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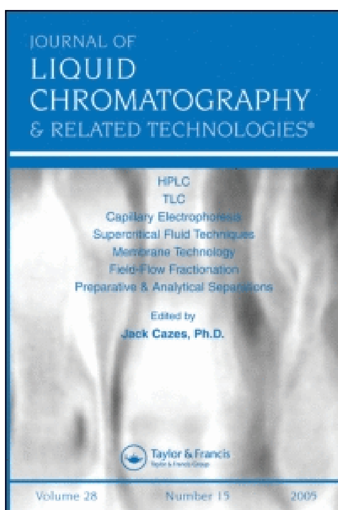

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PICOGRAM DETERMINATION OF AN AVERMECTIN ANALOG IN DOG PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION†

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ABSTRACT

A sensitive and automated method for the determination of a new avermectin analog (MK-324) in dog plasma has been developed and validated. The drug and internal standard were extracted from plasma via solid-phase extraction using an automated Gilson ASPEC XL system. The extracts were evaporated to dryness and reconstituted in a mixture of N,N-diethylmethylamine and acetonitrile. Automated pre-column derivatization of the reconstituted extracts using trifluoroacetic anhydride was performed prior to injection onto the HPLC system. The fluorescent derivatives of the drug and internal standard were chromatographed using a Zorbax RX-C18 column and a mobile phase composed of acetonitrile, water and tetrahydrofuran [6:1:1 (v/v/v)]. The assay was validated in the concentration range of 100 - 5,000 pg/mL. Its applicability, inter-day performance and specificity were demonstrated by analyzing plasma samples from six studies with MK-324.

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INTRODUCTION

The avermectins are a group of closely related 16-membered macrocyclic lactones.[1] Two of the members of this family of compounds, ivermectin[2] and abamectin,[2] were introduced as an antiparasitic drug and an agricultural pesticide, respectively. Their worldwide acceptance in the health care of animals and crop protection has made them major commercial successes. MK-324, a new avermectin, is a mixture of two homologous compounds, 22,23-dihydro-13-O-[(2-methoxyethoxy)methyl]avermectin-B_{1a}-aglycone (major component, >90%) (I, Fig. 1) and -B_{1b}-aglycone (minor component, <10%) (II). These two compounds differ by a single methylene group. MK-324 is a substituted ivermectin aglycone.[3,4]

To support studies designed to determine the potency of MK-324, there was a need for the determination of the drug in plasma at pg/mL concentrations to support a number of MK-324 studies in dogs. In addition, a large number of plasma samples needed to be analyzed and automation of some of the sample preparation steps was considered highly advantageous. The use of laboratory automation accessories was previously shown to be a viable alternative to manual sample preparation.[5,6] In order to determine plasma concentrations of MK-324 at pg/mL concentrations, a highly sensitive, automated, and validated assay was required and was developed. The assay was based on the determination of the major component of MK-324 (I, Fig. 1), and involved three steps:

(1) isolation of I from dog plasma; (2) derivatization to form a strongly fluorescent derivative; and (3) HPLC separation and fluorescence detection of the resulting derivative.

The detailed description of the assay methodology and its application to the analyses of dog plasma samples after dosing with MK-324 is the subject of this paper.

EXPERIMENTAL

Chemicals and Reagents

MK-324 (Fig. 1) and its analogue (IS, Fig. 1), used as an internal standard, were synthesized at the Merck Research Laboratories (Rahway, NJ, USA). Dog control plasma was available from the Department of Drug Metabolism I of Merck Research Laboratories (West Point, PA, USA).

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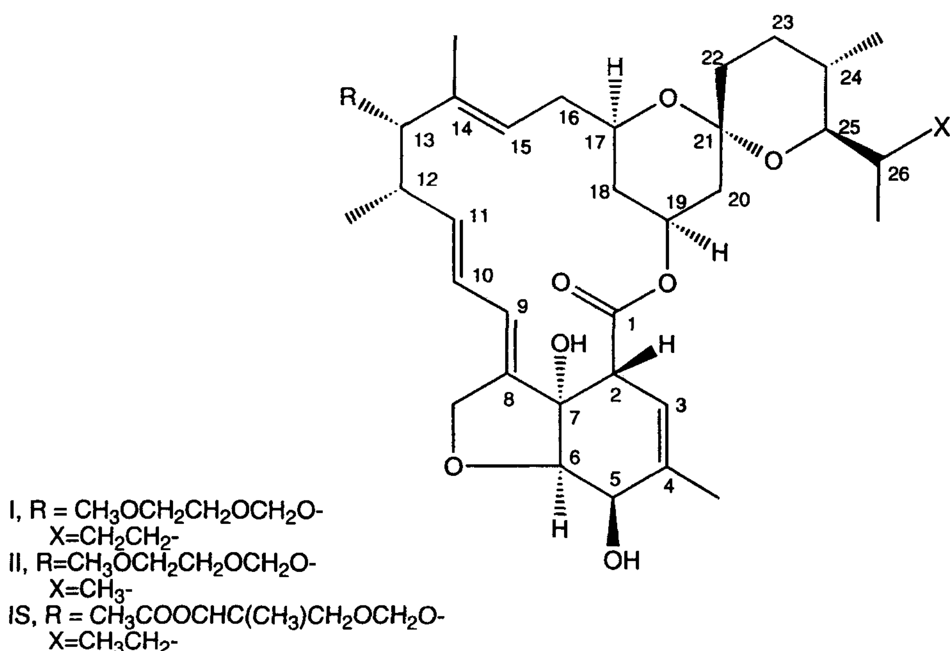

Figure 1. Chemical Structures of MK-324 and Internal Standard IS.

Acetonitrile, chloroform, hexane, and methanol were all HPLC OPTIMA grade, and tetrahydrofuran (certified grade) were all purchased from Fisher Scientific (Springfield, NJ, USA). Trifluoroacetic anhydride (TFAA) and N,N-diethylmethylamine (DEMA, >99%) were purchased from Aldrich (Milwaukee, WI, USA). De-ionized water(18 MΩ conductivity) was obtained through a Milli-Q-filtering system (Millipore, Milford, MA, USA).

Solid-phase extraction (SPE) C_{18} cartridges (3 mL, 200 mg) originated from J. T. Baker (Phillipsburg, NJ, USA).

Instrumentation

A Gilson ASPEC™ XL (Automatic Sample Preparation with Extraction Column) system (Fairview Heights, IL, USA) was used for automated sample preparation and solid-phase extraction. A Zymark TurboVap™ evaporator

(Hopkinton, MA, USA) was utilized for evaporating the chloroform eluent to dryness at 45°C. The HPLC system included a Perkin-Elmer Series 250 pump and ISS-200 programmable autosampler with 150-µL sample loop (Norwalk, CT, USA). A Zorbax RX-C18 analytical column (25 cm x 4.6 mm, 5 µm) (Chadds Ford, PA, USA) protected with a cyano guard column (1cm x 2mm, 5 µm) and a column inlet-filter purchased from Keystone (Bellefonte, PA, USA) were utilized. A Shimadzu RF-551 fluorescence detector (Kyoto, Japan) was employed as an HPLC detector. A Maxi Mix II vortex-mixer from Thermolyne (Dubuque, IA, USA) was also utilized.

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Chromatographic Conditions

The mobile phase consisted of a mixture of acetonitrile, tetrahydrofuran and water (6:1:1, v/v/v, pre-mixed), and was delivered isocratically at a flow rate of 1.2 mL/min. The fluorescence detector was set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm with the sensitivity setting at "high" and the gain setting at "2."

A Perkin-Elmer-Nelson ACCESS*CHROM laboratory data system was used for data acquisition, peak integration and quantification.

Preparation of Standards and Quality Control Samples

Stock solutions of MK-324 and IS at concentrations of 0.2 mg/mL were prepared in acetonitrile, and all subsequent dilutions were made using acetonitrile. Working solutions of MK-324 were prepared at concentrations of 50, 20, 10, 5, 2, 1, and 0.25 ng/mL and the internal standard was prepared at a concentration of 20 ng/mL.

An additional stock standard, independent from those used for preparing working standards, was used to make quality-control (QC) samples. QC samples were prepared by pooling control dog plasma and spiking them with the separately prepared stock standard solution of MK-324.

The low quality control (LQC) sample was prepared by diluting the high quality control (HQC) sample with control plasma in the ratio of 1:20 (v/v). The QC samples were divided into 1.25 mL aliquots in glass vials and stored at -15°C until they were analyzed.

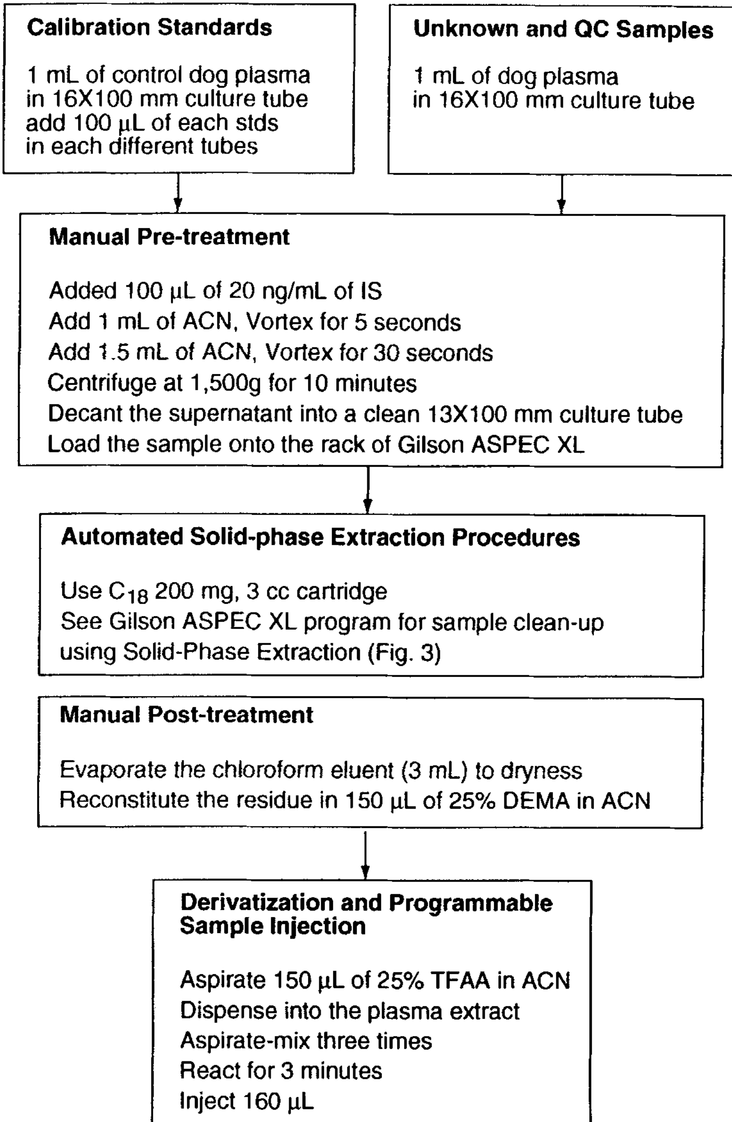

Figure 2. Flow Chart of Sample Preparation Procedure.

A six-point-calibration curve was constructed daily by spiking 1 mL of control plasma with 100 μL of the appropriate working standard and 100 μL of IS working standard solution (20 ng/mL). In addition, three sets of HQC and

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LQC plasma samples were analyzed daily, immediately after the analyses of standard line samples, in the middle of the analysis and, later, after the completion of analyses of dog plasma samples.

Sample Preparation

Dog plasma samples were thawed to room temperature and vortexed vigorously for 5 seconds on a vortex-mixer. Fifty to 1,000 μL of dog plasma diluted with 950-0 μL of control make-up plasma (total final volume equal to 1 mL for each sample) and 100 μL of IS (20 ng/mL) were pipetted into a disposable culture glass tube prior to protein precipitation. After the samples were vortex-mixed and centrifuged, the supernatants were transferred to glass tubes and placed onto a Gilson ASPEC™ XL system. The flow chart of sample preparation procedures using Gilson ASPEC™ XL system is presented in Figure 2. The SPE steps which were programmed on a Gilson system are listed below:

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Manually put all of the acetonitrile supernatant into culture tube

1. Begin loop
2. Condition SPE cartridge with 6 mL of methanol
3. Condition SPE cartridge with 3 mL of chloroform
4. Condition SPE cartridge with 3 mL of methanol
5. Condition SPE cartridge with 4.5 mL of water
6. Add 2 mL of water into culture tube
7. Mix by aspirate and dispense twice
8. Load 6 mL of the diluted supernatant onto SPE cartridge*
9. Wash SPE cartridge with 3 mL of water
10. Move arm from SPE cartridge to drain position
11. Switch LP valve (valve on)+
12. Wait 0.5 min
13. Switch LP valve (valve off)
14. Wait 0.1 min
15. Switch LP valve (valve on)
16. Move arm to SPE cartridge, stay on for 3 min
17. Switch LP valve (valve off)
18. Wash SPE cartridge with 5.0 mL of hexane
19. Elute sample with 3.0 mL of chloroform
20. End loop

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* draw 0.5 mL of excess volume to build up air gap in needle

+ LP = low pressure

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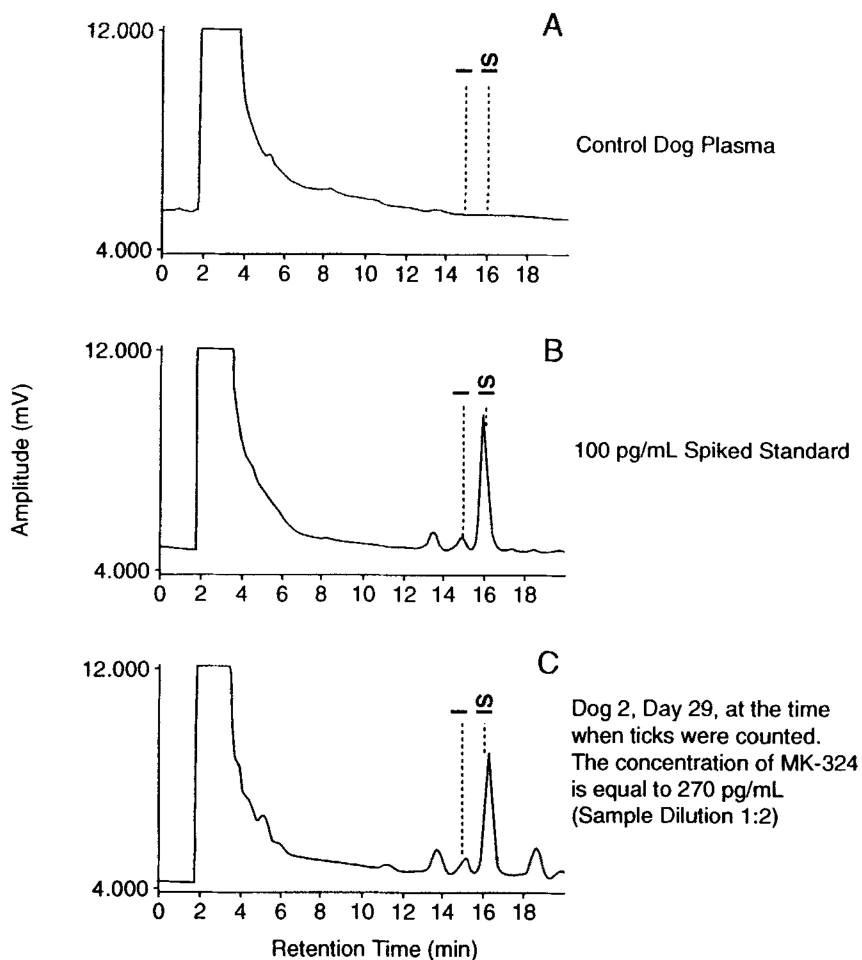

Figure 3. Representative chromatograms of MK-324 and internal standard IS in dog plasma (1 mL). (A) Control plasma, (B) Control plasma (1 mL) spiked with 100 pg of MK-324 and 2 ng of IS, (C) Dog 2, Day 29, the concentration of MK-324 was equal to 270 pg/mL (sample dilution was 1:2).

After the automated SPE sample clean-up steps were completed, the eluents from SPE cartridges were evaporated to dryness, dissolved manually in 150 µL of 25% DEMA in acetonitrile and derivatized with 150 µL of 25% TFAA in acetonitrile using automated pre-column derivatization capability of the autosampler. After derivatization, 160 µL of the reaction mixture was injected directly onto the HPLC system.

Precision, Linearity, Accuracy, Sensitivity, and Specificity

The precision of the method was determined by replicate analyses (n=5) of dog plasma containing MK-324 at all concentrations utilized for constructing the calibration curves. The linearity of each standard curve was confirmed by plotting the peak-height ratio of I to IS versus concentration of MK-324.

The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted (1/y) linear regression of the standard line. The standard curve was prepared and assayed daily with quality control and unknown samples.

The accuracy of the method was established by preparing quality control samples in control dog plasma at low and high concentrations on the standard line. These samples were frozen, stored with post-dose dog plasma samples and assayed daily with dog plasma samples. Samples with concentrations exceeding the linear calibration range were diluted with control plasma and re-assayed. The accuracy of the method was expressed by:

(mean observed concentration)/(spiked concentration) x 100%.

The limit of quantification (LOQ) was 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, C.V. ≤ 10 %) were obtained. Assay specificity was assessed by running blank control and pre-dose plasma samples from different dogs.

Peak heights of I and IS were measured and automatically processed using a PE Nelson ACCESS*CHROM laboratory data system. Concentrations of MK-324 in subject plasma samples were calculated from the daily constructed standard curves.

RESULTS

Assay Sensitivity and Precision

Typical chromatograms of control dog plasma, dog plasma spiked with MK-324 and IS (100 pg/mL and 2 ng/mL, respectively) and dog #2 plasma sample are shown in Figure 3. The total analysis time was 20 minutes. The specificity of the assay was demonstrated by the lack of endogenous

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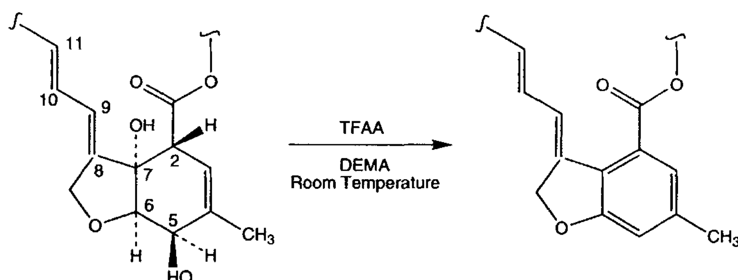

Figure 4. Chemical Derivatization Reaction of MK-324 in the Presence of TFAA.

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Table 1

Intra-Day Accuracy and Precision Data for Determination of MK-324 in Dog Plasma

Nominal Conc. (pg/mL)	Conc'n of MK-324 (pg/mL) 1	2	3	4	5	Mean	Accuracy[a]	Precision[b]
100	100.5	91.1	103.6	91.0	96.8	96.6	96.6	5.8
200	197.7	214.8	194.0	192.7	208.7	201.6	100.8	4.8
500	487.9	493.6	483.6	510.1	497.2	494.5	98.9	2.1
1000	1031.8	1011.4	1021.1	982.5	995.3	1008.4	100.8	2.0
2000	2010.7	2030.2	2012.3	1942.6	2013.5	2001.9	100.1	1.7
5000	4972.9	4902.6	4983.5	4821.3	4984.1	4932.9	98.7	1.4

[a] Expressed as [(mean calculated conc.)/(nominal conc.) X 100.
[b] Expressed as coefficient of variation (C.V. %).

interferences observed at the retention times of these compounds in any of the control and pre-dose plasma samples from dogs participating in trials. The assay has been validated in the concentration range of 100 to 5,000 pg/mL in plasma. The typical equation describing the standard line was Y = 0.000852 X - 0.0187 and the average correlation coefficient (r) was 0.9997. The limit of quantification (LOQ) of MK-324 was 100 pg/mL.

The precision of the assay was assessed by calculating the intra-day C.V.'s for calibration standards and QC samples. The precision (Tables 1 & 2), at all concentrations used for constructing the calibr'n curve, was < 10%.

Table 2

Intra-Day Reproducibility of Quality Control Samples during Analysis of MK-324 in Dog Plasma

Replicate	LQC[a] 125 pg/mL	HQC[b] 2500 pg/mL
1	121.4	2543.3
2	123.4	2505.2
3	113.8	2564.7
4	117.3	2570.8
5	123.9	2440.4
Mean	120.0	2524.9
S.D.[c]	4.3	53.8
C.V. %[d]	3.6	2.1

[a] LQC = low quality-control standard.
[b] HQC = high quality-control standard.
[c] Standard Deviation.
[d] Coefficient of Variation (C.V. %).

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The inter-day accuracy and precision data are presented in Table 3. The accuracy data for QC samples are also summarized in Tables 2 and 3. The data in Table 3 also indicate that MK-324 was stable in plasma during storage at -20°C for at least one year.

Application

The applicability of the method was demonstrated by analyzing dog plasma samples originating from six different studies with MK-324. As an example, the plasma concentration *vs.* time data after topical and oral administrations of MK-324 in one of these studies are presented in Table 4.

DISCUSSION

The method for the determination of MK-324 in dog plasma included three steps: (1) automated solid-phase extraction (SPE) of MK-324 from dog plasma using a Gilson ASPEC system; (2) automated pre-column derivatization to

Table 3

Inter-Day Precision and Accuracy of Determination of MK-324 in Plasma

Nominal Conc. (pg/mL)	Conc'n Found (pg/mL) Mean[c]	S.D.[c,d]	Precision[a] (%)	Accuracy[b] (%)
Standard				
100	95.8	2.5	2.6	95.8
200	199.8	7.3	3.7	99.9
500	500.5	17.2	3.4	100.1
1000	1049.2	43.2	4.1	104.9
2000	2008.0	36.2	1.8	100.4
5000	4945.7	47.5	1.0	98.9
Quality Control[e]				
125	124.2	3.52	2.84	99.3
2500	2530.1	94.1	3.72	101.2

[a] Coefficient of Variation (n=8).
[b] Expressed as [(Mean calculated conc.)/(nominal conc.) X 100.
[c] n=8.
[d] Standard Deviation.
[e] Three sets of quality control samples at each concentration were analyzed daily with the study samples on thirteen different days and over a period of six months.

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form a strongly fluorescent derivative using a Perkin-Elmer programmable autosampler; and (3) HPLC separation and fluorescence detection of the derivatives without further samples clean-up after derivatization. Extensive studies were performed toward the design of extraction conditions of MK-324 from plasma, optimization of the derivatization reaction and HPLC separation of derivatized I and IS from endogenous plasma impurities. The results of these studies are described below.

Extraction from Plasma

Several different approaches for isolating of MK-324 from plasma were studied. Direct liquid-liquid extraction at different pH's using various organic solvents gave low and variable recoveries and produced extracts containing

Table 4

Plasma Concentrations (ng/mL) of MK-324 in Dogs Following Different Treatments with MK-324

Pen #	Predose	2 Hr	4 Hr	8 Hr	24 Hr	Day 5	Day 15	Day 29
				Group 1 - Topical				
2	ND[a]	4.88	2.84	1.09	5.43	2.62	0.74	0.27
10	ND	2.32	3.20	1.30	3.53	4.01	2.14	0.44
18	ND	3.53	1.76	0.75	6.19	3.03	1.04	0.21
24	ND	0.37	0.78	2.59	3.99	4.47	1.00	0.40
30	ND	NQ[b]	0.19	0.51	1.11	0.85	0.46	0.14
				Group 2 - Oral				
6	ND	236.60	862.02	NS[c]	15.53	2.29	0.13	NQ
14	ND	373.57	129.53	46.64	16.05	2.87	0.20	NQ
16	ND	196.50	73.47	31.77	15.89	1.82	NQ	NQ
20	ND	299.31	96.07	35.38	12.82	2.77	0.31	NQ
26	ND	414.26	139.78	34.40	12.26	2.73	0.22	NQ

[a] Not detected.
[b] Below limit of quantification.
[c] No sample.

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several major interferences. Another approach was based on precipitation of plasma proteins with acetonitrile, followed by the removal and evaporation of the supernatant to dryness and derivatization of the residue. Although the derivatization reaction was effective, variation in the degree of dryness, presence of insoluble substances, and irreproducibility of derivatization prevented this direct method from being rugged and reliable enough for routine application. Therefore, isolation of MK-324 from plasma was attempted using solid-phase extraction. When applied from water or plasma, MK-324 was retained to varying degrees on a variety of SPE cartridges including Si, C-8, and C-18, but the recovery was irreproducible and was dependent on loading speeds and types of cartridges employed. To improve recovery, plasma samples were pre-treated with acetonitrile to denature plasma proteins prior to extraction using SPE. The careful control of sample loading speed and flow-rate of solvents during cartridge elution were found to be critical in achieving high recovery and reproducibility. Since the SPE steps were not only difficult to control manually but were also time consuming, repetitive, and tedious, it was decided to automate the SPE steps prior to HPLC analysis. After precipitation of proteins from a series of plasma samples with acetonitrile and

centrifugation, the supernatants were placed on the ASPEC™ XL system which was programmed (Fig. 2) to add water, load the mixed solution onto a C-18 SPE column and wash the column with water followed by hexane. The drug and IS were eluted with chloroform and, after evaporation of the eluent to dryness off line, the residue was derivatized with DEMA/TFAA in acetonitrile. The use of an automated system for sample clean-up provided a method that was both robust and efficient.

Chemical Derivatization

Due to the presence of the conjugated-diene chromophore in the ivermectin analogs, the ultraviolet (UV) absorption spectra of these molecules exhibit a strong absorption band with the maximum at around 245 nm and a molar absorption coefficient (ε) of approximately 30,000 M^{-1} cm^{-1}. The presence of this absorption band has been used as the basis for the development of several methods based on HPLC with UV detection.[7-11] However, these methods gave the limits of detection (LOD) of 1-2 ng/mL which was far above the LOQ required to support our studies with MK-324.

In order to increase assay sensitivity and specificity, the molecule of I was converted to a highly fluorescent analog using an acid anhydride. This dehydration reaction (Fig. 4), previously described and documented for a number of other avermectin analogs,[12-14] was extensively studied here to establish the best reaction conditions for derivatization of I at pg/mL concentrations of the analyte and compatible with automated pre-column derivatization. Use of acetic anhydride for derivatization[12] required high temperature (100°C) and an extended (one hour) reaction time for the reaction to be completed. Instead of acetic anhydride, the derivatization of I was performed in the presence of TFAA as described earlier for ivermectin.[15] The relative yield and the stability of the derivatized products was also investigated. The effect of presence of various tertiary amines as nucleophilic catalysts for acylation[13,16,17] on the yield and kinetics of reaction of I was evaluated.

Both DEMA and dimethylethylamine were found to improve the yield of the derivatized products. However, interferences in the blank prohibited the use of the latter base for the derivatization. By using DEMA as a base, the reaction of I and IS with TFAA produced stable products within a relatively short period of time (three minutes) which was desired for automated pre-column derivatization. The derivatives formed emitted fluorescence at 475 nm when excited at 365 nm.

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Automated Pre-column Derivatization and Chromatography

Automation of the derivatization step was necessary to eliminate the need for careful control of the reaction kinetics and to eliminate the problem associated with the potential chemical instability of the reaction products with high concentration of TFAA and DEMA. Instead of a typical batch pretreatment of many samples and subsequent analysis of these samples over an extended period of time (for example for 20 hours between the first and last sample injection), each sample was derivatized at precisely the same time prior to injection. This was accomplished utilizing the *Derivatization and Automix* capability of the autosampler. After SPE extraction, samples were reconstituted manually in acetonitrile (150 μL) containing 25% of DEMA, and placed in autosampler vials. I and IS were stable in these solutions for up to five days. The autosampler was then programmed to add 25% of TFAA in acetonitrile (150 μL) and, after three minutes, the mixture after derivatization was injected directly onto the HPLC system. While the chromatographic run (20 minutes) was performed on the derivatized sample, the next sample was derivatized in exactly the same manner as the previous one and injected. The derivatives of I and IS were separated from each other and any interfering peaks including the homolog II and IS using a Zorbax RX-C18 column protected with a cyano guard column, and a mobile phase composed of acetonitrile, water and tetrahydrofuran. The cyano guard column acted as a pre-column concentrator, leading to peak compression and a decrease in the baseline noise. The assay was specific, with the LOQ of 100 pg/mL.

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In conclusion, a highly sensitive, automated HPLC method for the determination of MK-324 in dog plasma, utilizing a Gilson ASPEC™ XL system for sample preparation and a Perkin-Elmer programmable autosampler for automated pre-column derivatization, has been developed. This method has been validated in the concentration range of 100-5,000 pg/mL. The linearity of the method in the extended concentration range of 25-5,000 pg/mL, (r=0.9997) has also been demonstrated. This method is rugged, reliable and was used routinely for the analyses of more than 1,000 dog plasma samples from six different studies with MK-324.

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