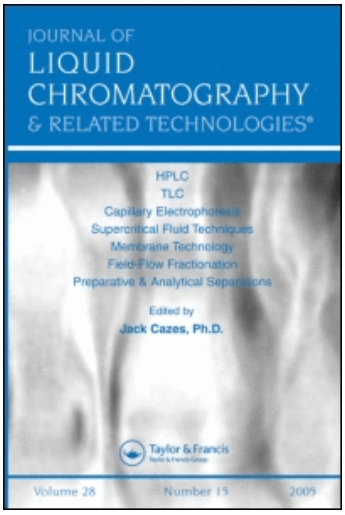

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Robotic Sample Preparation and HPLC Determination of the Major Component of Ivermectin in Human Plasma

J. Y. -K. Hsieh[a]; L. Lin[a]; W. Fang[a]; B. K. Matuszewski[a]
[a] Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania, USA

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Robotic Sample Preparation and HPLC Determination of the Major Component of Ivermectin in Human Plasma

J. Y.-K. Hsieh,* L. Lin, W. Fang, and B. K. Matuszewski

Department of Drug Metabolism, Merck Research Laboratories, West Point, Pennsylvania, USA

ABSTRACT

A robotic sample preparation method combined with high performance liquid chromatography (HPLC)-fluorescence detection for the determination of the major component of ivermectin in human plasma is presented. A Cyberlab C-300 workstation equipped with customized software and hardware was utilized to perform all the semi-automated liquid–solid phase extraction (LSPE) steps including acetonitrile (ACN) and water addition, sample loading, and hexane elution on Chem Elut™ cartridges. Under the automated pre-column derivatization conditions, both ivermectin and internal standard of the extracted samples were chemically converted to highly fluorescent derivatives and then monitored by a fluorescence detector. The assay was validated in the concentration range of 0.5–40 ng/mL, using 1 mL of human plasma. The application of

*Correspondence: J. Y.-K. Hsieh, Department of Drug Metabolism, Merck Research Laboratories, West Point, PA 19486, USA; E-mail: john_hsieh@merck.com.

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this assay was demonstrated by analysis of plasma samples from human subjects receiving oral doses of ivermectin.

Key Words: Robotic sample preparation; HPLC; Ivermectin; Automated pre-column derivatization.

INTRODUCTION

Ivermectin, 22,23-dihydroabamectin or 22,23-dihydroavermectin B_1 (Fig. 1, **I**), is a semi-synthetic derivative of abamectin containing at least 80% of 22,23-dihydroavermectin B_{1a} and not more than 20% of 22,23-dihydroavermectin B_{1b}. Ivermectin has broad-spectrum activity against different life-cycle stages of many, but not all, gastrointestinal and extraintestinal nematodes and arthropod parasites infecting humans.[1] It is active against the tissue microfilariae of onchocerca volvulus, but not against the adult form; its activity against strongyloides stercoralis is limited to the intestinal stages. Ivermectin has also been found to be moderately to highly effective against all human intestinal nematode parasites, except for hookworm. Due to its excellent safety profile, the clinical use of ivermectin for the treatment of head lice[2,3] has been reported.

Bioanalytical methods described for the determination of ivermectin in human and animal biological fluids involved enzyme-linked immunosorbent assay,[4] thin-layer chromatographic (TLC)[5] or high performance liquid chromatographic (HPLC) methods coupled to ultraviolet (UV) detection,[6–11] or fluorometric detection[12–22] of the derivatized product. The sample clean up and concentration was based on immunoaffinity columns,[11] liquid–liquid extraction[8,15,22] combined with, or solid-phase extraction (SPE),[10,12–17,21] and on-line SPE.[19] In spite of its high sensitivity (0.3 ng/mL in animal serum samples), enzyme-linked immunosorbent assay had a narrow calibration range of 0.3–10 ng/mL and possible cross-reactivities against metabolite(s) presented in the ivermectin-dosed biological samples. Generally, TLC or HPLC methods with UV detection of underivatized ivermectin did not provide the required sensitivity (1 ng/mL or lower) and selectivity for clinical plasma analyses. In most HPLC–UV detection methods, large sample volume (3–5 mL) and lengthy sample clean up procedures were required to achieve the sensitivity of low nanograms per milliliter. Since ivermectin does not possess strong chromophores for UV or fluorescence detection, it has to be chemically modified to enhance its detectability and selectivity to achieve the lower limit of quantitation (LLOQ) of <1 ng/mL, using 1 mL or less volume of biological samples. The conversion of ivermectin or its analogs to highly fluorescent derivatives (Fig. 2) using the dehydration reagent [acetic

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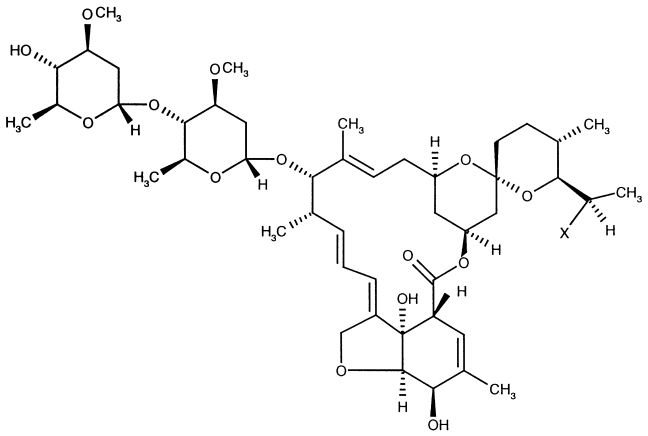
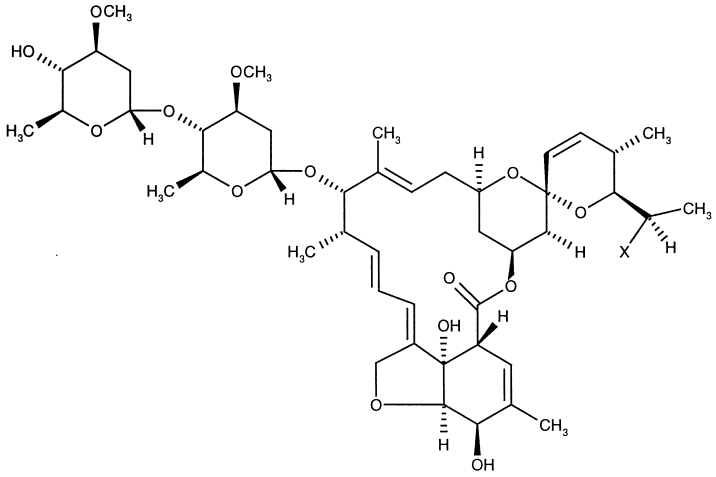


I



IS

B_{1a}: X = C_2H_5 (major component)
B_{1b}: X = CH_3 (minor component)

Figure 1. Chemical structures of ivermectin (**I**) and internal standard (**IS**).

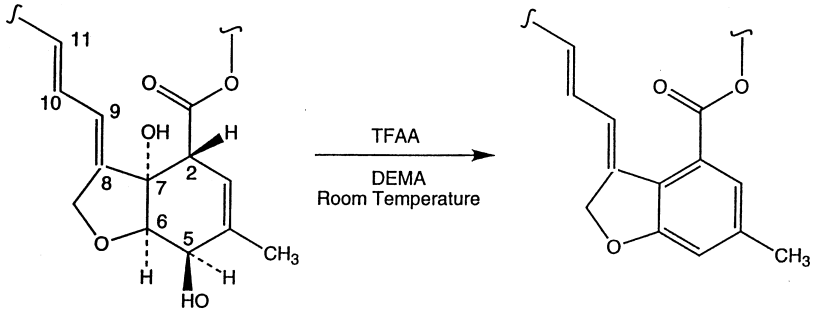


Figure 2. Fluorescent derivatization of ivermectin or its analogs (partial structure) with trifluoroacetic anhydride (TFAA) and *N*,*N*-diethyl methylamine (DEMA).

or trifluoroacetic anhydride (TFAA)] and different organic base catalysts (pyridine, 1-methylimidazole or trialkylamines) has been extensively studied in our[23] and other laboratories[14,16,20] to establish the best reaction conditions for derivatization of ivermectin or its analogs at pg/mL concentration.

Although, the detectability and selectivity of the determination of ivermectin after chemical derivatization and fluormetric detection has been greatly enhanced by a factor of 5–20 times, when compared to direct UV detection of the underivatized molecule, it usually required extensive and time-consuming sample clean up steps before and after the derivatization. These steps included double extraction procedures, such as an additional silica, florisil, or diol SPE following the first liquid–liquid or SPE extraction. It was highly desirable to eliminate these tedious and time-consuming sample preparation steps, especially in the clinical sample analysis laboratory environment. Automated on-line derivatization methods for ivermectin, or it analogs, have been reported[16,20,23] to eliminate the clean up procedure required after the derivatization, thus, increasing sample throughput and more constant derivatization yields. However, carefully controlled derivatization and automix conditions[23] should be throughly investigated in order to minimize the problem associated with the potential chemical instability of the reaction products with high concentration of dehydration and organic base reagents.

A more efficient, and less labor-intensive, means of preparing biological samples prior to analysis, evolved with the emergence of laboratory robotics in the early 1980s. It has been shown, that laboratory robotics[23–29] are adaptable to perform steps such as liquid–liquid and liquid–solid phase extraction (LPSE), which are generally required to isolate analytes from the biological matrices. Out of 20 bioanalytical methods for the determination of ivermectin or avermectin analogs in biological fluids, only two methods utilized Zymark Model Z100 robots[8] and Gilson ASPEC™ XL systems[23] to automate SPE

procedures for increasing assay efficiency and sample throughput. Due to single probe and serial (one sample was processed at a time) operations, both methods only achieved moderate increases in the total sample preparation throughput, e.g., less than 40 samples were prepared in an overnight run (16-hr). A semi-automated method[29] for the quantification of several investigational drugs in human plasma and urine using a Cyberlab C-300 workstation equipped with four probes, has been developed and demonstrated in our laboratories with equal or improved accuracy and precision, and increased sample throughput than manual procedures. In order to provide bioanalytical support for a large ivermectin clinical study, a sensitive, high throughput, and validated assay was needed and was developed. The assay was based on the determination of the major component of ivermectin (**I-B_{1a}**, Fig. 1) and involved three steps: (1) isolation of ivermectin and internal standard from human plasma; (2) derivatization to form strongly fluorescent derivatives; and (3) HPLC separation and fluorescence detection of the resulting derivatives.

The detailed description of the assay methodology and its application to the analyses of human plasma samples after oral dosing of human subjects with ivermectin is the subject of this paper.

EXPERIMENTAL

Materials and Chemicals

Ivermectin and internal standards (**IS**, Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ, USA). The standard of **I** was composed of ~80% of **I-B_{1a}** and ~20% of **I-B_{1b}**. Acetonitrile (ACN, HPLC-grade) and hexane (optima-grade) were purchased from Fisher Scientific (Spring Field, NJ). Trifluoroacetic anhydride, *N*,*N*-diethyl methylamine (DEMA) were obtained from Aldrich (Milwaukee, WI, USA). Heparinized human control plasma was supplied by Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received. Liquid–solid phase extraction was performed on Varian Chem Elut™ cartridges (3 mL, Walnut Creek, CA, USA).

Apparatus and Chromatographic Conditions

Cyberlab Model C-300 robotic liquid handling system (Brookfield, CT, USA) was used to perform liquid transfers of ACN, water, hexane, and plasma supernatant after protein precipitation. The system, along with customized software, included a power controller, two heads equipped with 4-channel non-expandable fixed probes and one head equipped with 4-channel expandable

disposable tips, six 16-tube centrifuge racks, two Chem Elut™ cartridge racks customarily made with similar spacing to Zymark TuboVap racks (Hopkton, MA, USA), tip box and disposal station, two wash stations, a multi-solvent reservoir station, a solvent switching station, and workstations for autosampler vials and 96-well plates. The layout of the Cyberlab C-300 robotic system used in the study, is shown in Fig. 3. The chromatographic system consisted of a Perkin-Elmer (Norwalk, CT, USA) LC Series 200 pump and a Model LC240 fluorescence detector. The analog output from the detector, was connected to a PE-Nelson (San Jose, CA, USA) Access*Chrom data acquisition system via a PE-Nelson Model 941 interface. An excitation wavelength of 365 nm and an emission wavelength using cut-off filters of 460 nm were used for fluorescence detection. The analytical column (25 cm × 4.6 mm, i.d.) was packed with Keystone BDS Hypersil C_{18} (5 μm, College Park, PA, USA) and protected by a SSI column inlet filter (1.5 mm × 0.5 μm, State College, PA, USA) and a Keystone BDS Hypersil C_{18} guard column (5 μm, 2 cm × 4.6 mm, i.d.). The mobile phase was prepared by mixing 5% (by volume) of deionized water and 95% of ACN, and delivered through the HPLC column at 35°C with a flow-rate of 0.9 mL/min.

Preparation of Standard Curve and Quality Control Samples

A stock solution of ivermectin (1 mg/mL), as a mixture of **I-B_{1a}** and **I-B_{1b}**, was prepared in ACN. Appropriate dilutions of the stock solution were made with 60% ACN in water (v/v) and the working standard solutions were used to spike into 1 mL of human control plasma at final concentrations of 0.5, 1.0, 2.5, 5.0, 10, 20, and 40 ng/mL. An additional stock standard, independent from those used for preparing working standards, was used to make quality control (QC) samples. Plasma QC samples were prepared separately in pools of 200 mL at final concentrations of 1.25, 7.5, and 25 ng/mL. All the QC samples were stored, along with subject samples, at −20°C until the time of analysis. A stock solution of internal standard (**IS**) was prepared at 100 μg/mL and further diluted to 100 ng/mL with a mixture of ACN and water (60 : 40, v/v). One hundred microliters of the working **IS** solution was pipetted into a standard curve, QC, and unknown subject plasma samples.

Clinical Sample Collection and Robotic Liquid–Solid Phase Extraction

Plasma samples were collected from healthy male subjects who received different oral doses (30, 60, 90, 120 mg) of ivermectin. Blood samples were collected at time intervals, as specified in the protocol. After separation, plasma samples were stored at −20°C until the time of analysis.

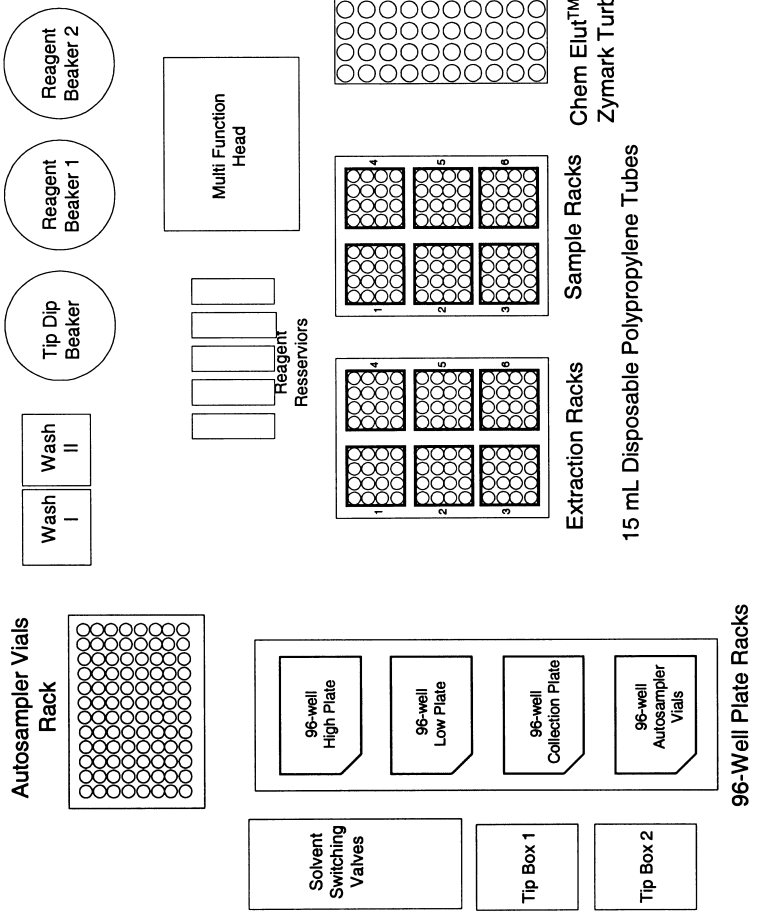


Figure 3. Layout of the Cyberlab C-300 robotic liquid handling system.

Subject and QC samples were thawed to room temperature and vortex-mixed vigorously for 15 seconds on a vortex-mixer. One hundred–1000 μL of subject plasma diluted with 900–0 μL of human control plasma (total final volume equal to 1 mL for each sample) and 100 μL of **IS** (100 ng/mL) were pipetted into a disposable polypropylene tube (15 mL) prior to sample extraction. The Cyberlab C-300 system executed protein precipitation (a), sample dilution (b), and LSPE (c–e) steps as follows:

a. Addition of 1 mL of ACN.*
b. Addition of 1.5 mL of water.*
c. Aliquoting of 2.3 mL of the supernatant after centrifugation at $2000 \times g$ for 10 min.
d. Loading the supernatant onto Chem Elut™ cartridge and waiting for 5 min.
e. Addition of 3×5 mL of hexane eluent.

*(Mixture was manually vortex-mixed for 15 seconds after each addition.)

After the above sample extraction steps were completed, the combined eluents were evaporated to dryness at 50°C, manually dissolved in 300 μL of 25% DEMA in ACN (v/v), and were automatically, as detailed in the flow chart presented in Fig. 4, pre-column derivatized with 100 μL of 50% TFAA in ACN (v/v) using Perkin-Elmer ISS 200 autosampler. After on-line derivatization, 30 μL of the reaction mixture was injected directly onto the HPLC system.

Data Acquisition and Analysis

Peak heights for **I** and **IS** derivatives were acquired and automatically processed using a PE-Nelson Access*Chrom laboratory automation system. Concentrations of **I** in study samples were calculated from the daily least-squares linear regression of peak height ratios (**I-B_{1a}/IS-B_{1a}**) vs. standard concentrations of **I** with reciprocal weighing on the concentrations. When a calculated concentration exceeded the standard curve range, the sample was diluted and reanalyzed.

RESULTS AND DISCUSSION

System and Liquid Solid Phase Extraction Characteristics

The laboratory unit operations (LUO), such as liquid aspirating and dispensing that were used in this assay, have been tested and validated by a weighing procedure.[29] The validation data for several LUOs are shown in

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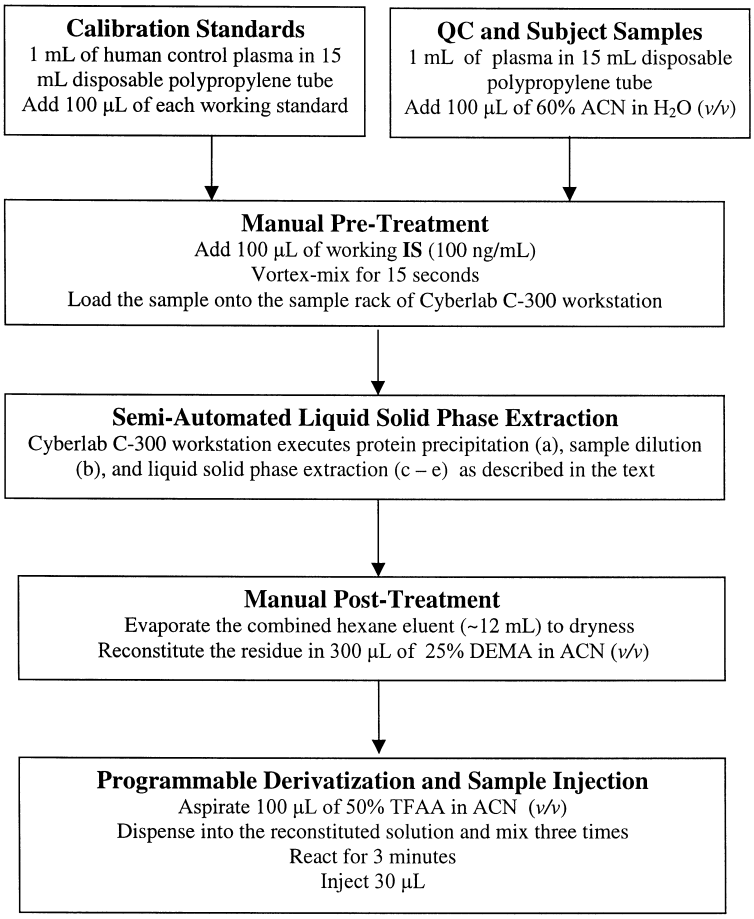

Figure 4. Flow chart of sample preparation, extraction, and derivatization.

Table 1, indicating acceptable precision and accuracy of all the pipetting operations utilized.

The Cyberlab C-300 workstation was equipped with three independent pipetting tools on the same head: four variable-spacing disposable tips for non carry-over transfers of human plasma and its supernatant after ACN protein precipitation, and two fixed-spacing subheads, each with four probes for handling large volume (5 mL or larger) of different organic eluents. Another important feature of the customized hardware included six square-shape aluminum racks, with each rack holding sixteen 15-mL tubes in the 4×4

Table 1. Validation of the laboratory unit operations (LUO).

Laboratory unit operations	% Coefficient of variation/accuracy[a] (%) $n = 12$
Dispensing 1 mL of water using four fixed probes and four 10 mL syringes	0.8/105.2
Dispensing 3 mL of water using four fixed probes and four 10 mL syringes	0.7/104.5
Dispensing 5 mL of water using four fixed probes and four 10 mL syringes	1.0/104.0
Aspirating and dispensing 0.2 mL of water using four disposable tips and four 2.5 mL syringes	1.8/99.5
Aspirating and dispensing 0.5 mL of water using four disposable tips and four 2.5 mL syringes	1.9/100.1
Aspirating and dispensing 1 mL of water using four disposable tips and four 2.5 mL syringes	1.5/101.4

[a]Expressed as $\left[\frac{\text{Mean determined volume}}{\text{Nominal volume}}\right] \times 100$.

format. The benefit of using each customized rack is that 16 sample tubes can be processed as a single batch through vortexer, and moved in and out of centrifuge buckets in a faster, more organized and less-error prone way. In addition to the fast processing of four samples, simultaneously, and more steps being automated in an unattended fashion, this semi-automated LSPE method using Chem Elut^{TM} cartridges greatly simplified the sample extraction procedure by simply loading plasma samples onto the Cyberlab system, and then eluting **I** and **IS** from the cartridge, directly, as opposed to more steps required for the conventional SPE that included loading, conditioning, and washing of multi-solvents, and elution with vacuum for solution flow between each step.

Sensitivity/Lower Limit of Quantification/Linearity

The lower limit of qualification, defined as the lowest concentration on the standard line for which acceptable accuracy (<10% of the nominal values) and precision (expressed as the coefficient of variation, CV < 10%) were obtained, was 0.5 ng/mL. This assay has been validated over the entire concentration range of 0.5–40 ng/mL. The linearity was confirmed by plotting the peak height ratios of **I-B$_{1a}$** to **IS-B$_{1a}$** vs. **I** concentrations. The unknown **I** concentrations were calculated from the equation $y = mx + b$, as determined by weighted $(1/y)$ linear least-square regression of the calibration line.

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Assay Selectivity

The selectivity of the assay was demonstrated by the absence of endogenous interferences at the retention time of derivatized **I** or **IS** in any of the human control (from five different lots) and pre-dose subject plasma samples. The total analysis time was 16 min. All components of the **I** and **IS** derivatives were baseline separated with the retention times of 14.5 and 10.2 min for **I-B_{1a}** and **IS-B_{1a}**, respectively. Representative chromatograms of human control human plasma, human control plasma spiked with **I** and **IS** (0.5 and 10 ng/mL, respectively), and a subject's post-dose plasma sample are shown in Fig. 5.

Intra-day Precision and Accuracy

An assessment of the intra-day variability of the assay was conducted in five different lots of human control plasma spiked with the analyte, over the calibration range of 0.5–40 ng/mL. The resulting assay precision and accuracy data are presented in Table 2. The intra-day precision of the assay, as measured by the coefficient of variation (% CV), was better than 5.9% for all points on the calibration curves. Assay accuracy was found to be within 2% of the nominal for all standards.

Inter-day Variability

Plasma QC samples at low (1.25 ng/mL), middle (7.5 ng/mL), and high (25 ng/mL) concentrations for **I** were prepared prior to the start of the study, and subjected to replicate within-day analyses ($n = 5$). Quality control samples were kept frozen at -20°C during the course of the study. Results are presented in Table 3. Two high (HQC), two middle (MQC), and two low (LQC) QC samples were analyzed daily, along with clinical samples, to assess inter-day variability. Results presented in Table 3, indicate that the intra-day variability of the assay was less than 4.2% CV and accuracy was in the range of 104.4–105.6% of the nominal standard concentration. The inter-day variability was determined by analyzing QC samples daily at the same three concentrations, in 31 separate runs, over a period of three months. Inter-day precision and accuracy (percentage deviation from nominal values) were below 7.9% CV from -0.8 to $+4.7$%, respectively.

Extraction

Recovery of the LSPE procedure for **I** and **IS** was determined by comparing the peak heights of standards spiked into human control plasma, extracted and derivatized as described in the Experimental section, to the peak heights of neat

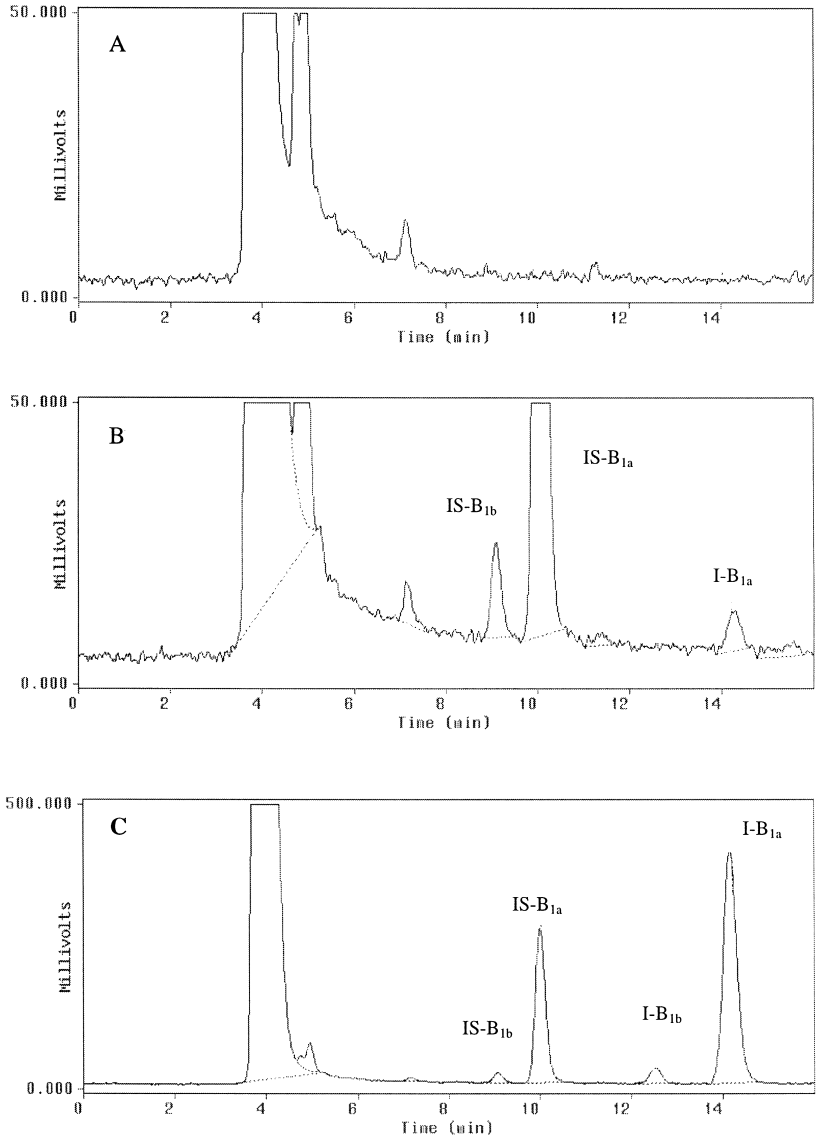

Figure 5. Representative HPLC chromatograms of: (A) human control plasma; (B) human control plasma spiked with 0.5 ng/mL of **I** and 10 ng/mL of **IS**; (C) subject's plasma (25.4 ng/mL) 12-h postdose after oral administration of 120-mg of **I** and spiked with 10 ng/mL of **IS**.

Table 2. Intra-day precision and accuracy data of the replicate analyses ($n = 5$) of **I** in human control plasma.

Nominal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy[a] (%)	Precision[b] (CV %)
0.5	0.51	102.0	4.9
1	1.02	101.8	2.7
2.5	2.48	99.0	3.7
5	4.97	99.4	2.2
10	9.86	98.6	4.0
20	19.9	99.3	3.1
40	40.4	101.1	5.9

[a]Expressed as $\left[\frac{\text{Mean determined concentration}}{\text{Nominal concentration}}\right] \times 100$ ($n = 5$).
[b]Coefficient of variation.

standards derivatized and injected directly into the HPLC system. A dilution factor (2.3/3.5) was also taken into consideration for calculating the extraction recoveries of both analyte and internal standard. The average recoveries for 1, 5, and 20 ng/mL were 94.1%, 102.9%, and 104.3%, respectively. The extraction recovery for **IS** at a concentration of 10 ng/mL was 99.6%.

Table 3. Intra-day and inter-day analysis of plasma quality control (QC) samples containing **I**.

Quality control nominal concentration	LQC (1.25 ng/mL)	MQC (7.5 ng/mL)	HQC (25 ng/mL)
Intra-day			
Initial mean ($n = 5$) determined concentration	1.32	7.89	26.10
Accuracy[a] (%)	105.6	105.2	104.4
Precision[b] (%)	4.2	1.7	1.7
Inter-day			
Mean ($n = 31$) determined concentration	1.24	7.89	25.64
Accuracy[a] (%)	99.2	104.7	102.6
Precision[b] (%)	7.9	6.1	4.7

[a]Expressed as $\left[\frac{\text{Mean determined concentration}}{\text{Nominal concentration}}\right] \times 100$.
[b]Coefficient of variation.

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Freeze-Thaw Stability

Freeze-thaw stability was examined by subjecting QC samples to three freeze-thaw cycles with freezer nominal temperature of −20°C. By comparing the initial mean values at three different (1.25, 7.5, and 25 ng/mL) concentrations of QC samples, after one freeze-thaw cycle, to the similar mean values, after two and three freeze-thaw cycles, the effect of freeze-thawing on the stability of **I** in human plasma was determined. There were no significant differences (99.0–103.8% of the initial mean analyzed concentrations, $n = 5$) in the assay concentrations following three freeze-thaw cycles, thus, indicating analyte stability through at least three freeze-thaw cycles.

Analysis of Clinical Samples

The applicability of this semi-automated HPLC assay was demonstrated by analyzing more than 1200 human plasma samples from a large clinical study. The mean plasma concentration vs. time profiles obtained from 12 healthy subjects, followed 30-mg of ivermectin under both fasted and fed administration, are shown in Fig. 6.

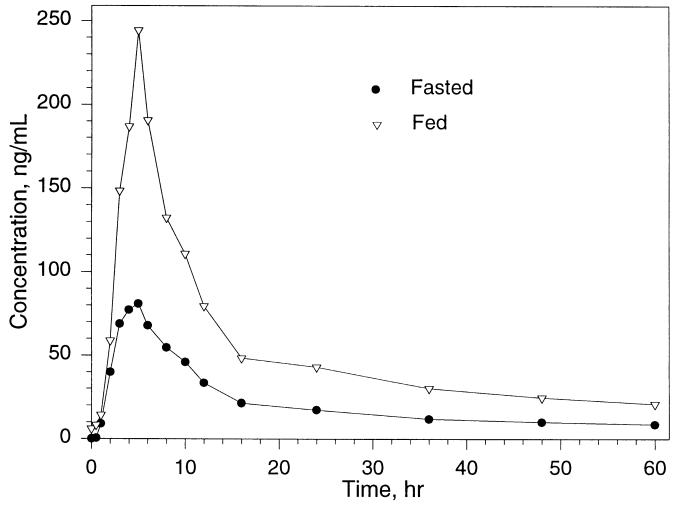

Figure 6. Mean plasma concentration (ng/mL) profiles of **I** of 12 subjects following single oral dose (fed and fasted administration) of 30 mg-ivermectin.

CONCLUSION

In conclusion, the assay for quantifying **I** in human plasma, using a Cyberlab C-300 workstation for ACN protein precipitation and LSPE of analyte and internal standard on Varian Chem Elut™ cartridges, followed by HPLC with fluorescence detection, has been developed for use in support of a clinical study. The method was fast, selective, precise, and accurate, and was validated in a concentration range of 0.5–40 ng/mL. Using this robotic sample preparation and extraction method, a minimum of 80 plasma samples were prepared and analyzed over a 24-h period in comparison to 24 animal samples robotically prepared and analyzed for the same period,[8] or 60 human plasma samples manually prepared and analyzed over a 50-h period.[14]

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