*Journal of Chromatography*, 617 (1993) 79–86
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6887

# Improvements in detection sensitivity for the determination of ivermectin in plasma using chromatographic techniques and laser-induced fluorescence detection with automated derivatization

S. R. Rabel and J. F. Stobaugh*

*Department of Pharmaceutical Chemistry, 3068 Malott Hall, University of Kansas, Lawrence, KS 66045 (USA)*

R. Heinig

*Institut für Klinische Pharmakologie, Bayer AG, Aprather Weg, W-5600 Wuppertal (Germany)*

J. M. Bostick

*Sandoz Pharmaceutical Corporation, 59 Route 10, East Hanover, NJ 07936 (USA)*

(First received February 19th, 1993; revised manuscript received April 17th, 1993)

ABSTRACT

Improvement in detection sensitivity for the analysis of ivermectin was observed through utilization of laser-induced fluorescence detection and by manipulation of chromatographic conditions. Gradient elution used in combination with narrow-bore chromatography and conventional fluorescence detection resulted in a limit of quantitation for the major homologue of ivermectin of 0.01 ng/ml in dog plasma. Laser-induced fluorescence detection with isocratic chromatographic conditions also resulted in a limit of quantitation of 0.01 ng/ml in dog plasma, which is a six-fold improvement over previously reported methods. Introduction of an automated procedure for the derivatization and injection of samples reduced the amount of sample handling, eliminated the potential for analyte/internal standard degradation and contributed to the overall ease of analysis.

## INTRODUCTION

The avermectins are a family of naturally produced macrocyclic lactones exhibiting a broad spectrum of anthelmintic activity [1,2]. Ivermectin (Fig. 1), which is an analogue of avermectin $B_1$, is obtained by selective hydrogenation at carbons 22 and 23 [3] and has proven to be a very potent antiparasitic agent used in both veterinary and medical applications. As a result of the high potency of ivermectin, efficacious doses given to cattle and sheep are 0.1 mg/kg and lower [4]. Administration of ivermectin in a single 0.2 mg/kg dose has been effective in treating human onchocerciasis, also known as "river blindness" [5]. The correspondingly low concentration of ivermectin in biological fluids necessitates a very sensitive analytical methodology capable of detecting low pg/ml concentrations of ivermectin.

Ivermectin is a mixture of two homologues,

* Corresponding author.

0378-4347/93/$06.00 

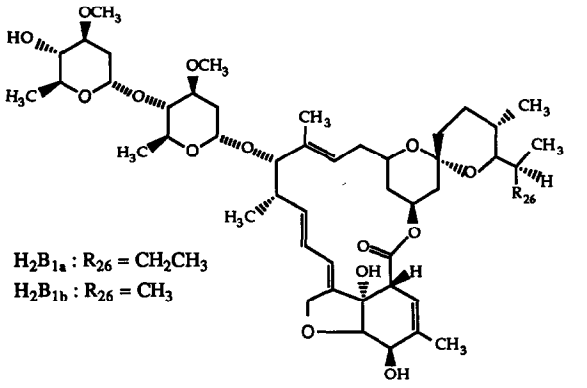


Fig. 1. Structures of the major ($H_2B_{1a}$) and minor ($H_2B_{1b}$) components of ivermectin.

22,23-dihydroavermectin 1a ($H_2B_{1a}$), which is present at $\geq 80\%$, and the minor component 22,23-dihydroavermectin 1b ($H_2B_{1b}$) at $\leq 20\%$. In the past, UV photometric detection [6–8], which gives detection limits on the order of 1–2 ng/ml, has been used as a method of detection. However, fluorescence detection [9–11] is more commonly used and gives lower detection limits. In the presence of a nucleophilic catalyst and an acylating agent, ivermectin undergoes an acylation–elimination reaction resulting in aromatization of the cyclohexene moiety. In a recent publication, N-methylimidazole and trifluoroacetic anhydride were used to effect this transformation [12]. The resulting fluorophore had a very large extinction coefficient and showed absorption and emission maxima at 365 and 475 nm, respectively [12].

The objective of the present work was to develop methodology and/or instrumentation that would result in an improvement in the signal-to-noise ratio, and therefore greater detection sensitivity, than currently exists for ivermectin. Presently, we describe chromatographic (gradient elution), detection (laser-induced fluorescence), and derivatization approaches, which may be optimized in order to achieve the stated objective.

## EXPERIMENTAL

### *Reagents*

All solvents were of HPLC grade. Acetonitrile, methanol and pentene-stabilized chloroform were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Tetrahydrofuran was purchased from Aldrich (Milwaukee, WI, USA). Water was filtered through a Milli-Q water system (Millipore, Bedford, MA, USA). Derivatization reagents of >99% purity were used. Trifluoroacetic anhydride and N-methylimidazole (as well as other catalysts) were obtained from Aldrich. In most cases N-methylimidazole was redistilled before use. Ivermectin and the ivermectin monosaccharide, which was used as an internal standard, were gifts from $Inter_x$/MSDRL. Dog plasma was purchased from Pel-Freeze Biologicals (Rogers, AR, USA).

### *Instrumentation*

The liquid chromatographic system consisted of an LC-6A liquid chromatograph, SCL-6B system controller, SIL-6B autoinjector, and C-R4A Chromatopac integrator (Shimadzu, Kyoto, Japan). An RF-535 fluorescence detector (Shimadzu) was used in those experiments requiring a fluorescence detector. The laser-induced fluorescence (LIF) detector, described by Bostick *et al.* [13], was built in house using a Model 4214NB He–Cd laser and 4200PS power supply (Liconix, Santa Clara, CA, USA). Fiber optic cables were supplied by General Fiber Optics (Cedar Grove, NJ, USA). Model 227 power supplies for the Model R1527 photomultiplier tubes (PMTs) (Hamamatsu, Bridgewater, NJ, USA) were purchased from Pacific Instruments (Concord, CA, USA). Bandpass filters with a center wavelength at 325 and 470 nm and half bandwidth of 10 nm were obtained from Omega Optical (Brattleboro, VT, USA). The long pass filter LL-400-F was manufactured by Corion (Holliston, MA, USA). An analog ratioing circuit [13] was constructed with AD515ALH operational amplifiers and an AD538 real-time analog computational unit from Analog Devices (Norwood, MA, USA). Linear regression of all the calibration curves was done using Minitab statistical software (Release 8, Macintosh Version, State College, PA, USA).

*Chromatographic conditions*

*Isocratic elution.* The composition of the mobile phase was acetonitrile–water (92:8, v/v) and a flow-rate of 1.0 ml/min was used. Several MOS-Hypersil-2 analytical columns of differing dimensions were obtained from Keystone Scientific (Bellefonte, PA, USA). For fluorescence detection, the column dimensions were 150 mm × 4.6 mm I.D., 5 μm particle size. However, for LIF detection, a different column (250 mm × 4.6 mm I.D. 3 μm particle size) was used with the same mobile phase. The injection volume was 100 μl. In a few of the initial optimization experiments, a $C_{18}$ column (5 cm × 4.6 mm I.D., 5 μm particle size) (Analytichem International, now available through Varian, Sunnyvale, CA, USA) was used. Excitation and emission wavelengths on the RF-535 were set at 365 and 475 nm, respectively. The 325-nm line of the He–Cd, which supplied *ca.* 2.5 mW output power, was utilized for excitation in LIF detection.

*Gradient elution.* Gradient elution was used in conjunction with a narrow-bore column (100 mm × 2 mm I.D., $d_p$ 3 μm, Keystone Scientific). A linear gradient from 72:28 (v/v) to 92:8 (v/v) acetonitrile–water was run over a period of 15 min. Flow was diverted from the detector prior to the elution of the analytes and subsequently channeled back past the detector. The flow-rate was 0.3 ml/min and the injection volume was 150 μl.

*Standard solutions*

All standard solutions of ivermectin and ivermectin monosaccharide were prepared in acetonitrile and stored at 4°C for up to thirty days. A 1.2 mg/ml stock solution of the $H_2B_{1a}$ component, which would be used for further dilutions, was prepared by dissolving 1.3 mg of ivermectin (91% in $H_2B_{1a}$) in acetonitrile. The concentrations included in the series of standard solutions were 12.0, 7.38, 3.66, 1.19, 0.73, 0.46, and 0.23 ng/ml. Working solutions of ivermectin monosaccharide were prepared by dissolving 0.3 mg of ivermectin monosaccharide (93% $B_{1a}$) in 10 ml of acetonitrile and diluted to 285 and 86 ng/ml in $B_{1a}$.

*Sample preparation*

The basic work-up procedure for dog plasma described by De Montigny *et al.* [12] was utilized with slight modifications. Dog plasma was thawed and centrifuged at 15 000 *g* for 5 min to remove precipitated proteins. Aliquots (1 ml) of the plasma were spiked with 50 μl of the respective ivermectin standard solutions resulting in plasma concentrations of 0.60, 0.37, 0.18, 0.06, 0.04, 0.02, and 0.01 ng/ml in $H_2B_{1a}$. The concentration of the internal standard solution used to spike plasma varied depending on the method of detection. The samples were vortex-mixed for 15 s followed by the addition of 1.0 ml of acetonitrile and centrifugation for 15 min at 1130 *g*. Water (1 ml) was added to each sample followed by centrifugation (15 min, 1130 *g*). Solid-phase extraction (SPE) cartridges ($C_{18}$, 3 ml) from J. T. Baker were conditioned before use by washing with the following sequence of solvents: 4 ml of acetonitrile, 5 ml of chloroform, 4 ml of acetonitrile, and 4 ml of water. The plasma supernatant was transferred onto the SPE columns. The pellet remaining after centrifugation was reconstituted in 3.5 ml of acetonitrile–water (1:2, v/v) and again vortex-mixed for 15 s and centrifuged at 1130 *g* for 15 min. The second supernatant was applied to the SPE columns and the sorbent was washed with 4 ml of the same acetonitrile–water mixture. The SPE sorbent beds were air-dried for 60 min with the aid of a vacuum manifold (Supelco, Bellefonte, PA, USA). Ivermectin and the internal standard were eluted from the SPE columns with 5 ml of acetonitrile–chloroform (1:1, v/v) rather than chloroform as described by De Montigny *et al.* [12]. The eluent was evaporated under nitrogen at 45°C in a Pierce Reacti-Therm heating module (Rockford, IL, USA). The residue from each sample was transferred into a 250-μl polypropylene autosampler vial (Sun Brokers, Wilmington, NC, USA) in two 100-μl aliquots of acetonitrile and again evaporated under nitrogen at ambient temperature.

*Automated derivatization*

The derivatization process was fully automated using the Shimadzu system controller and auto-

injector. Each sample was derivatized just prior to injection by the addition of 100 μl of N-methylimidazole–acetonitrile (1:1, v/v) followed by 150 μl of trifluoroacetic anhydride–acetonitrile (1:2, v/v). Although the derivatization reaction was complete after 30 s, the wait time prior to injection was predetermined by the lowest default setting in the pretreatment file (1.7 min).

## RESULTS AND DISCUSSION

Various chromatographic parameters were investigated in order to determine the optimal conditions which would result in minimal on-column band dispersion. As a result of the physicochemical properties of ivermectin, limitations were encountered with respect to the manipulation of these parameters. Due to the molecular shape and hydrophobic nature of ivermectin, small changes in the percentage organic modifier in the mobile phase have significant implications in retention behavior. The retention time of ivermectin on a $C_{18}$ column increased by over 7 min with a 2% decrease in methanol. When using a $C_{18}$ stationary phase, retention times in general were very long despite using large concentrations of organic modifier. Consequently, ivermectin eluted in a large peak volume and was quite broad. Shorter retention times with ample resolution and better peak shape were observed using a MOS-2 column with 92:8 (v/v) acetonitrile–water as the mobile phase. This combination of stationary phase and mobile phase was used in subsequent isocratic experiments.

According to previous protocols for sample preparation, chloroform was used to elute ivermectin from the SPE cartridges [12]. However, we observed low recoveries for the analyte and internal standard when eluting the SPE columns with strictly chloroform. This finding prompted investigations into the use of new solvent systems which would optimize recoveries. A binary solvent system of acetonitrile–chloroform (1:1) resulted in an maximum recovery of 94%.

One of the problems associated with the derivatization protocol of ivermectin is the nature of the injection matrix. Injection of a strong organic matrix consisting predominantly of acetonitrile leads to a disturbance of the column equilibrium in reversed-phase chromatography and consequently on-column peak dispersion. The problem of band spreading is more profound when a less hydrophobic stationary phase such as $C_8$ is used. In addressing this issue, several approaches to alter the injection matrix were evaluated. The injection matrix could be weakened by the addition of water or some solvent similar to that of the mobile phase. Ideally to prevent further dilution of the sample by the addition of water or mobile phase, the matrix should be evaporated followed by reconstitution in a different solvent. Therefore the activity of several catalysts of greater volatility than N-methylimidazole were examined with the hope of evaporating the matrix before dilution. The relative yield of fluorophore obtained using equimolar amounts of various catalysts is summarized in Table I. Dimethylethylamine was found to improve the yield of the derivative by a factor of 1.6 relative to N-methylimidazole. Although dimethylethylamine gave higher fluorophore yields relative to N-methylimidazole, interferences in the blank prohibited the use of this catalyst in conjunction with LIF detection. In order to make comparison studies of detection sensitivity, N-methylimidazole was used as the catalyst over the course of the remaining experiments. Prior to use, the N-methylimidazole was redistilled to remove impurities, which were found to vary among different lots obtained from the manufacturer. The second problematic fea-

TABLE I

RELATIVE YIELD OF FLUOROPHORE USING VARIOUS VOLATILE AMINES AS THE CATALYST

| Catalyst | Relative yield fluorophore (mean ± S.D.) |
|---|---|
| Pyridine | 0 |
| Triethylamine | 70.9 ± 6.9 |
| Methylimidazole | 100.0 |
| Diethylmethylamine | 115.9 ± 5.7 |
| Dimethylethylamine | 160.5 ± 4.9 |

ture of the derivatization process was the large tailing solvent front which was magnified as the sensitivity of the detection method increased. The detectability of the analyte peak was further complicated when situated on the tailing portion, however, several solutions to this problem were available. The retention time of the peak of interest could be increased to a point where the tailing had subsided or, alternatively, the solvent front could be shunted to waste which would reduce the background relative to the analyte. In the case of narrow-bore chromatography, the eluent containing the solvent front was diverted to waste while the analytes were allowed to pass through the detector. The solvent front in LIF detection became less of an issue since an increase in retention time of the analyte was required to resolve an interference.

The extreme hydrolytic lability of the trifluoroacetyl ester moiety present in the fluorophore precluded the use of a weaker injection matrix. Upon addition of water to the reaction solution, the fluorophore underwent hydrolysis to form a more hydrophilic species. Apparently excess trifluoroacetic anhydride was rapidly hydrolized in the presence of water releasing two equivalents of trifluoroacetic acid. As a consequence of the drop in pH, acid-catalyzed cleavage of the trifluoroacetyl ester groups proceeded relatively rapidly. The maximum rate of hydrolysis was observed when the water/trifluoroacetic anhydride ratio was 20. Under these conditions, hydrolysis of the ivermectin fluorophore was complete within 60 min. Therefore, dilution of the injection matrix with water was not a viable solution to the problem of weakening the matrix solvent strength.

Using the RF-535 fluorescence detector, a linear calibration curve was constructed from plasma concentrations of 0.06, 0.18, 0.37 and 0.60 ng/ml (each run in triplicate) *versus* the peak-area ratio of $H_2B_{1a}$ to the internal standard ($B_{1a}$). The equation of the line was $y = 0.004$ ($\pm 0.020$ S.D.) $+ 0.949$ ($\pm 0.056$ S.D.)$x$ ng/ml $H_2B_{1a}$ with a correlation coefficient of $r = 0.981$. The limit of quantitation using the RF-535 was 0.06 ng/ml. Representative chromatograms of spiked plasma and the plasma blank using conventional fluorescence detection are shown in Fig. 2.

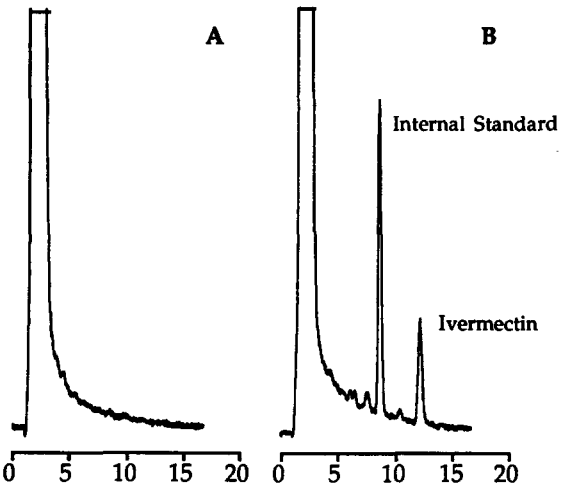


Fig. 2. Chromatograms of unspiked plasma (A) and a plasma sample spiked with 0.37 ng/ml ivermectin ($H_2B_{1a}$) and the internal standard ($B_{1a}$) (B); isocratic chromatography with conventional fluorescence detection.

An alternative chromatographic approach to increasing the mass sensitivity was to switch to the use of narrow-bore HPLC. In using a column of small inner diameter, dilution during the chromatographic process is reduced, however, a major disadvantage in using a narrow-bore column is decreased loading capacity. This problem was circumvented by the use of gradient elution in which the effective $k'$ value at the time of injection was infinity. The presence of a high percentage of water in the mobile phase at the time of injection forced hydrophobic analytes onto the stationary phase at the head of the column resulting in trace enrichment. Mass utilization of the sample was improved since the injection volume was increased from 100 to 150 $\mu$l with no significant deterioration in peak shape. The use of gradient elution also improved peak shape and enhanced detectability due to the zone compression which was inherent with this approach. The limit of quantitation obtained from plasma using narrow-bore HPLC and RF-535 detection was 0.01 ng/ml, corresponding to a six-fold improvement over the 4.6 mm I.D. column. A linear calibration curve represented by the equation $y = 0.003$

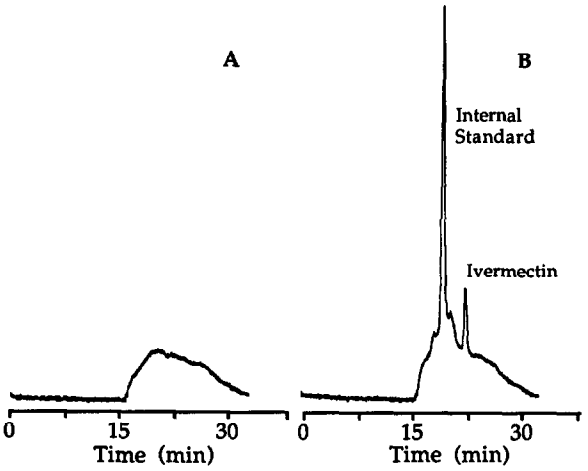


Fig. 3. Chromatograms of unspiked plasma (A) and plasma spiked with 0.18 ng/ml ivermectin ($H_2B_{1a}$) and the internal standard ($B_{1a}$) (B); gradient elution with a 2 mm I.D. column and conventional fluorescence detection.

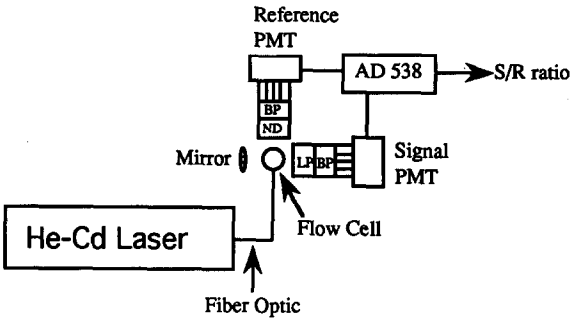


Fig. 4. Top view of laser-induced fluorescence detector. LP = Long-pass filter; BP = bandpass filter; ND = neutral density filter; PMT = photomultiplier tube; AD 538 = analog devices computational unit.

($\pm$0.003 S.D.) + 0.877 ($\pm$0.038 S.D.)$x$ ng/ml $H_2B_{1a}$ and a correlation coefficient of $r = 0.990$ was obtained using concentrations of 0.01, 0.02, 0.04, and 0.18 ng/ml. Chromatograms in Fig. 3 show spiked and unspiked plasma samples using narrow-bore HPLC with gradient elution and RF-535 detection.

The final approach taken to enhance the sensitivity of the ivermectin assay was to employ LIF detection. Although the maximum absorbance of the ivermectin fluorophore was at 365 nm [12], substantial absorbance in the 325-nm region suggested that use of the 325 nm line of a He–Cd laser as an source of excitation was feasible. Approximately 70% of the fluorophore absorbance was sacrificed due to the diminished molar absorptivity at 325 nm (estimated value *ca.* 21 000 l/mol-cm at 325 nm), however, one may argue that the additional power provided by the laser would more than compensate for this loss. A schematic diagram of the detector configuration, modeled after the design of Bostick *et al.* [13], is shown in Fig. 4. A dual-channel configuration was designed to correct for inherent variations in laser output. A 325-nm bandpass filter as well as a neutral density filter were positioned in front of the reference PMT, whereas a long-pass filter and a 470-nm bandpass filter were placed anterior to the signal PMT. Collimators were used to assure that only light at right angles was allowed to pass through the bandpass filter. The laser beam was delivered to the flow cell *via* a fiber optic cable. The reference and signal PMTs sampled light from the same portion of the flow cell. The collection efficiency of the signal PMT was increased almost two-fold by positioning a concave mirror on the opposite side of the flow cell, however, the scattering of light due to the presence of the mirror also increased. The respective currents were fed into a circuit which converted each current to a voltage and performed a real-time analog ratio of the two channels [13].

The detection sensitivity of the ivermectin assay was improved by using LIF detection, however, there were several issues associated with the method. Modifications in the isocratic chromatography conditions were required in order to resolve an interfering peak not previously seen by fluorescence detection. The appearance of the extraneous peak may be attributed to a decrease in selectivity by using 325 nm as the excitation wavelength, although this hypothesis is merely speculative. The interference eluted just prior to the peak of interest with a resolution of only 0.6–0.7. The simplest manner in which to achieve improved resolution was to increase the number of theoretical plates. Accordingly, the 15-cm MOS-2 column was replaced with a 25-cm col-

umn packed with 3-μm particles. The interfering peak was completely resolved at the expense of longer analysis times using the same composition of mobile phase with the longer column of smaller particle size. The resulting linear calibration curve utilizing LIF detection for ivermectin-spiked plasma samples was described by the equation $y = 0.074 (\pm 0.012 \text{ S.D.}) + 1.95 (\pm 0.117 \text{ S.D.})x$ ng/ml $H_2B_{1a}$ and a correlation coefficient of $r = 0.981$. Plasma concentrations of 0.01, 0.02, 0.06, and 0.18 ng/ml were used to obtain the linear plot and a limit of quantitation of 0.01 ng/ml. Chromatograms of typical plasma samples are shown in Fig. 5. An interference coeluting precisely with the analyte appears in the blank and is believed to be ivermectin carry-over from the work-up procedure. The interference, estimated at an equivalent concentration of 0.005 ng/ml, could not be eliminated from the assay. Therefore the limit of quantitation, taking into consideration the presence of the interference, may be estimated at 15 pg/ml. Whereas LIF detection was a feasible detection method in isocratic chromatography with several modifications of the chromatographic parameters, the role of LIF detection in conjunction with gradient elution was limited by an unstable baseline. Raman scattering, which originated from solvents in the mobile phase, was a source of baseline drift as the percentage modifier changed throughout the gradient program.

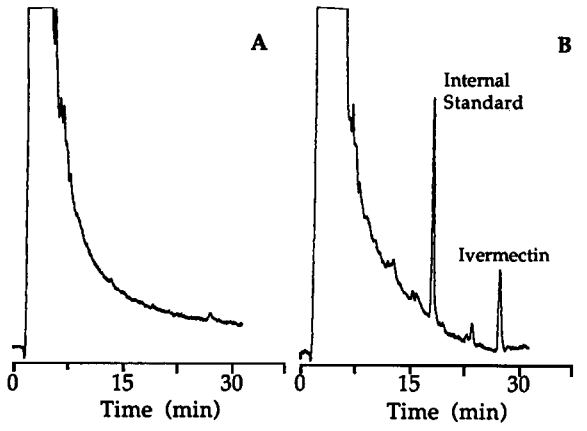


Fig. 5. Chromatograms of unspiked plasma (A) and plasma spiked with 0.18 ng/ml ivermectin ($H_2B_{1a}$) and the internal standard ($B_{1a}$) (B); isocratic chromatography utilizing LIF detection.

Detection sensitivity was compared by plotting the actual peak areas of ivermectin, rather than the ratio of areas, against the concentration of ivermectin in plasma. By examining the slope of the line for the three sets of conditions the relative sensitivity associated with each method was determined. A factor of 2 in sensitivity was gained by using a narrow-bore column in place of a 4.6 mm I.D. column with RF-535 detection. A notable improvement in sensitivity was seen when LIF detection is compared with the RF-535. Using the 4.6 mm I.D. column in both cases, the sensitivity of the LIF detector was 28 times greater than the conventional detector. However, it is important to note that the significant increase in sensitivity using LIF detection did not necessarily translate into the same extent of improved detectability. The magnitude of the noise increased along with the signal, therefore no advantage was gained in the process.

The incorporation of an automated derivatization process was advantageous for several reasons. Automated derivatization and injection required minimal sample handling leading to greater reproducibility and ease of analysis. The potential for analyte and internal standard degradation was removed since derivatization was carried out just prior to injection of the sample. The stability of the ivermectin ($H_2B_{1a}$) and internal standard ($B_{1a}$) in the derivatization matrix was investigated by De Montigny *et al.* [12] and according to their results both species degraded by 15% over a period of 18 h. However, the incorporation of automated derivatization eliminated any uncertainties regarding degradation and/or relative rates of degradation of ivermectin and the internal standard.

## CONCLUSIONS

The limit of detection for ivermectin in plasma was improved by a factor of 6 by using two different approaches. Narrow-bore chromatography with gradient elution allowed trace enrichment at the head of the column, thereby allowing

greater injection volumes and increased mass on the column. Versatility in the assay procedure was introduced in the form of automated derivatization and injection. The inconvenience of long analysis times, which accompanied narrow-bore chromatography with gradient elution, was eliminated by automation. The use of LIF detection was intriguing, however, the additional sensitivity gained was less than anticipated due to a simultaneous increase in baseline noise along with the response factor. The high noise level ultimately was one of the limiting factors in LIF detection at low levels. Additionally, the limit of detection was hindered by the presence of ivermectin carry-over from the work-up procedure, which was present at a concentration of 5 pg/ml. The cost and inconveniences associated with using LIF detection for this particular application did not seem justified considering the moderate improvements in the limit of detection. However, the role of LIF detection in the determination of ivermectin may be of greater significance in situations where minimal sample sizes prevail. For example, the analysis of ivermectin in specific tissues may be feasible utilizing LIF detection with a microseparation method such as micellar electrokinetic capillary chromatography. Furthermore, the specificity of the LIF detection system described in this paper should be applicable to the analysis of other members of the avermectin family which are of therapeutic interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of NCI Training Grant CA 09242 and Inter$_x$ Research Corp./Merck, Sharp and Dohme Research Laboratories.

REFERENCES

1 W. C. Campbell, M. H. Fisher, E. O. Stapley, G. Albers-Schonberg and T. A. Stapley, *Science*, 221 (1983) 823.
2 H. G. Davies and R. H. Green, *Nat. Prod. Rep.*, 3 (1986) 87.
3 J. C. Chabala, H. Mrozik, R. L. Tolman, P. Eskola, A. Lusi, L. H. Peterson, M. F. Woods and M. H. Fisher, *J. Med. Chem.*, 23 (1980) 1134.
4 J. R. Edgerton, D. A. Ostlind, L. S. Blair, C. H. Eary, D. Suhayda, S. Cifelli, R. F. Riek and W. C. Campbell, *Antimicrob. Agents Chemother.*, 15 (1979) 372.
5 E. W. Cupp, M. J. Bernardo, A. F. Kiszewski, R. C. Collins, H. R. Taylor, M. A. Aziz and B. M. Greene, *Science*, 231 (1986) 740.
6 D. D. Oehler and J. A. Miller, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 59.
7 J. V. Pivnichny, J.-S. K. Shim and L. A. Zimmerman, *J. Pharm. Sci.*, 72 (1983) 1447.
8 A. Fox and D. W. Fink, *Analyst*, 110 (1985) 259.
9 J. W. Tolan, P. Eskola, D. W. Fink, H. Mrozik and L. A. Zimmerman, *J. Chromatogr.*, 190 (1980) 367.
10 K. K. Kojima, K. Yamamoto, Y. Nakanishi and H. Katae, *J. Chromatogr.*, 413 (1987) 326.
11 R. Chiou, R. J. Stubbs and W. F. Bayne, *J. Chromatogr.*, 416 (1987) 196.
12 P. De Montigny, J.-S. K. Shim and J. V. Pivnichny, *J. Pharm. Biomed. Anal.*, 8 (1990) 507.
13 J. M. Bostick, J. W. Strojek, T. Metcalf and T. Kuwana, *Appl. Spectros.*, 46 (1992) 1532.