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Short communication

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

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Abstract

There is a need for an accurate, sensitive and selective high-performance liquid chromatography (HPLC) method for the quantitation of ivermectin in human plasma that separates the parent drug from metabolites. Ivermectin and the internal standard, moxidectin, were extracted from 0.2 ml of human plasma using Oasis HLB solid phase extraction cartridges. After extraction, fluorescent derivatives of ivermectin and moxidectin were made by reaction with trifluoroacetic anhydride and *N*-methylimidazole. Separation was achieved on a Alltech Ultrasphere C18 5μ column with a mobile phase composed of tetrahydrofuran–acetonitrile–water (40:38:22 v/v/v). Detection is by fluorescence, with an excitation of 365 nm and emission of 475 nm. The retention times of ivermectin and internal standard, moxidectin are approximately 24.5 and 12.5 min, respectively. The assay is linear over the concentration range of 0.2–200 ng/ml of ivermectin in human plasma ($r = 0.9992$, weighted by 1/concentration). Recoveries of ivermectin are greater than 80% at all concentrations. The analysis of quality control samples for ivermectin 0.2, 25, and 200 ng/ml demonstrated excellent precision with coefficient of variation of 6.1, 3.6 and 2.3%, respectively ($n = 6$). The method is accurate with all intra-day ($n = 6$) and interday ($n = 12$) mean concentration within 10% of nominal values at all quality control sample concentrations. Storage stability for 30 days at −80 °C and after three freeze–thaw cycles are within acceptable limits. The method separates ivermectin from multiple less and more polar unidentified metabolites. This method is robust and suitable for clinical pharmacokinetic studies. The analytical procedure has been applied to a pharmacokinetic study of ivermectin in healthy volunteers and to the analysis of plasma specimens from patients with disseminated strongyloidiasis.

Keywords: Ivermectin; Antifilarial; Antiparasitic; HPLC; Liquid chromatography

1. Introduction

Ivermectin is a macrocyclic lactone belonging to the avermectin family of compounds produced by the bacteria *Streptomycetes avermetilis*. The compound is an antiparasitic agent used in humans for the treatment of onchocerciasis and strongyloidiasis. Ivermectin is a broad-spectrum antiparasitic agent and is also used in veterinary applications to treat parasitic diseases in cattle, sheep, dogs, swine and horses. The drug is exceptionally potent with effective dosages' levels that are unusually low (0.15 mg/kg), and plasma ivermectin concentrations are corresponding low (<100 ng/ml). Ivermectin is a semi-synthetic derivative of avermectin B_1, consisting of a 80:20 mixture of the equipotent homologous 22,23-dihydroavermectin B_{1a} and B_{1b} derivatives. The structures of ivermectin and moxidectin are shown in Fig. 1.

A number of liquid chromatography and liquid chromatography/mass spectrometry methods have been described for determination of ivermectin in plasma [1–10]. Both liquid chromatographic methods using UV detection and fluorescence detection have been reported. Most of the analytical methods for determining ivermectin and other avermectins in plasma are based upon conversion of these compounds to fluorescent products based upon a double dehydration reaction leading to aromatization followed by high-performance liquid chromatography (HPLC) with fluorometric detection. The fluorescent bioanalytical applications has been extensively reviewed by Fink et al. [11]. The dehydration reaction uses trifluoracetic anhydride and *N*-methylimidazole form a highly fluorescent six-membered aromatic ring in conjugation with a butadiene unit as shown in Fig. 2.

Our laboratory has studied other avermectins and has developed a liquid chromatographic fluorescence assay of moxidectin

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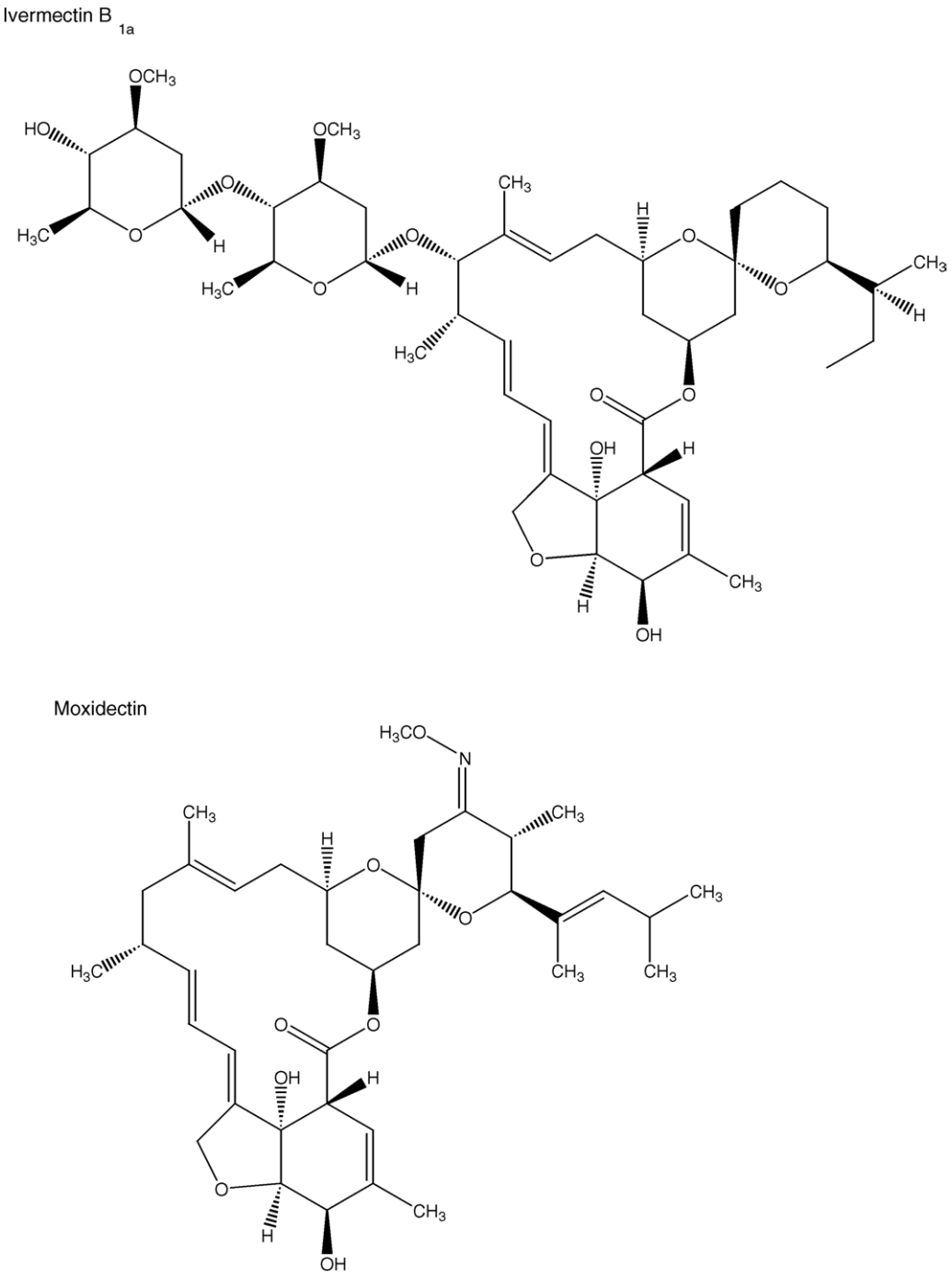

Fig. 1. Structures of ivermectin and the internal standard, moxidectin.

in human plasma for application to pharmacokinetic studies [12]. Moxidectin has a relatively large volume of distribution and a highly sensitive method is required for pharmacokinetic studies. The method for moxidectin uses ivermectin as an internal standard. We applied the moxidectin methodology to pilot ivermectin pharmacokinetic studies and were surprised to see multiple unidentified metabolite peaks in plasma samples beginning approximately 2 h post-dosing. We were intrigued by the presence of less- and more-polar metabolites, which were not reported by other analytical methods of human plasma [1–10]. The sensitivity of the methodology was greater than that required for most ivermectin pharmacokinetic studies, but high sensitivity seemed to be an advantage for detecting multiple metabolite peaks, which had lower peak heights than ivermectin. The chromatographic run time for this methodology was longer than most current methods; however, separation of ivermectin from multiple metabolites peaks seemed desirable and the run time could still be accomplished within 32 min. We changed to a Ultrasphere C_{18} column to obtain better peak shape. This report describes a sensitive, specific HPLC proce-

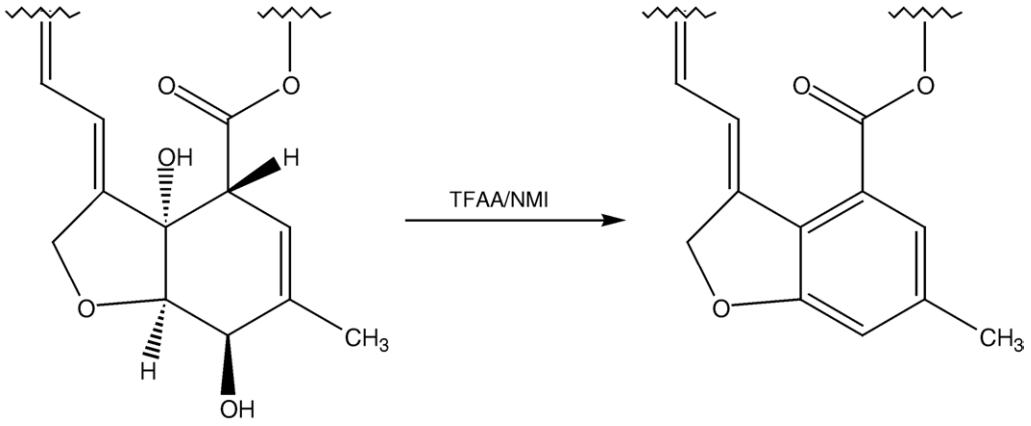

Fig. 2. Derivatization reaction of ivermectin and moxidectin with trifluoroacetic anhydride (TFAA) and *N*-methylimidazole (NMI).

dure with solid phase extraction and fluorescence detection for determining ivermectin in human plasma suitable for pharmacokinetic studies. The particular ivermectin component quantitated is 22,23-dihydroavermectin B_{1a}, which is the component of pharmaceutical interest.

2. Experimental

2.1. Solvents and chemicals

All solvents were HPLC grade. Organic solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). A Milli-Q Plus water system (Millipore Corporation, Bedford, MA) produced ultra pure analytical grade Type I water. A solution of tetrahydrofuran–acetonitrile–water (40:38:22 v/v/v) was used as the mobile phase. Water and a prepared 25% solution of methanol in water were used as wash solvents. For elution, 2-propanol was used. Trifluroacetic anhydride was obtained from Aldrich Chemical Co. (Milwaukee, WI). *N*-methylimidazole was obtained from Acros Co. (New Brunswick, NJ). Ivermectin was obtained from Sigma Chemical Co. (St. Louis, MO). Moxidectin was provided by Analytical Standards Distribution, American Cyanamid (Princeton, NJ).

2.2. Standard stock solutions

Stock solutions were made with ivermectin and moxidectin (Fig. 1). The chemicals were weighed on a Mettler-Toledo AG104 analytical balance (Mettler-Toledo, Inc., Hightstown, NJ, USA). The weighed amounts were dissolved in acetonitrile in volumetric flasks. The concentrations of stock solutions were: ivermectin (1.0 mg/ml), moxidectin (1.0 mg/ml). The initial stock solutions were further diluted in acetonitrile to produce solutions of ivermectin (0.01 mg/ml) and moxidectin (0.1 mg/ml). Working standards were prepared for each concentration in the standard curve. The working standards contained 0.4, 2.0, 10, 50, 200, and 400 ng of ivermectin per 100 μl of acetonitrile. A 40 ng/ml solution of moxidectin in acetonitrile was prepared as working solution for the internal standard. This concentration was chosen to give a peak height ratio of 1.0 near the midpoint of the ivermectin standard curve. Standard solutions were stored at −20 °C.

2.3. Instrumentation

Chromatography was preformed using a Waters 501 HPLC pump, a Waters 717 plus Autosampler (Waters, Milford, MA), a Shimadzu RF10AxL fluorescence detector Shimadzu, Kyoto, Japan), and an Alltech Ultrasphere C_{18} 5 μm 4.6 × 250 mm column (Alltech, Deerfield, IL). Results were plotted and processed using a Shimadzu CR501 Chromatopac. The mobile phase (see Section 2.1) flow rate was 1.0 ml/min. The fluorescence detector settings were: excitation 365 nm and emission 475 nm, and sensitivity high.

2.4. Extraction procedure

This procedure was validated using 200 μl of spiked human plasma. 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 ivermectin and moxidectin was preformed by solid phase extraction (SPE). Waters Oasis HLB 30 mg 1 ml reservoir cartridges (Waters Corporation, Milford, MA) and an SPE vacuum manifold (Alltech, Deerfield, IL, USA) were used for the procedure. Pipetman® precision microliter pipettes (Woburn, MA, USA) were used throughout the assay.

Validation samples were prepared in control plasma by adding ivermectin dissolved in acetonitrile. Standard and QC samples were prepared from separately weighed master solutions of ivermectin. Calibration curves were prepared daily with each set of samples. Calibration curves consisted of a plasma blank and spiked plasma samples with final concentrations of ivermectin of 0.2, 1.0, 5.0, 25, 100, and 200 ng/ml. Spiked plasma was prepared by adding 10 μl of the working solution for each point in the standard curve and 10 μl of internal standard to 200 μl of human plasma in a 10 ml borosilicate 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 cartridge was activated by aspirating with 1 ml

of methanol followed by 1 ml of HPLC grade water. Samples were transferred to the activated cartridges and aspirated at a vacuum of 3 mm of Hg. Each cartridge was washed with 2 ml of water followed by 2 ml of 25% methanol. After washing, samples were eluted with 2 ml of 2-propanol under a vacuum of 4 mm of Hg into clean borosilicate glass 10 ml test tubes. The elutant was evaporated at 50 °C in a N-EVAP® (Berlin, MA, USA), under a gentle stream of nitrogen. Dried samples were derivatized (Fig. 2) with 100 μl of *N*-methylimidazole in acetonitrile (1:1 v/v) and 150 μl of trifluroacetic anhydride in acetonitrile (1:2 v/v). The derivatized samples were transferred to vials containing 250 μl polypropylene conical inserts and placed on the autosampler where 20–100 μl (100 μl for ivermectin concentrations ≤1 ng/ml; 20 μl if for ivermectin concentrations >1 ng/ml) was injected. Varying the injection volume was necessary to avoid exceeding the detector's detection limit.

2.5. Quality controls

QC samples for autosampler stability and for intra- and inter-day assays were individually prepared by spiking 200 μl of plasma with ivermectin. QC samples were prepared at concentrations of 0.2, 25, and 200 ng/ml of ivermectin. QC samples for freeze–thaw, stability and subject analysis were prepared in bulk. Bulk QCs were prepared at concentrations of 0.6, 25, and 200 ng/ml of ivermectin. Bulk QCs were divided into 2.5 ml Nalgene cryovials and stored at −80 °C.

2.6. Data regression

Chromatographic data were collected using a Shimadzu CR501 Chromatopac. Peak area ratios of ivermectin/moxidectin were calculated. Equations for the calibration curves were obtained by 1/concentration weighted linear regression analysis. The equations were used to calculate the concentrations of ivermectin in samples and QC samples by peak area ratios.

2.7. Selectivity, precision, accuracy and recovery

Six human plasma samples from six individual donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent detector response at the retention time of ivermectin and moxidectin was compared to the lower limit of quantification.

Precision, accuracy, and recovery were evaluated by analyzing six spiked plasma samples at each concentration level for ivermectin (0.2, 25 and 200 ng/ml). The coefficient of variation at each concentration was calculated to determine the precision of the method. The accuracy of the method was determined by comparing the measured concentrations of the extracted plasma samples to the true concentration spiked into the sample. Recovery was assessed by comparing the peak area obtained for extracted spiked samples to the peak area obtained for unextracted standard mixtures representing 100% recovery.

2.8. Stability

Freeze and thaw stability, storage stability at −80 °C and autosampler stability experiments were conducted. QC samples containing 0.6 and 200 ng/ml of ivermectin in plasma were subjected to three freeze–thaw cycles. Samples were frozen at −80 °C for at least 24 h and thawed unassisted at room temperature. When completely thawed, the samples were transferred back to the freezer and refrozen for at least 24 h. Autosampler stability was conducted by repeated injection of extracted spiked ivermectin (200 ng/ml) plasma samples.

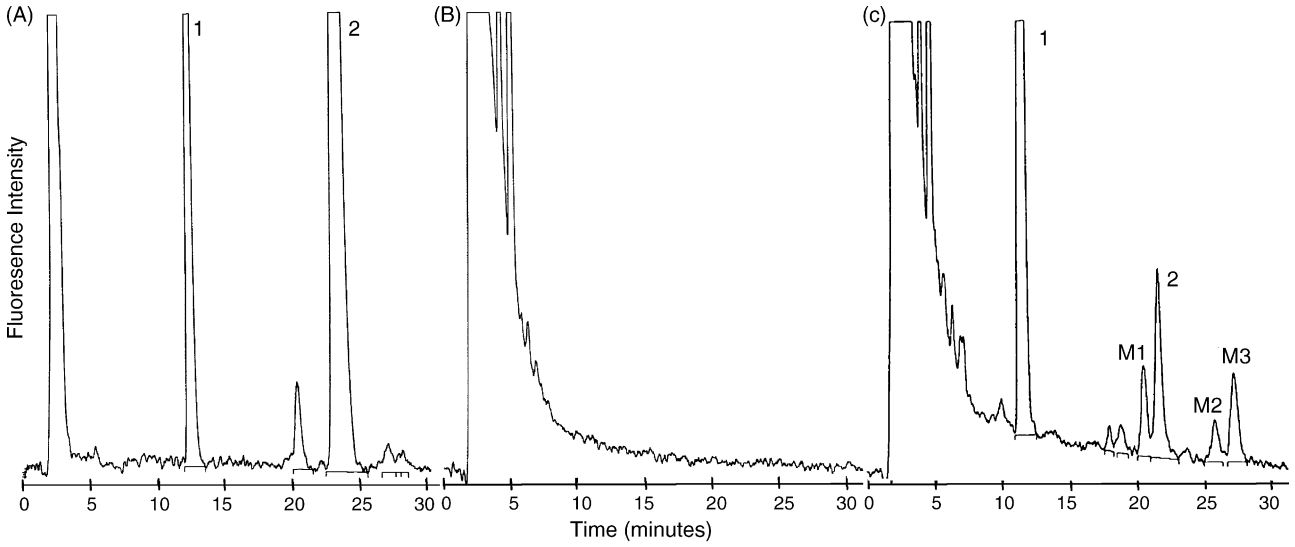

Fig. 3. Chromatograms of (A) ivermectin (peak 2, r.t. = 23.378 min) 100 ng/ml and moxidectin (peak 1, r.t. = 12.380 min) 20 ng/ml, (B) blank (analyte-free) human plasma and (C) 48 h post-dose plasma sample from a subject given a 150 μg/kg oral dose of ivermectin. Calculated level is ivermectin 2.5 ng/ml (peak 2, r.t. = 23.602 min), moxidectin peak (r.t. = 12.507), and metabolites M1 (r.t. = 22.418), M2 (r.t. = 28.09), and M3 (r.t. = 29.458) (r.t., retention time).

3. Results and discussion

3.1. Separation

The molecular structures of ivermectin and moxidectin (internal standard) are shown in Fig. 1. Chromatographic run times are 32 min per sample. Fig. 3(A) shows an injection standard containing 100 ng/ml of ivermectin and 20 ng/ml of moxidectin. A representative chromatogram of a plasma blank is shown in Fig. 3(B), which shows that the chromatogram is free from interfering peaks at the retention times of ivermectin and moxidectin. Fig. 3(C) shows a representative chromatogram from a healthy subject who took a single oral dose of ivermectin. The retention times of ivermectin and the internal standard are approximately 12 and 23 min, respectively (note metabolite peaks at 22, 28 and 29.5 min). Fig. 4 shows a chromatogram from a patient with disseminated strongyloidiasis who received subcutaneous ivermectin. This chromatogram shows an ivermectin peak at 23 min, separated from metabolite peaks at 22, 28 and 30 min. The chromatograms in Figs. 3C and 4 show good baseline separation of the chromatographic peaks of interest.

3.2. Linearity

Calibration curve parameters for ivermectin are shown in Table 1. Results were calculated using peak area ratios. Calibration curves were linear using 1/concentration weighted linear regression in the concentration ranges of 0.2–200 ng/ml for ivermectin (Table 2). The calibration curves cover the range of expected concentrations for analyzing the plasma of subjects given either a 150 μg/kg oral dose or a 200 μg/kg subcutaneous dose of ivermectin.

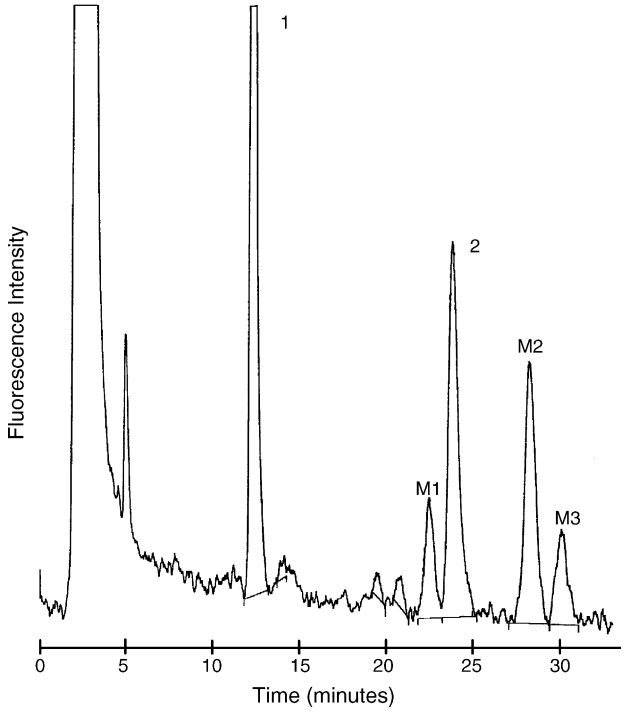

Fig. 4. Chromatogram of a plasma sample from a patient with disseminated strongyloidiasis who received subcutaneous ivermectin showing an ivermectin peak (r.t. = 23.053), moxidectin peak (r.t. = 12.336), and metabolites M1 (r.t. = 22.408), M2 (r.t. = 28.224), and M3 (r.t. = 29.994).

Table 1
Calibration curve parameters and statistics in human plasma

Curve	Ivermectin		
	Slope	*y*-Intercept	Correlation coefficient
1	0.02866	0.00188	0.9998
2	0.02987	−0.00042	1.0000
3	0.02995	0.00180	0.9989
4	0.02883	0.00088	0.9999
5	0.02879	0.00258	0.9998
6	0.02740	0.00087	0.9996
Mean	0.02892	0.00127	0.9997
S.D.	0.00093	0.00105	
C.V. (%)	3.23		

S.D., standard deviation; C.V., coefficient of variation.

The limit of quantification (LOQ) in human plasma for ivermectin was accepted as 0.2 ng/ml. This was greater than five times the baseline noise seen at the retention time of ivermectin. Plasma samples were spiked to a nominal concentration of 0.2 ng/ml with working solutions and internal standard and carried through the extraction procedure. At the LOQ, the coefficient of variation ($n = 6$) of the measured concentration was 6.1%, and the deviation of the mean of the measured concentration from the nominal value was 4.3%.

3.3. Selectivity, precision and accuracy

To demonstrate selectivity of the assay in human plasma, control plasma from six lots were compared with whose of plasma fortified with ivermectin or internal standard. The control plasma from all six lots were free of interference at the retention times of the analyte and internal standard. The data on precision and accuracy are shown in Table 3. Precision values varied from 2.3 to 7.3% for intra- and inter-day analyses. Accuracy varied from 94.9 to 108.6%. The results indicate that there is good reproducibility and accuracy for the determination of ivermectin for samples determined on the same or different days.

Table 2
Ivermectin in human plasma, back calculated calibration standard values

Ivermectin	
Concentration (ng/ml)	Calculated concentration (ng/ml)
0.2	0.19
1.0	1.0
5	4.5
25	28.8
100	103
200	193

Slope, 0.0246; Intercept, 0.00138; *r*, 0.9987.

Table 3
Intra-day and inter-day precision and accuracy for ivermectin in human plasma

Theoretical concentration (ng/ml)	Ivermectin		
	0.2	25	200
Intra-day run			
Overall mean ($n = 6$)	0.21	24.6	190
S.D.	0.01	0.89	4.3
C.V. (%)	6.1	3.6	2.3
Accuracy (%)	104.9	98.3	94.9
Inter-day run			
Overall mean ($n = 12$)	0.22	25.2	198
S.D.	0.02	0.91	9.9
C.V. (%)	7.3	3.6	5.0
Accuracy (%)	108.6	100.7	99.2

S.D., standard deviation; C.V., coefficient of variation; DMT, deviation of mean value from nominal.

3.4. Determination of the extraction efficiency

Recovery was tested at low and high concentrations of ivermectin, and moxidectin. Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for ivermectin and moxidectin were 86 and 83%, respectively (Table 4). The results demonstrate that the extraction efficiency is relatively constant over the range considered.

3.5. Stability

No significant degradation was detected after three freeze–thaw cycles of QC samples of ivermectin in human plasma at the concentrations tested (Table 5).

Autoinjector stability was carried by repeated injections of extracted plasma samples containing ivermectin (200 ng/ml) at room temperature. The ivermectin measurements were >95% of the initial values during storage in the autoinjector for 24 h.

Samples containing 0.6 and 200 ng/ml of ivermectin in plasma were subjected to storage at −80 °C for 30 days. Plasma samples ($n = 5$) were taken for analysis at 0, 5, 15 and 30 days. The mean concentrations of ivermectin in plasma did not change significantly within this time period under the indicated storage conditions.

3.6. Interference by other drugs

Ketoconazole, fluconazole and verapamil have been tested for potential interference with the assay. No interferences were found.

Table 5
Freeze and thaw stability of ivermectin in plasma specimens during three freeze–thaw cycles

Theoretical concentration (ng/ml)	0.6	200
Cycle 3	$n = 6$	$n = 6$
Mean	0.52	210.7
S.D.	0.08	9.72
C.V. (%)	15.1	4.6
Accuracy (%)	−85.9	105.4

S.D., standard deviation; C.V., coefficient of variation.

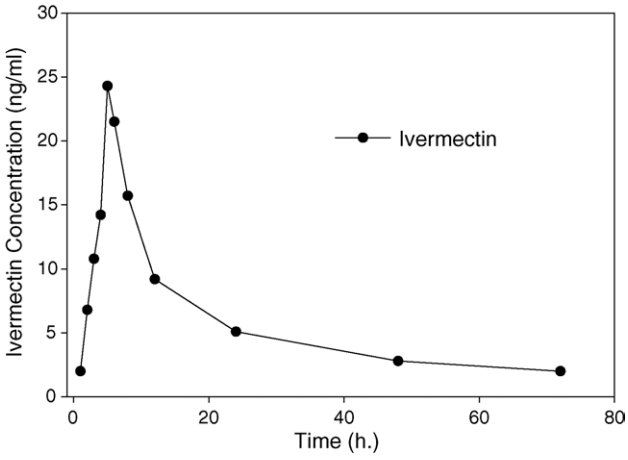

Fig. 5. A plasma concentration–time profile for a male subject who received an oral dose of 150 μg/kg ivermectin.

3.7. Application of the method

This method was successfully applied to a clinical pharmacokinetic study of 16 healthy subjects who received an oral dose of ivermectin (150 μg/kg). The method appears to be robust as more than 300 plasma samples have been successfully analyzed. A plasma concentration–time profile for a subject who received an oral dose of 150 μg/kg ivermectin is presented in Figs. 5 and 6. Fig. 3(C) shows a chromatogram from the same healthy volunteer showing three prominent metabolite peaks labeled M1, M2 and M3. Two additional peaks appear at 19.6 and 20.5 min, which may also be less polar metabolites of ivermectin. This analytical method has also been applied to clinical specimens from two patients with disseminated strongyloidiasis who received veterinary subcutaneous ivermectin [13,14]. Fig. 4 shows a chromatogram from a patient with disseminated strongloidiasis, which reveals multiple prominent metabolite peaks that are well separated from ivermectin and have similar retention times to Fig. 3(C). It is apparent from Fig. 7 that metabolite M2 accumulates in plasma with multiple dosing. It is likely that

Table 4
Recovery of ivermectin and moxidectin (internal standard) from human plasma

	Concentration (ng/ml)	Mean extract area	*N*	C.V. (%)	Mean pure area	*N*	C.V. (%)	Mean % recovery
Ivermectin	0.6	226374	5	15.1	257366	5	12.8	88
	200	12536600	5	9.0	14955999	5	4.8	84
Moxidectin	20	2392252	5	11.0	2891444	5	5.8	83

C.V., coefficient of variation.

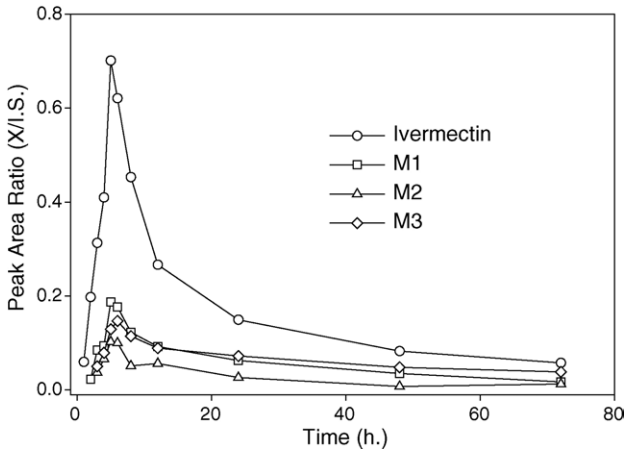

Fig. 6. Profiles of the peak area ratios of analyte/internal standard (X/I.S.) vs. time for ivermectin and metabolites M1, M2 and M3 for male subject in Fig. 5 (I.S., internal standard).

this less polar metabolite accumulates in the body because of a longer elimination half-life of the metabolite.

Fink and Porras reported radioactive metabolites after administration of ^{3}H-ivermectin to healthy volunteers after an oral dose [15]. The peak plasma concentrations of radioactive metabolites were about twice those of parent drug and occurred at about 7 h, somewhat later than the peak ivermectin concentration at 4 h post-dose. Some of the metabolites were longer lived than the parent drug and disappearance of the radioactively metabolites in plasma had an apparent half-life of about 3 days. Plasma metabolites that were less polar than the parent drug were tentatively characterized as fatty acid ester conjugates of the monosaccarides or aglycone of ivermectin [16]. Positive identification of more polar metabolites in urine and feces include 3″-0-desmethyl-22,23-dihydroavermectin B_{1a}, and 22,23-dihydroavermectin B_{1a} monosaccharide.

Because of the potency and known CNS toxicity of ivermectin [17], accumulation of lipophilic metabolites may be of toxicological concern with multiple dosing. We could not locate information on the efficacy or toxicity of ivermectin metabolites. Guzzo et al. have recently reported a safety, tolerance and pharmacokinetic study in healthy volunteers given 30 or 60 mg of ivermectin three times per week [18]. They found that the drug was generally well tolerated with minimal accumulation on a regimen given every fourth day. However, they reported only parent drug quantitated using and HPLC method with fluorescence detection and did not mention ivermectin metabolites.

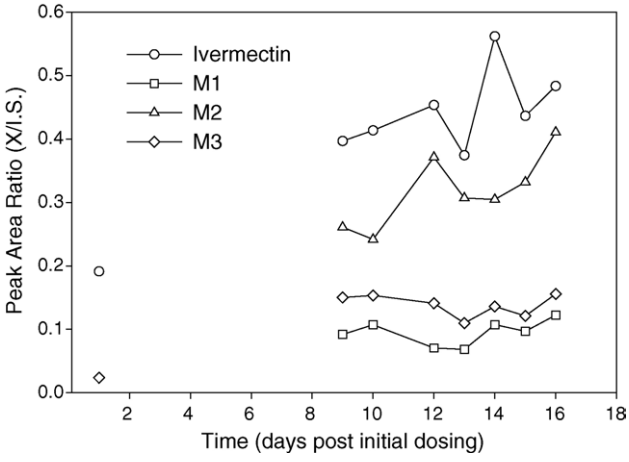

Fig. 7. Profile of peak area ratios of analyte/internal standard (X/I.S.) vs. time for ivermectin and metabolites M1, M2 and M3 for male patient with disseminated strongyloidiasis who received subcutaneous ivermectin daily for 14 days (200 μg/kg) (I.S., internal standard).

Of the other published ivermectin analytical methods, we have reviewed for the analysis of human specimens we did not find any mention metabolite peaks found in the analysis. While our method has a longer run time than other reported methods, we believe the metabolite information gained by the methodology is advantageous for clinical pharmacokinetic studies. The high sensitivity of our methodology undoubtedly contributes to the ability to detect the multiple small metabolite peaks, which can be detected over 72 h following a single standard dose of oral ivermectin. We cannot rule out the possibility of co-eluting metabolites under the peaks we have labelled as M1, M2 and M3. Clinical specimens have been run using the methodology described in this paper, both with and without the addition of internal standard, and no interference has been found at the retention time of the moxidectin internal standard. Recently developed LC-MS/MS methods offer specificity and the potential to identify these significant ivermectin metabolites.

4. Conclusions

A HPLC assay procedure using solid phase extraction for the specific and quantitative analysis of ivermectin in human plasma samples is described. The extraction procedure and the chromatographic set-up are simple, reliable and consistent. The assay uses moxidectin as an internal standard and has a run time of approximately 32 min. The chromatograms show a clear separation parent drug from unidentified ivermectin metabolites and has no interfering peaks in plasma specimens. The assay has been validated and the results of validation demonstrate that the standard curve is linear over the concentration range 0.2–200 ng/ml. The assay is reproducible and accurate. The analysis has a quantitation limit of 0.2 ng/ml for ivermectin, which is as good or superior to that reported in other papers [2,5]. Furthermore, the proposed method uses low volumes of plasma (0.2 ml), which can be advantageous in clinical pharmacokinetic studies. Autoinjector stability has been demonstrated for up to 24 h. The high sensitivity and ability to provide metabolite information are the primary advantages of this methodology.

Acknowledgements

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