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An automated method for the determination of subnanogram concentrations of eprinomectin in bovine plasma

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Abstract

Eprinomectin is a potent anthelmintic compound that kills certain parasitic nematodes and arthropods of cattle. A sensitive and automated bioanalytical assay was developed for quantitation of eprinomectin in bovine plasma in support of clinical development of eprinomectin for use in all classes of cattle. This assay determined the concentration of eprinomectin in plasma by reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection. Plasma sample preparation included liquid extraction performed by the Packard MultiPROBE robotics workstation, followed by solid phase extraction performed by the Gilson ASPEC XL automated workstation. The HPLC assay included automated pre-column derivatization with a fluorogenic reagent system which included trifluoroacetic anhydride and *N*-methylimidazole as the catalyst. This reversed-phase chromatographic analysis was based on the fluorescence detection of derivatized eprinomectin and an internal standard, L-648 548, which was similarly derivatized by the fluorogenic reagents. The assay was automated and validated for two concentration ranges of 0.05–10 and 0.5–200 ng ml^{-1}. The lower limit of quantitation of eprinomectin in plasma was 0.05 ng ml^{-1}. The %RSD of the assay was 10% or better at all concentrations. This automated analysis of eprinomectin was used for high-throughput clinical assays with acceptable accuracy and precision. Published by Elsevier Science B.V. All rights reserved.

Keywords: Eprinomectin; Automated plasma assay; Packard MultiPROBE 204DT robotics workstation; Gilson ASPEC XL workstation; Reversed-phase chromatography; Pre-column derivatization; Fluorescence detection

1. Introduction

Eprinomectin (4″-epiacetylamino-4″-deoxyavermectin, Fig. 1) is a macrocyclic lactone which belongs to the avermectin family of compounds [1]. Eprinomectin is a mixture of two homologous compounds, B_{1a} (major component, $\geq 90\%$) and B_{1b} (minor component, $\leq 10\%$) that differ by a single methylene group. It has antiparasitic activity against a variety of endo- and ectoparasites in cattle at a dose of 500 μg kg^{-1} [2]. Eprinomectin is minimally metabolized; eprinomectin B_{1a} residue is the major component of the total drug residue in cattle liver (N. Narasimhan, personal communication, Merck, NJ) and plasma. The

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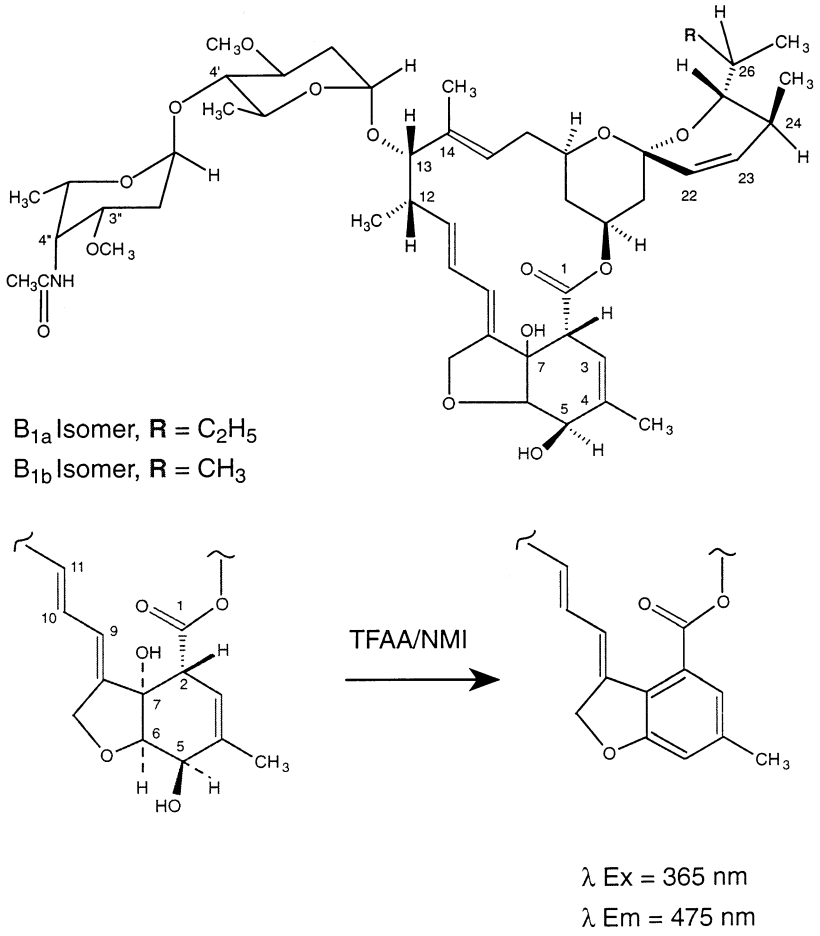

Fig. 1. Chemical structure of eprinomectin and the fluorescent reaction product following dehydration.

concentration of eprinomectin B_{1a} in cattle plasma was therefore measured in support of clinical studies including the determination of the pharmacokinetics and bioavailability of the drug. To support several clinical studies for eprinomectin, a quantitative and automated bioanalytical method was required with specificity, sensitivity, and yet with high throughput.

The assay for the determination of the B_{1a} component of eprinomectin (marker residue) in cattle plasma involved three steps: (1) liquid extraction of eprinomectin from cattle plasma; (2) solid phase extraction; and (3) pre-column derivatization to form a strongly fluorescent derivative for HPLC separation and fluorescence detection of the resulting derivative. This assay method was similar to a previously reported procedure for ivermectin in milk, yet with full automation [3]. This automated assay was validated with respect to specificity, range and linearity, limit of quantitation, intraday and interday accuracy and precision, and sample stability.

This is the first analytical paper on determination of eprinomectin in plasma. Previous analytical papers on ivermectin, a related compound, describe both fluorescence and UV quantitation with HPLC analysis [3,7,8]. Analytical procedures using UV detection at 245 nm allowed detection limits of about 2 ng ml^{-1}. UV quantitation of ivermectin suffers from high signal to noise ratios

and therefore would limit the quantitation in complex biological matricies such as animal plasma and tissue samples using validated assays [7]. Lower detection limits for ivermectin were reported by dehydrative derivatization with acetic anhydride which produced an intensely fluorescent product. This reaction was carried out for 24 h at 100°C in pyridine which served as both the catalyst and the solvent [8]. An improved fluorescence reagent system using trifluoroacetic anhydride and *N*-methylimidazole, the catalyst, in acetonitrile for determination of ivermectin in animal plasma was reported from our laboratories [3]. The derivatization with these reagents required no additional sample clean up prior to HPLC and significantly increased the detection limit. Therefore, this reaction scheme was used for derivatization of eprinomectin to a fluorescent product. Also, since the reaction occurs immediately upon mixing of reagents with the analyte it was possible to automate the precolumn derivatization step within the autosampler.

In developing assays with high specificity and sensitivity where the biological matrix cannot be analyzed directly, the speed of sample preparation usually becomes the rate-limiting step in achieving high throughput clinical assays. In addition to the use of automated analytical instrumentation, there is also a great need for automation of the sample preparation steps. The current trends in automation of bioanalytical assays require robotics systems/workstations which perform specific automated bioextraction tasks with minimal manual intervention between tasks. This paper describes the automation and validation of a high throughput assay for the determination of eprinomectin B_{1a} in cattle plasma. Two separate assays with different ranges and sensitivities were validated to correspond with the concentration ranges of the clinical samples. Both liquid and solid phase extractions were performed for the more sensitive assay while only liquid extraction was required for the higher concentration range assay. Solid phase extraction was performed on samples containing low levels of eprinomectin B_{1a} to remove matrix interferences and for enrichment of the analyte.

2. Experimental

2.1. Chemicals and reagents

Trifluoroacetic anhydride (99%) (TFAA), 1-methylimidazole (99%+) (NMI), and triethylamine (TEA) were obtained from Aldrich. HPLC grade methanol, acetonitrile, and *o*-phosphoric acid, 85% were obtained from Fisher. Hydro water purification system (18 MΩ cm^{-1}) was used. Eprinomectin and L-648 548 (Fig. 2) analytical standards and bovine plasma were obtained from Merck (Rahway, NJ). Solid phase extraction was performed using C18 cartridges (200 mg, 3 ml) obtained from Waters.

2.2. Standard solutions of eprinomectin and L-648 548

The analytical standard used for eprinomectin was 95.3% pure (90.4% B_{1a} and 4.9% B_{1b}). The analytical standard for the internal standard was 89.5% pure (85.7% B_{1a} and 3.8% B_{1b}). The analytical stock solutions (200 μg ml^{-1}) of eprinomectin and L-648 548 (internal standard) were prepared by dissolving the compound in acetonitrile. Serial dilutions were made from the stock solutions to obtain final concentrations of 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng ml^{-1} of eprinomectin. The internal standard working solutions were similarly prepared from a stock solution by diluting with acetonitrile to 20 and 2 ng ml^{-1}.

2.3. Derivatizing reagents

30% NMI and 30% TFAA in dry acetonitrile were prepared, stored in a desiccator and used within a month. The acetonitrile which was used for reagent preparation was kept over molecular sieve 4A beads at all times.

2.4. Packard multiPROBE 204DT robotics workstation

The use and validation of various types of automated assays with the MultiPROBE workstation was previously described [4]. In this study,

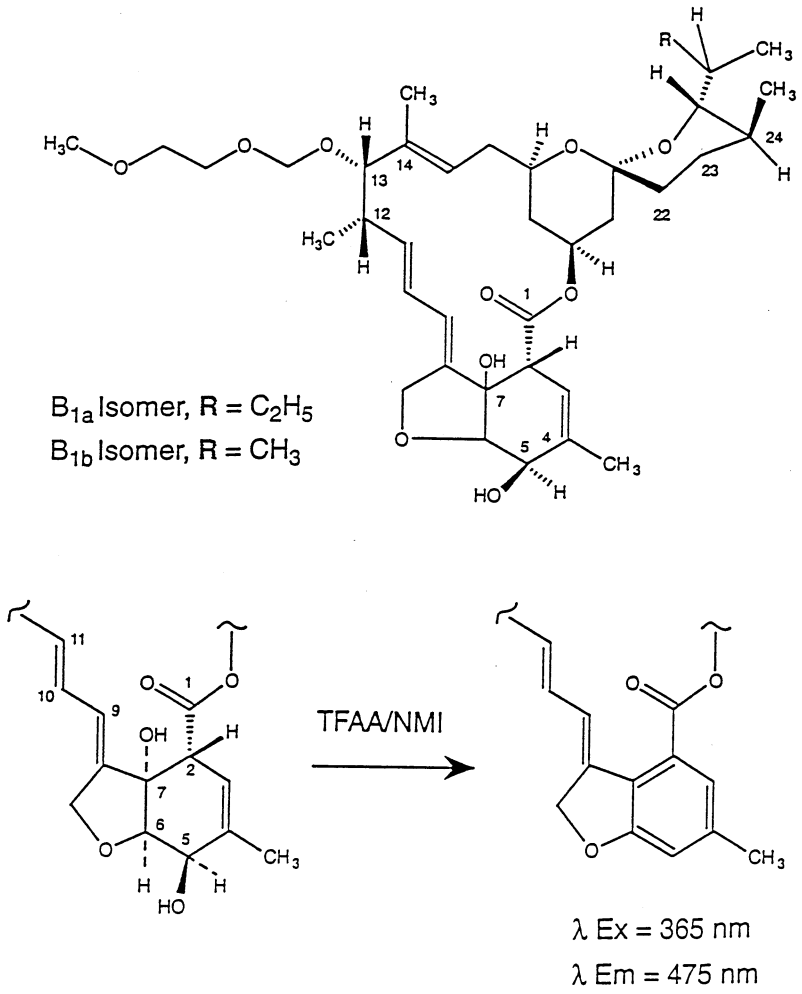

Fig. 2. Chemical structure of L-648 548 and the fluorescent reaction product following dehydration.

the liquid extraction step of the clinical plasma samples and preparation of the calibration standards were performed using the MultiPROBE 204DT robotics workstation. Various configurations of racks were defined on the workstation for the assay. Several protocols were programmed and then were linked to create the complete liquid extraction steps of this assay.

The instrument was set up with the 1 ml disposable tip configuration. Using the conductive 1 ml disposable tips, the instrument pipetted 1 ml of plasma, quality control (QC) samples, and control plasma. The instrument aliquoted the working standards of eprinomectin into 15 ml disposable polypropylene centrifuge tubes which had received 1 ml of control plasma. The calibration standards, QC samples, and clinical samples were then spiked with the internal standard. All plasma samples received an additional 3 ml of acetonitrile. Then, they were removed from the workstation and vortex-mixed for 60 s and centrifuged at 3000 rpm for 12 min. The samples were returned onto the workstation where they were diluted with 1 ml of water. Additional precipitate was removed by a second centrifugation. The samples were again placed on the workstation and the superna-

tant was quantitatively aspirated into individual receiving test tubes. These final extracts were then transferred to the Gilson solid phase extraction workstation.

2.5. Gilson ASPEC XL solid phase extraction workstation

Solid phase extraction (SPE) was required only for the more sensitive assay with a concentration range of 0.05–10 ng ml^{-1}. The C18 SPE columns were processed sequentially. Each column was conditioned sequentially with 4 ml acetonitrile, 4 ml acetonitrile-chloroform (1:1) containing 0.1% NMI, 4 ml acetonitrile, and 4 ml 0.1M sodium phosphate buffer. The sample was then loaded onto the cartridge and was washed twice with 4 ml of acetonitrile–water (1:2). The column was dried under positive N_2 pressure for 3 min. The drug and the internal standard were eluted with 5 ml of acetonitrile–chloroform (1:1) containing 0.1% NMI. The eluted samples were then evaporated to complete dryness under a stream of nitrogen for at least 55 min in a Zymark TurboVap evaporator set at 45°C. The dry residues were reconstituted in 100 μl of 30% NMI in acetonitrile, vortex mixed, and were transferred to autosampler vials and placed in the HPLC autosampler.

2.6. Chromatographic equipment

Gradient reverse-phase liquid chromatography was performed using a Shimadzu HPLC system consisting of an LC-10AS pump and a SIL-10 A autosampler. The injection volume was 100 μl, and the total derivatization time was 7 min (the first 6 min included addition of TFAA, air-mixing, syringe washes, with a final 1 min of waiting). The Shimadzu SCL10A system controller was programmed to add 150 μl of 30% TFAA in acetonitrile to the reconstituted residue, air-mix three times, and then inject 100 μl onto the HPLC column. The flow rate was 1.0 ml min^{-1}. A Shimadzu RF-551 spectrofluorometer was used. The detector was set at an excitation wavelength of 365 nm and the emission was measured at 475 nm. The chromatograms and all data were acquired and processed by EZChrom software.

2.7. Chromatography conditions

The fluorescent derivatives of eprinomectin B_{1a} and B_{1b} and L-648 548 B_{1a} and B_{1b} were resolved on a Zorbax RX-C18 column (4.6 × 25 cm) which was fitted with an ODS guard column and was maintained at 30°C. The gradient HPLC method consisted of 100% B from $T=0$ to $T=12$ min, 100 B–100% A from $T=12$ to $T=14$ min, 100% A from $T=14$ to $T=25$ min, and equilibration with 100% B to $T=28$ min. The mobile phases for the gradient system were: solvent A (CH_3CN:CH_3OH:H_2O:TEA:H_3PO_4/50:44:6:0.2:0.2) and solvent B (CH_3CN:CH_3OH:H_2O:TEA:H_3PO_4/50:40:10:0.2:0.2). The retention time of eprinomectin B_{1a} derivative was 11.5 min and for L-648 548 B_{1a} derivative was 24 min.

3. Results and discussion

3.1. Fluorescence derivative of eprinomectin

Derivatization of ivermectin with trifluoroacetic anhydride in the presence of the base catalyst, *N*-methylimidazole, was described earlier [3]. Similar to ivermectin, eprinomectin contains a tertiary hydroxyl group at C7 and a secondary hydroxyl group at C5. When eprinomectin reacts with trifluoroacetic anhydride in the presence of a base catalyst these hydroxyl groups are thought to be acylated with subsequent deacetylation of the intermediate. The derivatized fluorescent product contains an aromatic ring which is conjugated with the butadiene system in the macrocyclic lactone ring (Fig. 1). The fluorescence excitation and emission maxima for the conjugated derivative are at 365 and 475 nm, respectively, similar to that reported for the fluorogenic ivermectin derivative. L-648 548, the internal standard, similarly was derivatized with TFAA and NMI and had the same fluorescence characteristics (Fig. 2).

3.2. Plasma analysis and assay automation

The plasma assay for eprinomectin was automated using various robotics and workstations to

Table 1
Precision and accuracy of measurement of standard concentrations of eprinomectin B_{1a} in bovine plasma

Nominal (ng ml^{-1})	Mean[a] (ng ml^{-1})	RSD (%)	Accuracy[b] (%)	Recovery[c] (%)
Standard curve 1				
0.05	0.054	6.3	108.0	95.9
0.1	0.104	9.8	104.2	101.3
0.5	0.507	3.4	101.4	103.3
1	0.976	10.0	97.6	105.7
2	1.852	8.0	92.6	95.8
5	5.343	5.3	106.9	99.9
10	9.931	2.6	99.3	101.1
Standard curve 2				
0.5	0.543	7.9	108.6	93.8
1	0.959	8.5	95.9	91.6
2	1.860	2.6	93.0	84.9
5	5.020	3.6	100.4	95.3
10	10.18	3.3	101.8	99.0
20	19.59	3.9	98.0	105.0
50	53.05	2.3	106.1	96.2
100	100.6	4.9	100.6	107.3
200	196.6	2.8	98.3	94.0

For standard curve 1 both liquid extraction and solid phase extraction were performed at all concentrations; for standard curve 2 only liquid extraction was performed at all concentrations. Calibration curves were obtained from the weighted $(1/x)$ linear least-squares regression curves constructed using the values at each concentration.
[a] Mean concentrations were the average of five determinations.
[b] Accuracy (%) was expressed as (mean calculated concentration/nominal concentration) $\times 100$.
[c] Recovery (%) was defined as (spiked plasma/reference sample) $\times 100$.

increase the throughput for the analysis of clinical samples (Fig. 4). Each robotics systems/workstation performed a specific bioextraction task with some manual intervention between tasks. Typically, in this assay 70 samples were processed batchwise for the liquid extraction step using the Packard MultiPROBE workstation. This batch liquid extraction process, depicted in Fig. 4, took 4–5 h. The tasks included preparation of standard curve samples, QC samples, and clinical samples using a number of linked assay protocols which were programmed using the Easyprep software of the Packard MultiPROBE workstation.

For clinical studies with plasma levels exceeding 0.5 ng ml^{-1} of the drug, the SPE step was bypassed. All clinical samples containing low levels ($\approx$0.05–3 ng ml^{-1}) of eprinomectin required solid phase extraction. After liquid extraction, these samples were loaded onto a Gilson ASPEC XL workstation and were further purified using C18 SPE cartridges. This process was carried out sequentially for the 70 samples. Solid phase extraction for a typical assay (70 samples) took 29 h.

Samples were then dried as described in the experimental section and were reconstituted in 30% NMI in acetonitrile. They were then placed in the autosampler of an HPLC. Pre-column derivatization and chromatographic separation and quantitation of the drug was programmed through the HPLC software.

3.3. Assay validation and application to plasma analysis

The validation of this bioanalytical assay was performed in compliance with the general guidelines established in a joint industry, academia, FDA conference on analytical method validation [6]. Two separate standard curves were set-up for analysis of clinical samples with high and low nanogram concentration ranges of eprinomectin.

As shown in Table 1, standard curve 2 was the less sensitive assay range. Standard curve 1 was the more sensitive assay. Removal of endogenous matrix interferences and enrichment of the analyte at low levels required an additional clean-up step using solid phase extraction. Therefore, solid phase extraction of plasma samples further improved the sensitivity of the assay to 0.05 ng ml^{-1}. Therefore, a lower concentration range of 0.05–10 ng ml^{-1} was validated by inclusion of the SPE step. Typical chromatograms of control bovine plasma, a plasma sample spiked with eprinomectin and the internal standard (0.5 and 2 ng ml^{-1}, respectively) and a clinical sample spiked with the internal standard (2 ng ml^{-1}) are shown in Fig. 3. The B_{1a} isomers of eprinomectin and the internal standard eluted at 11.5 and 24 min, respectively.

Selectivity (specificity): control plasma from six different cattle were prepared and assayed by the method shown in Scheme 1, except that these control samples were not spiked with the analyte or the internal standard. The specificity of the assay was demonstrated by the lack of interfering peaks at the retention times of eprinomectin B_{1a} and L-648 548 B_{1a} in any of the control plasma samples (Fig. 3).

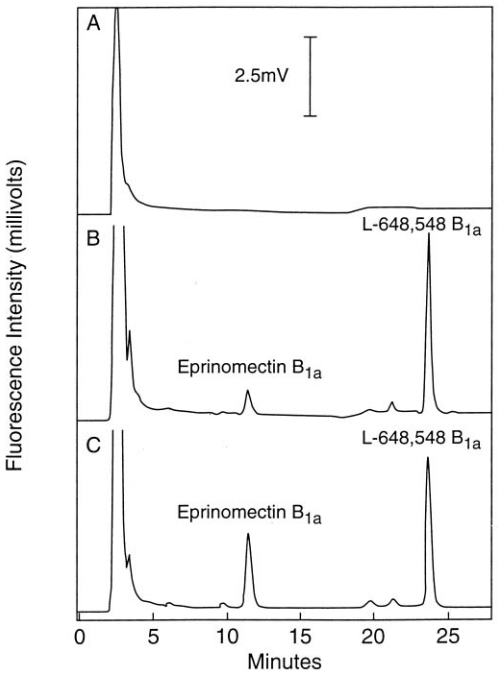

Fig. 3. Representative chromatograms of (A) blank bovine plasma; (B) plasma spiked with eprinomectin (0.5 ng ml^{-1}) and internal standard (2 ng ml^{-1}); and (C) a clinical sample which was spiked with 2 ng ml^{-1} internal standard.

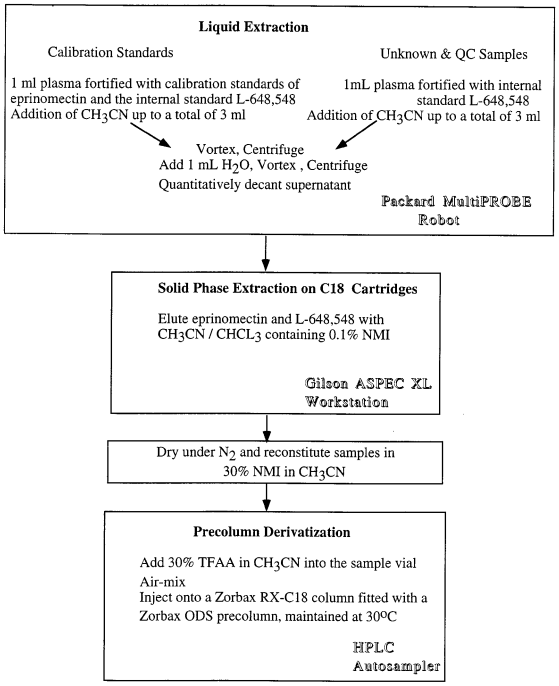

Fig. 4. Automated sample preparation method using various automated workstations. Liquid extraction was performed on all clinical samples. Solid phase extraction was performed on samples with low levels of eprinomectin to remove matrix interferences and for enrichment of the analyte.

Assay Range: two calibration curves were constructed for eprinomectin over concentration ranges of 0.05–10 and 0.5–200 ng ml^{-1}. Various concentrations of calibration standards and the internal standard were spiked into control plasma to establish the calibration curves (Table 1). The concentration of the internal standard was 2 ng ml^{-1} for the more sensitive assay and 20 ng ml^{-1} for the higher concentration range curve. Standard curves for eprinomectin were constructed by plotting peak area ratios (eprinomectin B_{1a}/L-648 548 B_{1a}) versus drug concentrations. The calibration curve was fitted using a weighted $(1/x)$ linear least-squares regression analysis. The aver-

Table 2
Interday variability for the analysis of quality control plasma samples spiked with eprinomectin B_{1a}

Nominal concentration (ng ml^{-1})	Mean concentration (ng ml^{-1})	n	RSD (%)
0.35	0.35	18	18.0
5.0	5.14	15	7.9
150	153.7	15	3.8

n refers to the number of clinical assays where the quality control samples were used. For each clinical assay ($n = 1$), the QC samples at each concentration were determined in quadruplicate. The QC samples were stored at −20°C and were thawed and used throughout the 15–18 assays which were performed over a three and one-half month period.

age ($n = 5$) correlation coefficients (r^2) of 0.9977 and 0.9982 were obtained for the low and high concentration range curves, respectively. The average values for the slope and intercept for the low concentration range curves ($n = 5$) were 0.026 ± 0.004 and $2.6 \times 10^{-5} \pm 1.3 \times 10^{-5}$, respectively. The average values for the slope and intercept for the high concentration range curves ($n = 5$) were 0.029 ± 0.002 and $2.4 \times 10^{-5} \pm 0.3 \times 10^{-5}$, respectively.

Precision, accuracy, and linearity of calibration standards: five individual calibration curves were constructed for each concentration range using plasma samples from five different cattle. The accuracy and percent coefficient of variation (%RSD) at each concentration level established the accuracy and precision of the calibration curves (Table 1). The %RSD at each concentration level for the five replicate curves did not vary by greater than 10%, including at the limit of quantitation. The accuracy measured as percent mean/nominal value at each concentration level was within $100 \pm 9\%$ at all concentration levels. Absolute recoveries of eprinomectin were determined by comparing the area ratios of eprinomectin B_{1a} to L-648 548 B_{1a} from extracted plasma to those of directly injected standards (Table 1). Over the range of the two calibration curves, the recovery of eprinomectin B_{1a} was $> 84.9\%$. The high recoveries for eprinomectin B_{1a} for standard curve 1 ranged from 95.8 to 105.7% ($100.4 \pm 3.6\%$) which indicated that the drug could be efficiently extracted even with the addition of a SPE step to the sample clean-up procedure.

Interday accuracy and precision: the interday variability was determined by analyzing QC samples which were prepared at high and low concentrations and which were within the range for the two calibration curves. The QC samples were stored at −20°C until used. The nominal concentrations of the QC samples were 0.35, 5, and 150 ng ml^{-1}, and their interday variability (%RSD) were 18.0, 7.9, and 3.8%, respectively (Table 2). The measured concentration of the three QC samples averaged 0.35, 5.14 and 153.7 ng ml^{-1} as shown in Table 2. The data in Table 2 also demonstrated the stability of eprinomectin in plasma samples during storage at −20°C for three and a half months.

4. Conclusions

The pre-column derivatization of eprinomectin to a fluorescent derivative allowed low level determination of this drug in biological fluids. Similar to the derivatization of ivermectin and abamectin with TFAA and NMI [3], the derivatization of eprinomectin was complete in less than 30 s at room temperature. Similarly, because there were a few reagent by-products, no additional purification of the mixture was necessary. This pre-column derivatization was therefore programmed within the autosampler. The extraction and SPE steps of the assay for eprinomectin could very well be performed manually as reported for avermectins [3]. However, such sample preparation is quite labor intensive. The automation of sample preparation provided the high throughput that was required for performing clinical assays in support of the development of eprinomectin. Approximately 2400 plasma samples including clinical samples, QC samples, and calibration

standards were processed within 3 months using the Packard and Gilson robotics workstations and an HPLC equipped with an autosampler. Furthermore, automation of the assay freed the analyst from exposure to biological fluids and organic solvents. Especially notable is the versatility of Packard MultiPROBE for automation of different types of assays such as that described in this paper and for radioinmmnoassays described in a previous report [4].

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References

[1] W.L. Shoop, P. DeMontigny, D.W. Fink, Int. J. Parasitol., in press.
[2] W.L. Shoop, J.R. Egerton, C.H. Eary, H.W. Haines, B.F. Michael, H. Mrozik, P. Eskola, M.H. Fisher, L. Slayton, D.A. Ostlind, B.J. Skelly, R.K. Fulton, D. Barth, S. Costa, L.M. Gregory, W.C. Campbell, R.L. Seward, M.J. Turner, Int. J. Parasitol., in press.
[3] P. DeMontigny, J.S.K Shim, J.V. Pivnichny, J. Pharm. Biomed. Anal. 8 (6) (1990) 507–511.
[4] L. Antonian, M.D. Iacolina, R.R. Proux, Clin. Lab. Sci. 8 (1995) 117–120.
[5] V.P. Shah, J. Pharm. Sci. 81 (3) (1992) 309–312.
[6] S. Braggio, J. Pharm. Biomed. Anal. 14 (1986) 375–388.
[7] J. Fischer, M.T. Kelly, M.R. Smyth, P. Jandera, J. Pharm. Biomed. Anal. 11 (3) (1993) 217–223.
[8] J.W. Tolan, P. Eskola, D.W. Fink, H. Mrozik, L.A. Zimmerman, J. Chromatogr. 190 (1980) 367–376.

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