HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF IVERMECTIN IN PLASMA

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Abstract. A simple, sensitive, selective and reproducible method based on reversed-phase chromatography was developed for the determination of ivermectin in human plasma. The internal standard (moxidectin) was separated from ivermectin on a Hypersil Gold C18 column (150 x 4.6 mm, 5 μm particle size), with retention time of 3.7 and 7.0 minutes, respectively. Fluorescence detection was set at an excitation and emission wavelength of 365 and 475 nm, respectively. The mobile phase consisted of acetonitrile, methanol and distilled water (50:45:5, v/v/v), running through the column at a flow rate of 1.5 ml/minute. The chromatographic analysis was operated at 25ºC. Sample preparation (100 μl plasma) was done by a single step protein precipitation with acetonitrile, followed by derivatization with 100 μl of *N*-methylimidazole solution in acetonitrile (1:1, v/v) and 150 μl of trifluoroacetic anhydrous solution in acetonitrile (1:2, v/v). Calibration curve over the concentration range of 20-8,000 ng/ml plasma was linear with correlation coefficient better than 0.995. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 15% (coefficient of variation) Good accuracy was observed for both intra-day and inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (below +15%). Limit of quantification was 0.02 ng using 100 μl sample. The mean recovery for ivermectin and the internal standard was greater than 90%. The method was free from interference from endogenous substances and commonly used drugs. The method appears to be robust and has been applied to the investigation of plasma concentration *vs* time profile of ivermectin in five healthy Thai volunteers following a single oral dose of 200 μg ivermectin/kg body weight.

INTRODUCTION

Ivermectin is a semi-synthetic 22, 23-dihydro derivative of avermectin B1 (abamectin) which is the major and most important product of the fermentation of *Streptomyces avermitilis* (Campbell, 1991). The drug consists of an 80:20 mixture of the equipotent homologous 22, 23 dehydro B_{1a} and B_{1b}. Ivermectin is widely used since the introduction to the market in 1981, as an antiparasitic agent against endo- and ectoparasites of domestic animals and is considered the drug of choice for lymphatic filariasis and river blindness (onchocerciasis) in humans (Burg *et al*, 1979; Soll *et al*, 1987).

A number of analytical methods have been reported for the determination of ivermectin in human and animal biological fluids as well as tissue organ extracts. These methods involve enzyme-linked immunosor-

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bent assay (Mitsui *et al*, 2001), immunobiosensor (Samsonova *et al*, 2002), thin-layer chromatography (TLC) (Taylor *et al*, 1994) and high performance liquid chromatographic techniques (HPLC) with ultraviolet (UV) (Pivnichny *et al*, 1983; Schnitzerling and Nolan, 1985; Picnichny *et al*, 1987; Dickinson *et al*, 1990), fluorescence (Tolan *et al*, 1980; Alivinerie *et al*, 1981; Tway *et al*, 1981; Mrozik *et al,* 1982; Alvinerie et al, 1987, 1993; Chiou *et al*, 1987; Kojima *et al*, 1987; de Montigny *et al*, 1990; Nordlander and Johnsson, 1990; Fisher *et al*, 1993; Krishna and Kolz, 1993; Fink *et al*, 1996; Sutra *et al*, 2001) or mass-spectrometry (MS) (Croubels *et al*, 2002) detection. Enzyme-linked immunosorbent assay is sensitive but has a narrow calibration range and possible cross-reactivities against metabolite(s) presented in the ivermectin-dosed biological samples. Since ivermectin does not possess strong chromophores for UV or fluorescence detection, the HPLC techniques involve either pre- or post-column derivatization, with UV or fluorometric detection of the derivatized product. Recently, two HPLC methods with fluorescence detection have been reported for assay of ivermectin and moxidectin in human plasma (Chen *et al*, 2002; Kitzman *et al*, 2006). Although the methods are sensitive, they require expensive sample preparation procedure using solid phase extraction (SPE) before derivatization. In addition, run time was long (within 20 minutes). HPLC with MS or MS/MS detection is highly sensitive and allows detection of ivermectin without derivatization following a simple and rapid sample preparation step. Nevertheless, the instruments are not yet readily obtainable in most laboratories. Sample clean-up and concentration are based on immunoaffinity chromatography (Li and Zhang, 1996), protein precipitation and/or liquid-liquid extraction combined with solid-phase extraction (Tolan *et al*, 1980; Chiou *et al,* 1987; Kojima *et al*, 1987; Picnichny *et al*, 1987; Dickinson *et al*, 1990; Montigny *et al*, 1990; Krishna and Klolz, 1993), and on-line solid phase extraction.

TLC or HPLC methods with UV detection of underivatized ivermectin is considered not sensitive nor selective for clinical plasma analysis. Furthermore, large sample volumes (3-5 ml) and lengthy sample clean up procedures are required for most HPLC-UV methods to achieve a sensitivity in the low nanogram per milliliter range. The previously reported HPLC with fluorescence detection methods provide improved sensitivity. However, most of these methods have at least one of the following limitations, namely, requirement of large sample volumes (1-2 ml), inadequate sensitivity [limit of quantification (LOQ) of 0.1-1 ng using 1 ml of plasma], lengthy derivatization reaction (1 or 24 hours), and laborious and costly sample clean-up procedures (requirement of SPE). A more efficient and less labor-intensive means of preparing biological samples prior to analysis with improved sensitivity is highly desirable.

MATERIALS AND METHODS

Chemicals

All solvents (acetonitrile, methanol) were HPLC grade. Organic solvents were purchased from Fison Scientific Equipment (Bishop Meadow Road, Loughborough, UK). Ivermectin (Fig 1a) and moxidectin (Fig 1b) were obtained from Sigma (St Louis, MO, USA). The derivatizing agents, *N*-methylimidazole and anhydrous trifluoroacetic were from Aldrich (Milwaukee, WI, USA).

Standard stock solutions

Stock solutions were made with ivermectin and the internal standard (moxidectin). Appropriate amounts of chemicals were dissolved in methanol in volumetric flasks. Stock solutions for ivermectin and internal standard were prepared at the concentration of 1,000 ng/µl. The stock solutions were further diluted with metha-

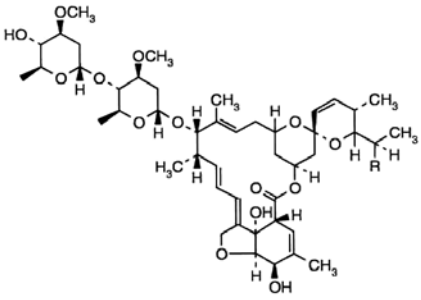
nol to make working solutions at concentration of 0.01 ng/µl for ivermectin and 1 ng/µl for the internal standard. Standard solutions were stored at -20ºC until used.

Chromatography

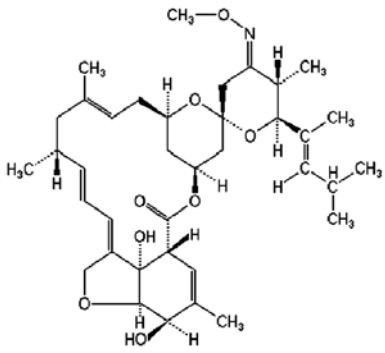
The method was developed using a chromatographic system consisting of a Waters 600 HPLC solvent Delivery/Controller, equipped with a Rheodye 7125 injector with a 100-µl loop (Rheodyne, Berkeley, CA, USA), a fluorescence detector (FP 1520, Jusco Corporation, Tokyo, Japan), and Millennium 32 Software for data integration. Fluorescence detection was set at an excitation and emission wavelength of 365 and 475 nm, respectively. The separation was carried out on a reversed phase Hypersil Gold C18 column (150 x 4.6 mm, 5 µm particle size; Wellington House, Waterloo Street West, UK). The elution solvent consisted of a mixture of acetonitrile, methanol and distilled water at the ratio of 50:45:5 (v/v/v), eluting through the column at a flow rate of 1.5 ml/minute. The chromatographic analysis was conducted at 25ºC. All solvents were vacuum filtered and degassed through 0.2 µm pore size polymeric PTFE filters.

Fluorogenic derivatization and sample preparation

Fluorogenic derivatization of ivermectin. Since ivermectin does not possess strong chromophore for UV or fluorescence detection, chemical modification is required to enhance its detectability and selectivity to achieve the lower limit of quantification of < 1 ng, using small volume of biological samples. Trichloroacetic acid and *N*-methylimidazole in acetonitrile were used as a derivatizing agent as previously described by Montigy *et al* (1990). This method allowed the rapid conversion of ivermectin to a fluorescent derivative (Fig 2) within 30 seconds. Furthermore, fewer reagent by-products were formed compared with the method that used acetic anhydride as the derivatizing agent, thereby eliminating the need for further sample preparation after derivatization.



(a)



(b)

Fig 1–Chemical structures of (a) ivermectin and (b) internal standard (moxidectin).

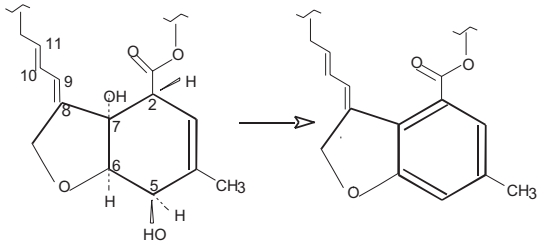


Fig 2–Fluorescent derivatization of ivermectin with trifluoroacetic anhydride and *N*-methylimidazole.

Sample preparation. This procedure was validated on specimens using 100 µl of spiked

human plasma. Outdated human plasma was obtained from the Blood Bank of Thammasat Chalermprakiet Hospital, Thammasat University, and stored frozen in aliquots at -20ºC. Five ng of the internal standard were added to 100 µl of plasma, followed by 500 µl of acetonitrile to precipitate proteins. The samples were vortex mixed for 2-3 seconds and centrifuged at 12,000*g* for 12 minutes (4ºC). The supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 30-40ºC. The residue was subjected to derivatization with 100 µl of *N*-methylimidazole solution in acetonitrile (1:1, v/v) and 150 µl of anhydrous trifluoroacetic solution in acetonitrile (1:2, v/v) and 100 µl were injected into the HPLC column. The derivatization reaction of ivermectin and moxidectin (internal standard) in acetonitrile was complete within 30 seconds at 25ºC. No sign of significant instability was observed for the fluorescent derivative in the reaction mixture.

Calibration curves

Detector linearity. Solutions of ivermectin in methanol at concentrations ranging from 0 to 16 ng/100 µl plasma were injected into the HPLC system in order to assess detector linearity. Peak height was plotted against the quantity of ivermectin injected. Ivermectin was linear ($r^2 > 0.995$) over the concentration range observed.

Plasma. Calibration curves were prepared by replicate analysis of ten plasma samples (100 µl each) spiked with various concentrations of ivermectin (20, 40, 80, 200, 400, 800, 1,000, 2,000, 4,000 and 8,000 ng/ml plasma) and a fixed concentration of the internal standard (5 ng/100 µl plasma). Samples were analysed as described above.

Data analysis. Concentrations of ivermectin were determined from the peak height ratios (peak height of ivermectin/peak height of internal standard), which corresponded to the known ivermectin concentrations in a calibration curve as described above. Peak detection, peak height integration, peak height ratio calculation, calibration curve fitting (least square regression without weighting) and calculation of sample concentrations were performed by the Millennium 2000 Chromatograph® software.

Method validation

Precision. The precision of the method based on within-day repeatability was determined by replicate analysis of six sets of samples spiked with ten different concentrations of ivermectin (20, 40, 80, 200, 400, 800, 1,000, 2,000, 4,000 and 8,000 ng/ml plasma). The reproducibility (day-to-day variation) of the method was validated using the same concentration range of plasma as described above, but only a single determination of each concentration was made on six different days. Coefficient of variation (CV) was calculated from the ratio of standard deviation (SD) to the mean and expressed as percentage.

Accuracy. Accuracy of the method was determined by replicate analysis of six sets of samples spiked at ten different levels of ivermectin (20, 40, 80, 200, 400, 800, 1,000, 2,000, 4,000 and 8,000 ng/ml plasma) and comparing the difference between the spiked value and that actually found.

Recovery. The analytical recovery of sample preparation procedure for ivermectin was estimated by comparing the peak heights obtained from samples (plasma) prepared as described above with those measured with equivalent amounts of ivermectin in methanol. Triplicate analysis was performed at the concentrations of 20, 200, and 4,000 ng/ml plasma for ivermectin and 5 ng/100 µl plasma for moxidectin.

Selectivity. The selectivity of the assay was demonstrated by checking for the absence of (i) endogenous interferences at the retention times of the derivatized products of ivermectin

and moxidectin in human blank plasma obtained from six different lots, and (ii) the interference by commonly used drugs and drugs that are likely to be coadministered with ivermectin (paracetamol, dimenhydrinate, praziquantel, albendazole and albendazole sulphoxide), after subjecting them to sample preparation procedures.

Limit of quantification. The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of ivermectin (in spiked plasma sample) that produced a peak height ten times the baseline noise at a sensitivity of 0.005 f.u.f.s. (fluorescence unit full scale) in a 100 µl sample, which also produced acceptable accuracy (<20% of the nominal values) and precision (expressed as the coefficient of variation, CV < 20%).

Stability. The stability of ivermectin was determined by storing spiked plasma samples (at the concentrations of 20, 200, and 4,000 ng/ml plasma; triplicate analysis for each concentration) in a -20ºC freezer (Sanyo, Japan) for six months. Concentrations were measured periodically (1, 2, 3 and 6 months). For freeze and thaw stability, samples were frozen at -20ºC for at least 24 hours and thawed at room temperature (25ºC). When completely thawed, the samples were transferred back to the original freezer and refrozen for at least 24 hours. The process was repeated three times.

Quality control. Quality control (QC) samples for ivermectin were made up in plasma using a stock solution separate from that used to prepare the calibration curve, at the concentrations of 20, 200, and 4,000 ng/ml plasma. Samples were aliquoted into cryovials and stored frozen at -20ºC for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

Application of the method to biological samples. The method was applied to the investigation of plasma concentration-time profile of ivermectin in five healthy Thai volunteers (aged 20-25 years, weighing 50-54.5 kg) following a single oral dose of 200 µg ivermectin/kg body weight. Written informed consent was obtained from the volunteer prior to study participation. Venous blood samples (3 ml) were collected into heparinized-coated plastic tubes at the following time points: 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 48, 60, 72, 84, 96, 108, 120, 144 and 168 hours of dosing. Plasma samples were obtained through centrifugation at 1,500*g* for 15 minutes and stored at -70ºC until analysis.

RESULTS

Chromatographic separation

A number of HPLC chromatographic systems were investigated to optimise the separation of ivermectin and the internal standard (moxidectin). Retention maps were generated for both compounds as a function of stationary phase (the Hypersil Gold C18, MicroBondapak C18, Supelcosil C18, and C8 reversed phase column) and mobile phase. The Hypersil Gold C18 C18 column was found to produce the best peaks with relatively short run time compared to the others. For the elution solvents, the mixture of either acetic acid (0.2% in distilled water), methanol and acetonitrile, or acetonitrile, tetrahydrofuran and distilled water, or distilled water and acetonitrile resulted in similar capacity factors (K') of the internal standard (1.0-2.0) and ivermectin (1.5-3.0). The elution solvent consisting of acetonitrile, methanol and distilled water at the ratio of 50:45:5 (v/v/v) was chosen as an appropriate elution solvent as it resulted in optimal separation. The retention time of the internal standard and ivermectin was approximately 3.7, and 7.0 minutes, respectively. The

chromatograms showed a good baseline separation. However, the possibility that ivermectin peak may contain co-eluting metabolites cannot be excluded. Chromatogram of standard solution of internal standard and ivermectin is shown in Fig 3.

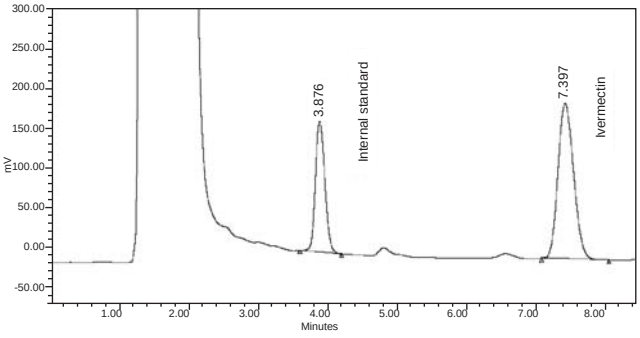


Fig 3–Chromatogram of a standard solution of internal standard (5 ng) and ivermectin (20 ng), with retention times of 3.7 and 7.0 minutes, respectively.

Fluorogenic derivatization and sample preparation

Sample preparation (100 µl plasma) was done by a single step protein precipitation with acetonitrile, followed by derivatization with 100 µl of *N*-methylimidazole solution in acetonitrile (1:1, v/v) and 150 µl of trifluoroacetic anhydrous solution in acetonitrile (1:2, v/v). Derivatization conditions to produce fluorescent product that were reported previously include those using acetic anhydride and pyridine (catalyst) (Tolan *et al*, 1980), as well as dimethylformamide and *N*-methylimidazole (catalyst) (Tway *et al*, 1981; de Montigny *et al*, 1990; Lin and Matuszewski, 1995; Fink *et al*, 1996). The former reaction conditions however, require extensive sample preparation before and after derivatization with a long reaction period of 24 hours (Tolan *et al*, 1980). Although the detectability and selectivity for the determination of ivermectin after chemical derivatization and fluorometric detection have been greatly enhanced when compared to direct UV detection of the underivatized molecule, it usually requires extensive and time-consuming sample clean up steps (SPE) before and after the derivatization (double extraction procedure, *eg* involving additional silica, florisil or diol SPE following the first liquid-liquid or SPE extraction) (Alvinerie *et al*, 1981,1987, 1993; Schnitzerling and Nolan, 1985; de Montigny *et al*, 1990). In the present procedure, no sample clean-up procedure was required following the derivatization step with trifluoroacetic acid and tri-methylimidazole and therefore short and simple analysis time could be obtained.

Chromatograms of blank plasma, plasma spiked with ivermectin at concentrations of 20 and 2,000 ng/ml (with a fixed concentration of internal standard of 5 ng/100 µl plasma) and plasma collected from a volunteer after a single oral dose of 200 mg ivermectin/kg body weight are shown in Fig 4.

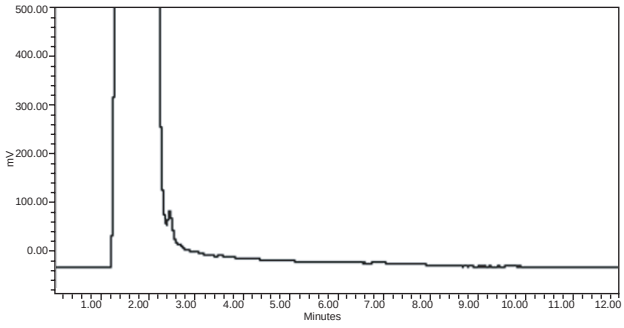
Calibration curves

Plasma analysis was calibrated using concentration range of 0-8,000 ng/ml plasma. All calibration ranges yielded linear relationships with correlation coefficients of 0.995 or better. The linear regression equation obtained from the mean of the six calibration curves was $y = 1.17 (\pm 0.02) x - 1.12 (\pm 0.04)$, where y is the peak height ratio and x is the analyte concentration in ng/ml.

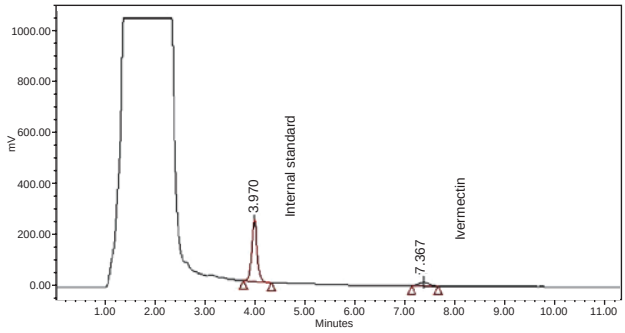
Method validation

Precision. Little variation of ivermectin assays was observed; coefficients of variation (CV) for six analysis at the concentration range observed were all below 15%. The intra-assay (within-day) and inter-assay (day-to-day) variation for ivermectin assay at the concentration range 0-8 ng/100 µl plasma are summarized in Table 1. Intra- and inter-day assay variation varied between 0.4 and 7.1%, and 0.5 and 11.3% (% CV), respectively.

Accuracy. Good accuracy was observed for both the intra-day or inter-day assays, as indicated by the minimal deviation of mean val-



(a)



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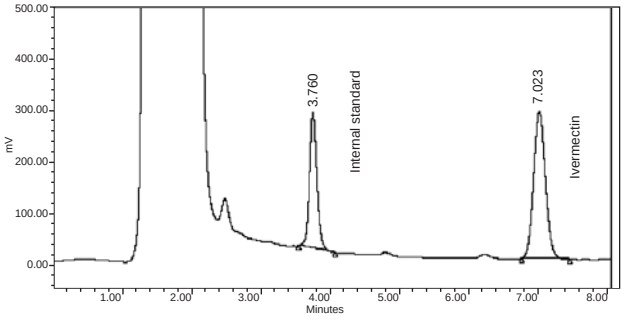
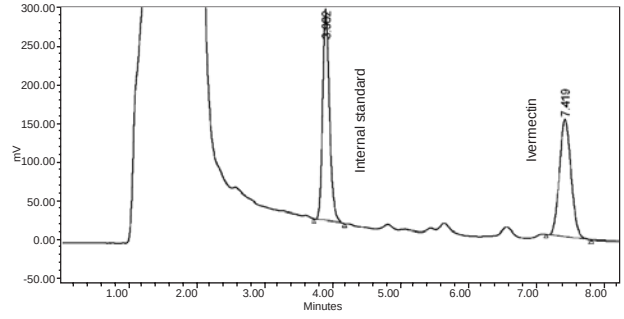


(c)



(d)

ues found with measured samples from that of actual amount added. The intra-assay (within-day) and inter-assay (day-to-day) accuracy for ivermectin assay at the concentration range 0-8 ng/100 µl plasma are summarized in Table 1. Intra- and inter-day assay accuracy, expressed as the mean deviation from the actual amounts varied between -9.0 and +9.0%, and -12.0 and +13.0%, respectively.

Recovery. The mean recoveries for ivermectin in plasma at the concentrations of 20, 200 and 4,000 ng/ml plasma including the internal standard (5 ng/100 µl plasma) in all cases were greater than 93%. The results reflect essentially 100% recovery from the spiked plasma and indicate lack of interference from the sample preparation procedure.

Selectivity. Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks in plasma at the retention times of the internal standard and ivermectin (3.7 and 7.0 minutes, respectively). Fig 4 (a, b, c) illustrates typical chromatograms for blank plasma and plasma spiked with ivermectin and internal standard.

Limit of quantification. The limit of quantification (LOQ) in human plasma for ivermectin was accepted as 0.02 ng using 100 µl of plasma.

Stability. Plasma samples containing ivermectin at concentrations of 20, 200,

Fig 4–Chromatogram of (a) blank plasma, (b) plasma spiked with 20 ng (with 5 ng internal standard per 100 µl plasma), (c) plasma spiked with 2,000 ng/ml ivermectin (with 5 ng internal standard per 100 µl plasma) and (d) plasma collected from a volunteer at 2 hours after a single oral dose of 200 µg ivermectin/kg body weight.

and 4,000 ng/ml plasma were found to be stable when stored in a -20ºC freezer for a minimum of six months without significant decomposition of the drug. Long-term storage of the spiked samples for up to 6 months did not appear to affect the quantification of the analytes (Table 2a). Mean deviation (%) of measured concentrations after storage at the observed periods (1, 2, 3 and 6 months) varied between -4.0 and +4.4%. Freezing and thawing for three successive cycles did not affect the measured concentrations. Mean deviation from the theoretical values varied between +1.7 to + 2.0% (Table 2b).

Quality control. Three validated analysts conducted the plasma analysis. A standard curve and quality control specimens were included with each analysis. Control samples with nominal concentration of 20, 200 and 4,000 ng/ml plasma ivermectin were analyzed at the beginning and the end of the analytical run. All results were within the acceptable limit (± 20% of their respective nominal values).

Application of assay and analysis of specimens

To demonstrate the clinical applicability of the method, plasma concentration levels of ivermectin were determined in 5 healthy Thai volunteers following a single oral dose of 200 µg ivermectin/kg body weight. Plasma concentration-time profiles of ivermectin are shown in Fig 5.

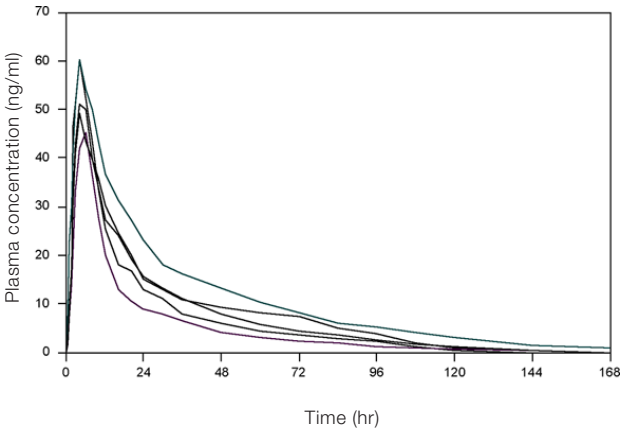


Fig 5–Plasma concentration-time profiles of ivermectin in five healthy Thai volunteers following a single oral dose of 200 µg ivermectin/kg body weight.

Table 1
Summary of assay precision and accuracy (intra- assay and inter-assay) for ivermectin assay in plasma.

Concentration added (ng/ml plasma)	Precision (%CV)		Accuracy (%DMV)[a]	
	Intra-assay (N=6)	Inter-assay (N=6)	Intra-assay (N=6)	Inter-assay (N=6)
20	7.1	11.3	0.0+	+13.0
40	0.4	0.5	0.8-	-6.1
80	4.1	7.3	0.6-	+7.2
200	4.9	7.1	1.7-	-7.7
400	4.0	4.4	1.6-	+4.4
800	3.4	6.6	0.4-	+7.1
1,000	9.4	13.5	6.7-	-12.0
2,000	4.8	10.2	2.5-	+10.4
4,000	4.6	8.1	9.0+	+8.2
8,000	3.8	5.2	9.0-	+5.3

[a] %DMV = deviation of mean value from theoretical value (%)

Table 2
Storage stability data of ivermectin in plasma at concentrations 20, 200, and 4,000 ng/ml plasma.

(a) Long-term stability at 1, 2, 3 and 6 months

Time period(month)	Concentration(ng/ml plasma)	Mean (SD)(N=3)	% DEV[a]
1	20	20 (2.5)	-4.0
	200	200 (10.1)	+0.3
	4,000	3,970 (13.2)	+0.7
2	20	20 (2.1)	+3.8
	200	200 (7.5)	+0.5
	4,000	3,970 (6.8)	+0.9
3	20	20 (3.2)	0.0
	200	200 (10.2)	+0.3
	4,000	4,120 (13.3)	-3.1
6	20	20 (1.8)	-2.3
	200	200 (1.7)	-1.7
	4,000	3,820 (14.2)	+4.5

[a] %DEV = deviation of single value from theoretical value (%)

(b) Freeze and thaw stability

Concentration Added (ng/ml plasma)	Mean (SD) (N=3)	% DMV[a]
20	20 (1.5)	+2.0
200	200 (2.8)	+2.2
4,000	3,930 (27.2)	+1.8

[a] %DMV = deviation of mean value from theoretical value (%)

DISCUSSION

We have described in this report, a simple, sensitive, selective and reproducible HPLC method with fluorescence detection for determination of ivermectin in plasma. The method is based on a single step protein precipitation with acetonitrile, followed by derivatization of ivermectin with trifluoroacetic anhydride and *N*-methylimidazole. No sample clean-up step after derivatization is required. Although HPLC with MS or MS/MS detection is highly sensitive and allows detection of ivermectin without derivatization following a simple and rapid sample preparation step, the instruments are expensive and not yet readily obtainable in most laboratories. The present method could be used as an alternative method to LC/MS or LC/MS/MS. Its advantages over the previously reported HPLC methods lie with the combination of the following performances: (i) improved sensitivity using small volume of plasma sample (LOQ 0.02 ng using 100 µl plasma); (ii) shorter analysis time (no sample clean-up step is required); and (iii) simple and inexpensive sample preparation procedure (no SPE is required). The analytical method for the determination of ivermectin in plasma established in this study meets the criteria for application to routine

clinical drug level monitoring or pharmacokinetic study.

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