadzu Integrator CR-501 Chromatography System. Calibration graphs are constructed using peak area ratios as a function of analyte concentration. The calibration curves were obtained by weighted (1/concentration) least-squares linear regression analysis. The equations of the calibration curves were then used to calculate the concentrations of moxidectin in the samples and QC samples by their peak area ratios.

2.7. Precision, accuracy, and recovery

Precision, accuracy, and recovery were evaluated by conducting repeated analysis ($n = 6$) of spiked plasma samples at three different concentration levels: 0.2, 400, and 1000 ng/ml. The relative standard deviation (RSD) of each concentration was calculated to determine the precision of the method. Comparing the measured concentrations of extracted plasma samples, and the true concentration in the spiked samples determined the accuracy of the method. Recovery was assessed by comparing the detector response from an amount of moxidectin added to and extracted from drug free human plasma, compared to the detector response obtained for unextracted standard mixtures representing 100% recovery.

2.8. Stability

Freeze/thaw, long-term, and autosampler stability experiments were conducted. For stability studies, plasma was harvested from freshly drawn blood collected by venipuncture from a healthy donor. The freeze/thaw stability study was carried out at two concentration levels by spiking drug-free plasma to 15 and 600 ng/ml with storage in a −20 °C freezer (Model ULT 2540-7-A12 freezer, Revco Scientific Inc., Asheville, NC, USA). The freeze/thaw experiment was carried out over three freeze/thaw cycles. The long-term stability experiment of moxidectin in human plasma was assessed over a period of 12 weeks. The long-term stability was carried out at three concentration levels: 1, 400 and 1000 ng/ml with storage in both −20 and −80 °C freezer (Model ULT 2586-5-A14 freezer, Revco Scientific Inc., Asheville, NC, USA). Autosampler stability was conducted under normal analytical conditions by repeated injection of an extracted spiked 50 ng/ml plasma sample.

3. Results and discussion

3.1. Separation

The molecular structures of moxidectin and ivermectin B1a are shown in Fig. 1. Blank human plasma was tested for endogenous interference. Of the lots tested, all were clear of interference in the moxidectin and internal standard regions. A representative chromatogram of the plasma blank selected for use in the validation procedure is shown in Fig. 2(A). Fig. 2(B) shows a chromatogram calibration standard containing 0.2 ng/ml of moxidectin in human plasma. A moxidectin standard (50 ng/ml) is shown in Fig. 3(A) and Fig. 3(B) shows a chromatogram of a calibration standard containing 50 ng/ml of moxidectin in human plasma. The retention time for moxidectin is 10.7 min and for the internal standard, ivermectin is 18.6 min, respectively. A run time of approximately 30 min per sample was used. Compared to another analytical method described by Alvinerie, this LC procedure results in much cleaner chromatograms [8].

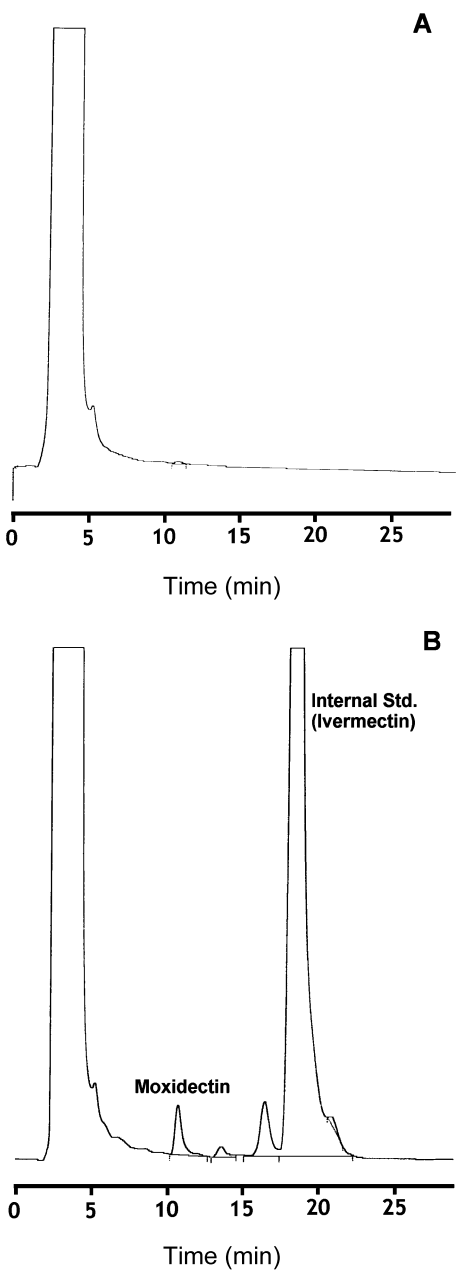

Fig. 2. Chromatograms of 200 μl injection blank human plasma (A), calibration standard containing 0.2 ng/ml moxidectin in human plasma, 200 μl injection (B).

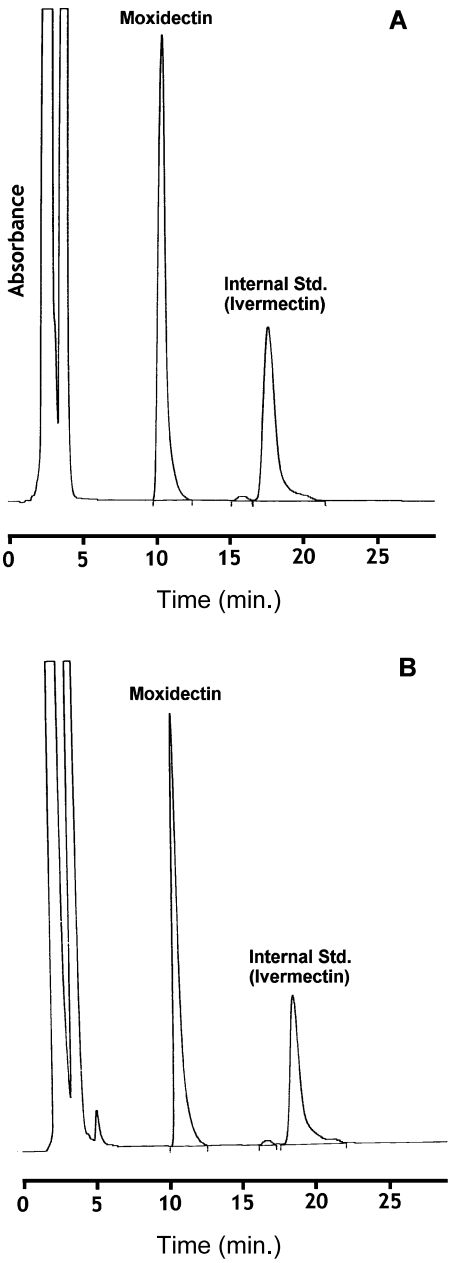

Fig. 3. Chromatograms of 50 ng/ml moxidectin standard, 20 μl injection (A), calibration standard containing 50 ng/ml moxidectin in human plasma, 20 μl injection (B).

3.2. Linearity

Calibration curve parameters for moxidectin are shown in Table 1A. Results were calculated using peak area ratios. Calibration curves for moxidectin in human plasma were linear using weighted (1/concentration) linear regression in the

Table 1A
Calibration curve parameters and statistics for moxidectin in human plasma

Curve	Slope	y-Intercept	Correlation coefficient
1	3.118E−02	3.552E−03	0.9994
2	3.163E−02	5.587E−02	0.9996
3	3.036E−02	6.290E−03	0.9999
4	3.101E−02	2.875E−03	0.9999
5	3.237E−02	1.100E−02	0.9997
6	3.256E−02	1.162E−02	0.9997
Mean ($n=6$)	3.152E−02		
S.D.	8.411E−04		
RSD (%)	3%		

S.D., standard deviation; RSD, relative standard deviation.

concentration range of 0.2–1000 ng/ml, with correlation coefficients greater than or equal to 0.9994 for all curves. The calibration curve precision and accuracy is presented in Table 1B, demonstrating the accuracy is 15% or less for all concentration points. We believe this concentration range is appropriate for analyzing the body fluids of subjects given anticipated doses of moxidectin. The limit of quantitation (LOQ) has been accepted as the lowest point on the standard curve, 0.2 ng/ml, with a RSD of less than 15%. The ratio of signal to noise at the LOQ is $>5{:}1$. Greater sensitivity can be achieved using a larger plasma volume.

Table 2
Intra- and inter-day precision and accuracy for moxidectin in human plasma

	Theoretical concentration (ng/ml)		
	0.2	400	1000
Intra-day			
Overall mean ($n=6$)	0.21	385.5	1008.7
S.D.	0.03	22.0	27.6
RSD (%)	11.9	5.7	2.7
DMT (%)	5.0	−3.6	0.9
Inter-day			
Overall mean ($n=18$)	0.20	385.9	1011.1
S.D.	0.04	19.2	28.2
RSD (%)	18	5	2.8
DMT (%)	0	3.7	1.1

S.D., standard deviation; RSD, relative standard deviation; DMT, deviation of mean value from nominal.

3.3. Precision and accuracy

Data on precision and accuracy are shown in Table 2. For an intra-day run ($n=6$), the RSD of moxidectin at 0.2, 400, and 1000 ng/ml has been shown to be 11.9, 5.7, and 2.7%, respectively. The inter-day precision is also shown in Table 2. The RSD results for inter-day precision at the same concentrations at 0.2, 400, and 1000 ng/ml has been shown to be 18.0, 5.0, and 2.8%, respectively ($n=18$). These results indicate there is good reproducibility and accuracy for the determination of moxidectin for samples determined on the same or different days.

Table 1B
Calibration curve precision and accuracy ($n=6$)

Moxidectin concentration (ng/ml)	Calculated concentration (ng/ml)	S.D.	RSD (%)	Accuracy (%)
0.2	0.17	0.03	16.2	85.0
0.6	0.64	0.09	14.4	107.2
5	4.9	0.4	8.1	97.8
50	54.5	3.9	7.2	109.1
200	205.8	10.5	5.1	102.9
400	389.8	12.4	3.2	97.4
600	573.4	50.7	8.8	95.6
1000	919.8	124.8	13.6	92.0

S.D., standard deviation; RSD, relative standard deviation.

Table 3
Recovery of moxidectin and ivermectin from human plasma

Concentration (ng/ml)	Moxidectin		Ivermectin (internal standard)	
	Mean extract area	Mean % recovery	Mean extract area	Mean % recovery
0.2	25,132	94.0	136,2594	73.5
RSD (%) ($n = 6$)	5.9		8.1	
400	151,9939	74.5	120,128	64.8
RSD (%) ($n = 6$)	5		3.2	
1000	401,3948	70.7	122,230	66.0
RSD (%) ($n = 6$)	3		3.7	
Overall recovery ($n = 18$)		79.7		68.1

RSD, relative standard deviation.

3.4. Absolute recoveries

Recovery was tested at low, medium and high concentrations of moxidectin and internal standard. Absolute recoveries were determined by comparing the peak areas of extracted quality control (QC) samples with the peak areas of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for moxidectin and the internal standard, ivermectin were 80 and 68%, respectively (Table 3).

3.5. Stability

Autoinjector stability was carried out for over 24 h by hourly injection of the same extracted plasma sample, spiked with moxidectin to 50 ng/ml, at room temperature (nominally 25 °C). The mean moxidectin concentration was 47.9, with a RSD of 3%, and no trend with respect to time indicating degradation. The results demonstrated the extracted specimens remained stable over the course of the study.

QC samples containing 15 and 600 ng/ml moxidectin in plasma were subjected to three freeze/thaw cycles. Samples were frozen at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were transferred back to the original freezer and kept refrozen for another 24 h. Freezing and thawing of the QC samples appeared to have no effect on quantitation of the analyte (Table 4).

QC samples containing 1, 400, and 1000 ng/ml moxidectin in plasma were subjected to storage at −20 and −80 °C for 12 weeks. Plasma samples ($n = 3$) were taken for moxidectin analysis at 0, 2, 4, 8, and 12 weeks (Table 5). The QC samples stored in a freezer set to maintain −20 and −80 °C remained stable for the duration of the study period.

3.6. Features and application of assay

The new aspects of this assay include: validation of methodology for human plasma; use of

Table 4
Freeze/thaw stability of moxidectin in plasma specimens during three freeze/thaw cycles

	Theoretical concentration (ng/ml)	
	15 ($n = 3$)	600 ($n = 3$)
Time = 0		
Mean	15.0	605.2
S.D.	0.27	2.34
RSD (%)	1.8	0.4
Cycle 2		
Mean	14.7	691.9
S.D.	0.27	96.40
RSD (%)	1.8	13.9
Cycle 3		
Mean	13.7	619.2
S.D.	0.63	37.75
RSD (%)	4.6	6.1

S.D., standard deviation; RSD, relative standard deviation.

Table 5
Storage stability of 1, 400 and 1000 ng/ml moxidectin in human plasma at −20 and −80 °C ($n = 3$)

Time (weeks)	0	2	4	8	12
−20 °C					
1 ng/ml					
Mean	1.04	1.13	0.91	1.00	0.85
S.D.	0.06	0.12	0.03	0.06	0.07
RSD (%)	5.9	10.2	3.6	5.7	8.1
400 ng/ml					
Mean	378.7	384.5	315.58	374.8	363.9
S.D.	22.10	21.27	15.62	5.63	6.99
RSD (%)	5.8	5.5	4.9	1.5	1.9
1000 ng/ml					
Mean	981.7	1002.5	895.82	1016.8	1010.9
S.D.	59.75	38.80	41.53	56.5	23.73
RSD (%)	6.1	3.9	4.6	5.6	2.3
−80 °C					
1 ng/ml					
Mean	1.04	0.87	0.97	0.99	0.79
S.D.	0.06	0.02	0.02	0.016	0.039
RSD (%)	5.9	1.8	1.7	1.6	4.9
400 ng/ml					
Mean	378.7	377.5	330.5	365.3	360.69
S.D.	22.10	7.26	8.95	3.76	14.7
RSD (%)	5.8	1.9	2.7	1.0	4.1
1000 ng/ml					
Mean	981.7	1054.0	943.40	1067.3	1042.5
S.D.	59.75	47.17	30.07	17.67	14.47
RSD (%)	6.1	4.5	3.2	1.7	1.4

S.D., standard deviation; RSD, relative standard deviation.

200 μl of plasma, compared with 1 ml for an earlier plasma method [9]; use of an internal standard in the methodology; development of a clean-up procedure which results in cleaner chromatograms than an earlier method [9], and validation of the assay over a concentration range which is relevant to concentrations that will be encountered in clinical pharmacokinetic studies.

The described assay has been validated using dog (data not shown) and human plasma and has been applied to dog and human pharmacokinetic studies. The chromatograms and elution profiles for dog and human plasma are nearly identical in our hands. Fig. 4 shows the mean plasma concentration versus time profiles for moxidectin after administration of 250 and 1000 μg/kg orally in Beagle dogs infected with *B. pahangi*. The moxidectin plasma concentration–time profile show a pronounced distribution with a prolonged elimination phase and is similar to observations in cattle, sheep and horses [14–17]. It is clear from these data that a broad concentration range is an assay requirement for pharmacokinetic studies. We have applied this method to the analysis of over 3000 plasma specimens from a dog pharmacokinetic study and have found that the method is reliable and robust.

3.7. Interference with other drugs

Diethylcarbamazine and doramectin have been tested for possible interference with the assay and were found not interfere with the assay. Plasma specimens were spiked with diethylcarbamazine (1

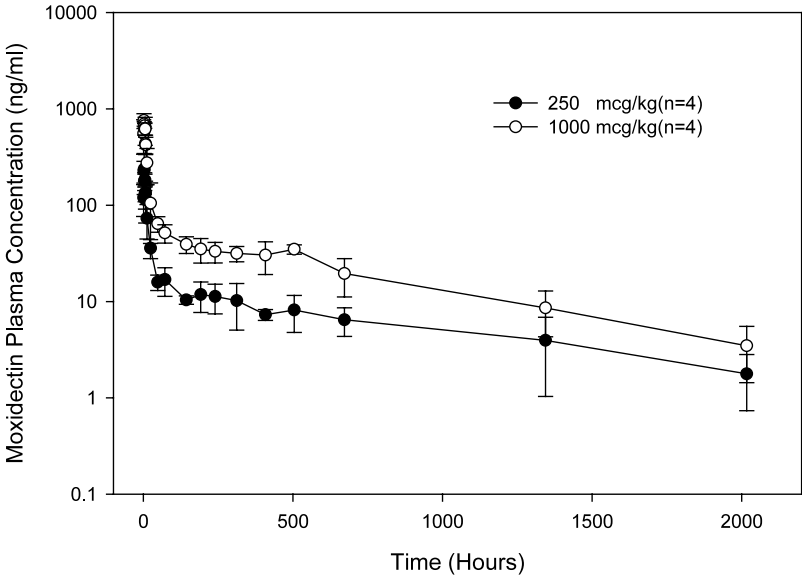

Fig. 4. Plasma concentration (mean and SD) versus time profile for moxidectin after administration of 250 or 1000 μg/kg orally in dogs ($n = 4$ each).

μg/ml) and or doramectin (50 ng/ml) and carried through the assay procedure. Under the described conditions, ivermectin is separated from doramectin and elutes at 16.1 min. Fresh blood was drawn into Vacutainers® containing either heparin or disodium E.D.T.A. The plasma was harvested, and carried though the assay procedure. Neither anticoagulant interferes with the assay.

4. Conclusions

The objective of this study was to validate a liquid chromatographic assay procedure using SPE for the specific and quantitative analysis of moxidectin in human plasma. The assay uses ivermectin as an internal standard and has a run time of approximately 30 min. Use of a SPE procedure results in chromatograms free of interference at the times of interest. The assay has been validated and the results of validation demonstrate that the standard curve is linear over the concentration range of 0.2–1000 ng/ml. The assay is reproducible and accurate, with recovery of the analyte in the range of 71–94%. The analysis requires 200 μl of plasma and has a detection limit of 0.2 ng/ml. The procedure has a sample throughput of at least 40 specimens per day, and sufficiently robust to support of clinical pharmacokinetic studies. The assay meets the guidelines for bioanalytical methods validation for human studies [18].

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