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DETERMINATION OF AVERMECTINS IN PLASMA AT NANOGRAM LEVELS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION[*]

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SUMMARY

An analytical method is described for the determination of the avermectins in plasma based upon high-performance liquid chromatography of fluorescent derivatives of these compounds. The analyte is isolated by adsorption chromatography on Florisil, dehydrated in an acetic anhydride–pyridine mixture, and the fluorophore is further separated by chromatography on silica gel in advance of introduction into a reversed-phase system. This method, which can be applied to samples containing as little as 0.2 ng drug per ml, has an accuracy of 5% mean relative error and a precision of 8% relative standard deviation. A study and discussion of several factors which affect the analytical reaction are included.

INTRODUCTION

The avermectins are a family of new anthelmintic compounds which appear to be the most potent broad-spectrum agents yet reported. Avermectin B_{1a}, for example, is effective against a wide range of helminths in sheep and cattle in single oral or parenteral doses of 0.1 mg/kg (ref. 1). The structures of these compounds are shown in Fig. 1[2].

The unusually low dosages of these drugs require an extremely sensitive and selective analytical method for their determination in the plasma of dosed animals in support of pharmacokinetic studies. Preliminary radiotracer studies using tritium-labeled drug predict peak plasma concentrations of about 100 ng/ml for 0.3–0.5 mg/kg doses. A reversed-phase high-performance liquid chromatographic (HPLC) analytical method with UV detection which was previously developed to determine avermectins in fermentation broth and formulations[3] does not provide the required sensitivity for plasma analyses.

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$R_1 = CH_3$: Components A   (X) = $-CH=CH-$: Components 1

$R_1 = H$:   Components B

$R_2 = C_2H_5$: Components a   (X) = $-CH_2-\overset{OH}{\underset{H}{C}}-$: Components 2

$R_2 = CH_3$: Components b

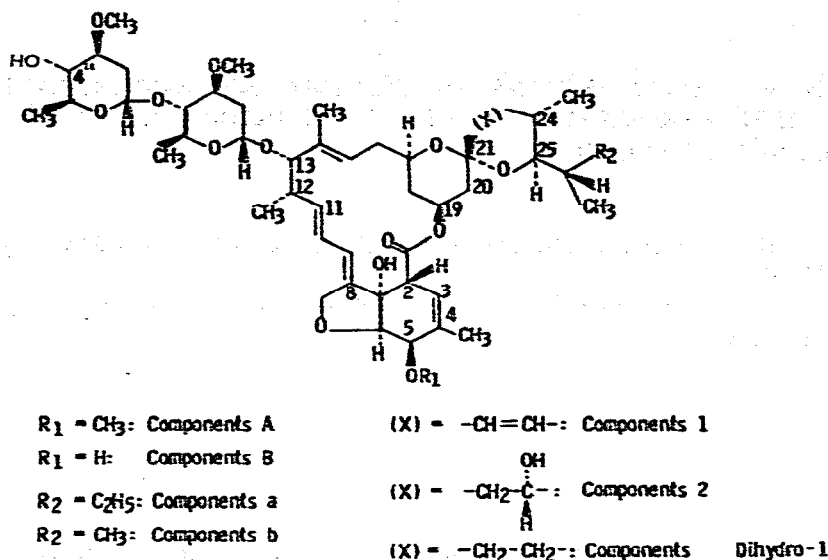   (X) = $-CH_2-CH_2-$: Components   Dihydro-1

Fig. 1. Structure of the avermectins.

Previously Johnson *et al.*[4] demonstrated the sensitivity and selectivity of fluorescence detection in liquid chromatography, which make this technique the optimum method of detection. The present paper describes a procedure for the isolation, derivatization and fluorescence–HPLC determination of the B-series avermectins in plasma. Although most of the data presented are for the dihydro-B_{1a} compound, the method is generally applicable to the avermectins as a group and can be applied to mixtures of avermectins as well as to their hydrolytic degradates. The isolation procedure is based on column adsorption chromatography on Florisil, and the fluorogenic reaction is a dehydration in acetic anhydride–pyridine. A detection limit of about 0.2 ng/ml is obtained using 5-ml plasma samples.

EXPERIMENTAL

Reagents and apparatus

Avermectins. The production, isolation and purification of the avermectins have been previously described[3]. The purity of avermectin reference material was confirmed by reversed-phase HPLC with UV detection at 245 nm.

Stock solutions. Stock solutions were prepared in anhydrous methanol.

Acetic anhydride–pyridine reagent. 99 % acetic anhydride (Aldrich, Milwaukee, Wisc., U.S.A.) and reagent-grade anhydrous pyridine (J. T. Baker, Phillipsburg, N.J., U.S.A.) were purified by distillation. A 2-ml volume of the acetic anhydride and 6 ml of pyridine were combined in a 15-ml centrifuge tube containing about 1 g Type 4A molecular sieve pellets (Union Carbide, Linde Division, South Plainfield, N.J., U.S.A.). A fresh reagent should be prepared before each use.

Florisil columns. Glass columns (10 × 0.7 cm I.D.) were prepared with 6.5 cm diameter funnel tops, and small plugs of silanized glass wool were inserted into the constricted ends of the columns. Each column was packed with 0.40 g of 100–200

mesh Florisil (Floridin, Pittsburgh, Pa., U.S.A.). The Florisil was washed thoroughly with chloroform–ethyl acetate (3:1), dried, washed with water and heated overnight at 120°. Each prepared column is rinsed with 10 ml chloroform immediately before use.

Silica gels column. Small plugs of silanized glass wool were inserted into the constricted end of 5½ in. disposable Pasteur pipets (Kimble, Toledo, Ohio, U.S.A.). Each column was packed with 0.25 g of 40–140 mesh silica gel (J. T. Baker) which was heated overnight at 120°.

Liquid scintillation counting. Counting of tritium-labeled avermectins was performed on a Packard Model 3255 liquid scintillation counter using Packard Instagel counting cocktail (Packard Instrument, Downers Grove, Ill., U.S.A.). Counts were calculated using the internal standard method.

HPLC instrumentation. A Spectra-Physics Model 740 pump, a Model 740c pump controller and a Model 714 pressure monitor comprised the basic chromatograph. The detector was a Perkin-Elmer LC-1000 fluorometer with flow-through cell. The column was a DuPont (Wilmington, Del., U.S.A.) Zorbax ODS (25 cm × 4.6 mm), operated under ambient temperature conditions. A 10-cm precolumn packed with DuPont Permaphase ODS was used to prevent contamination of the analytical column; the precolumn packing should be changed whenever a significant pump pressure increase occurs, indicating clogging.

Extraction procedure (1-ml samples)

In a 15-ml tube, 1.0 ml of plasma was treated with 3.0 ml of ethanol–water (1:1), mixed, placed in an ultrasonic bath 10 min and extracted with three 8-ml portions of ethyl acetate by shaking and centrifuging.

Extraction procedure (5-ml samples)

In a 50-ml centrifuge tube, 5.0 ml of plasma was treated with 10.0 ml of ethanol–water (1:1), mixed, placed in an ultrasonic bath 10 min and extracted with three 10-ml portions of ethyl acetate by shaking and centrifuging.

Isolation procedure

The ethyl acetate extracts were combined in a 50-ml centrifuge tube and evaporated to dryness in a 50° water bath under a stream of dry nitrogen. The residue was redissolved with 1 ml chloroform and transferred by Pasteur pipet to the top of the Florisil adsorption column. The tube was rinsed twice with additional 1-ml portions of chloroform, and these washings were applied to the column in the same manner. When the solvent top reached the Florisil bed, the column was rinsed with 3 ml of chloroform and the effluent discarded. After the chloroform flow stopped, the column was eluted with 25 ml of chloroform–ethyl acetate (3:1). The effluent was collected in a 50-ml centrifuge tube and evaporated to dryness in a 50° water bath under a stream of dry nitrogen.

Derivatization reaction

The residue in the 50-ml centrifuge tube was dissolved with about 0.25 ml acetone and transferred to a silanized 1-ml Reactivial (Pierce, Rockford, Ill., U.S.A.). Two additional 0.25-ml washings were performed and combined in the vial and

evaporated under a stream of nitrogen gas at room temperature. A 0.1-ml aliquot of the acetic anhydride–pyridine reagent was added and the vial tightly closed with a PTFE-lined screwcap and placed on a 100° Reactiblock heater (Pierce) for 20–24 h. The excess reagent was then removed by evaporation under a stream of dry nitrogen at room temperature.

Derivative isolation

The residue in the vial was dissolved with about 0.2 ml chloroform and transferred to a silica gel chromatographic column. The vial was rinsed twice with additional 0.2-ml portions of chloroform which were added to the column. When the solvent reached the silica gel bed, the elumn was eluted with 10 ml of chloroform. Pressure was applied to the top of the column using a rubber pipet bulb to speed the elution. All eluate was collected in a 15-ml centrifuge tube and evaporated to dryness in a 50° water bath under a stream of nitrogen.

HPLC procedure

The residue in the centrifuge tube was dissolved with 0.20 ml of mobile phase (see below) and applied to the HPLC column using a 0.10-ml injection loop. The chromatographic conditions were: mobile phase, acetonitrile–methanol–water (100:15:1); flow-rate, 2.4 ml/min. The detector configuration was: 364-nm bandpass excitation filter, 390-nm short wavelength cut-off emission filter, 480-nm emission monochromator setting; wide slit width.

RESULTS AND DISCUSSION

Fluorogenic reaction

Spectral properties. Fig. 2 presents the ultraviolet absorption spectrum of dihydro-avermectin B_{1a} in methanolic solution. This compound exhibits one absorption band at λ_{max} 244 nm, with shoulders at 238 and 254 nm. It is transparent at $\lambda > 310$ nm. Upon treatment with acetic anhydride in pyridine, two low energy
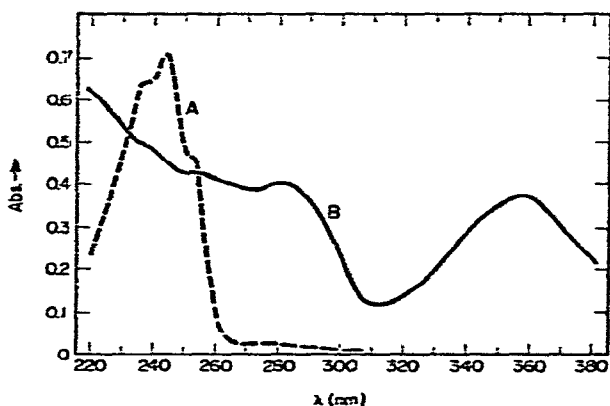

Fig. 2. UV absorption spectra. A, 19 μg/ml dihydro-avermectin-B_{1a} in methanol; B, derivative of dihydro-avermectin-B_{1a} in methanol.

bands appear at λ_{max} 284 and 360 nm (Fig. 2B), which are accompanied by the appearance of visible fluorescence in this same medium. Fig. 3 presents the fluorescence emission and excitation spectra in methanolic solution which result from this treatment. The near coincidence of the long-λ absorption λ_{max} and the 370-nm excitation peak (considering the instrumentation limitations) is confirmation that the fluorescence does result from the same derivative molecule which is measured in Fig. 2. The drug *per se* is not fluorescent.
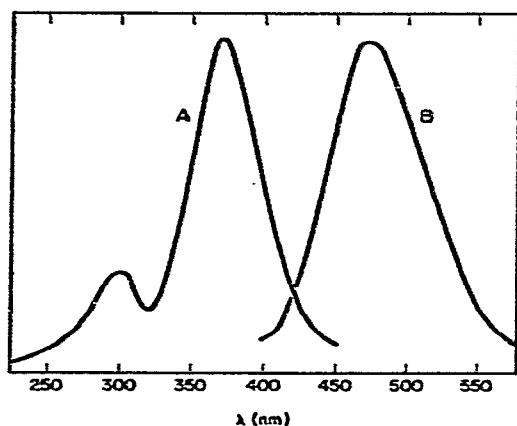

Fig. 3. Fluorescence spectra of dihydro-avermectin-B_{1a} derivative in methanol. A, Excitation spectrum, λ_{emit}: 475 nm; B, emission spectrum, λ_{excite}: 360 nm.

Reaction product. Heating the B-series avermectins in acetic anhydride–pyridine causes elimination of both hydroxyl groups and the protons *trans* to them on the 2–7 ring, as well as acetylation of the hydroxyl at the 4″ position and at the 23 position when present[5]. This dehydration produces an aromatic system which is conjugated with the 8–11 diene to furnish the fluorophore measured in Fig. 3. The reaction scheme is shown in Fig. 4. Dehydration under acetylation conditions has also been reported as an undesired side-reaction during the acetylation of hydroxy-steroids[6].
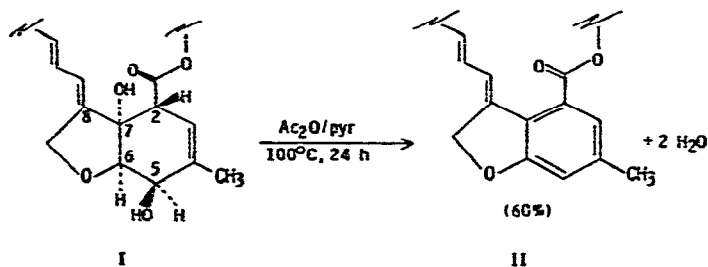

Fig. 4. Derivatization reaction (partial structures). Dehydration of avermectin B-component dihydroxycyclohexene ring (I) to fluorescent aromatic derivative (II).

For characterization of the fluorescent product by 300 MHz nuclear magnetic resonance (NMR) and by mass spectrometry (MS), a mixture of 1.0 ml of anhydrous pyridine, 0.5 ml of acetic anhydride and 100 mg of 22,23-dihydro-avermectin-B_{1a} was heated in an oil bath of 100° for 24 h. The reaction mixture was poured on ice water and extracted with diethyl ether. The ether extract was washed with 2.5 N aqueous HCl, aqueous dilute $NaHCO_3$ solution, and water, was dried with $MgSO_4$ and concentrated *in vacuo* to *ca.* 100 mg light foam. Further separation of the reaction products was achieved by chromatography on silica gel GF plates (20 × 20 cm, 1500 μm thickness, Uniplate, Analtech) developed with dichloromethane–tetrahydrofuran–ethanol (94.9:5.0:0.1). Two UV-absorbing bands with R_F values of ≈0.5 and 0.3 were extracted to give 28 mg of A and 71 mg of B, respectively, as amorphous, white materials. Compound B was identified as 4″,5-di-O-acetyl-22,23-dihydro-avermectin-B_{1a} by direct comparison with authentic material. Compound A corresponded to a highly fluorescent spot on thin-layer chromatography, UV λ_{max}. in methyl alcohol 360 nm ($\varepsilon \approx 1.5 \cdot 10^4$) and 284 nm ($1.7 \cdot 10^4$); MS 880 ($M^+$), 550, 331, 307, 299, 243, 187, 127. Loss of the disaccharide including the 4″-O-acetyl group gives the aglycone fragment of mass 550 which cleaves between the la   ne oxygen and C-19 and between C-12 and C-13, giving the two fragments 307 (C_{13} through C_{29}) and 243 (C_1 through C_{12}, including the aromatic ring). The disaccharide including the 4″-acetyl group appears in the fragment 331, 299 (331 — methanol), 187 (331 — oleandrose) and 127 (187 — acetic acid). The 300 MHz proton NMR spectrum (obtained on a Varian SC-300 spectrometer) of compound A corresponds to that of 22,23-dihydro-avermectin-B_{1a} except for the following significant changes: two somewhat broadened singlets at $\delta 6.90$ and $\delta 6.74$ represent the aromatic C_3 and C_5 protons; the expected *meta*-splitting is obscured through weak coupling with the C_4-methyl protons, but is observed upon irradiation of the C_4CH_3 protons at $\delta 2.32$ with $J = 1.2$ Hz. The C_4CH_3 is shifted from $\delta 1.82$ to 2.32, a monoacetylmethyl appears at $\delta 2.10$, and the triplet representing the C_4-H is shifted from $\delta 3.18$ to $\delta 4.69$ upon acetylation. Significant downfield shifts are also observed for C_9H and C_8CH_2; the signals assigned to C_2H, C_6H and C_7OH in the starting material have disappeared. This evidence led to the proposal of 4″-O-acetyl-2,5,6,7-tetradehydro-5,7-dideoxy-22,23-dihydro-avermectin-B_{1a} as compound II, and the low energy absorption and fluorescence bands which result from this reaction reflect the extension of conjugation of this compound relative to the starting material. These results also suggest that the mechanism of this analytical reaction involves acetylation of the hydroxyl groups prior to dehydration.

Optimization of the analytical reaction. In the present method, 4″-acetylation occurs rapidly, and the reaction is continued under selected conditions to maximize the yield of the fluorescent dehydration product. The effects of temperature, reaction time and reagent composition were studied to optimize the reaction for analytical applications. Although reaction rate increases with temperature, the range of 105–110° was selected for this analytical reaction because of the appearance of several extraneous fluorescent by-products at temperatures > 110 °C. Fig. 5, which presents the effect of reaction time on the derivatization, reveals that maximum yield is realized in 22–24 h. The decrease at longer time probably results from the formation of other by-products. Studies of the amount and proportions of the acetic anhydride–pyridine reagent demonstrated that 0.1 ml of a 1:3 mixture gave the optimum results: reaction yield decreases with lower volumes and lower acetic anhydride concentration, and
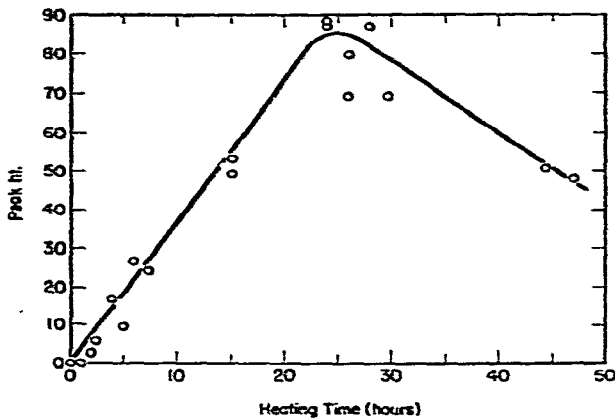
Fig. 5. Effect of reaction time at 100 °C on yield of fluorescent derivative, measured by fluorescence-HPLC.

interfering peaks appear with larger volumes and increased reagent. The fluorescent derivative in the final methyl alcohol solution is stable for at least 24 h.

Derivatization of radiolabeled (tritiated at C_5) dihydro-avermectin-B_{1a} (43 ng) provided HPLC fractions for liquid scintillation counting. The structure of the fluorescent product (II) indicates that radioactivity introduced at this position will be quantitatively retained through the fluorogenic reaction. The total HPLC recovery of the radioactivity was 100%: early-eluting peaks accounted for 37% of the radioactivity and 61% eluted simultaneously with the fluorescent derivative (II), with <2% appearing at longer retention times. These results indicate that a 61% chemical yield has been realized under the optimized conditions for this derivatization reaction. Although the reaction is not stoichiometric, it is sufficiently reproducible so that it can be used as an analytical reaction by comparison with an analytical reference standard which is treated similarly.

HPLC chromatography

Based on the results in Fig. 3, an analytical emission wavelength of 480 nm under 364-nm excitation was selected to optimize HPLC fluorescence detection. When combined with the reversed-phase separation, these wavelengths serve to resolve the analyte from the products of extraneous fluorogens as well as from endogenous fluorophores which are expected to absorb and emit at higher energies. A typical chromatogram from a plasma sample containing 1.4 ng/ml of drug is presented in Fig. 6.

This chromatographic system also resolves the individual avermectin derivatives, all of which exhibit identical fluorescence spectra. Fig. 7 shows the chromatogram resulting from the derivatization of a mixture of avermectins. The more saturated compounds (22,23-dihydro forms) elute at later times. That the fluorescent derivative of compound B_{1a} elutes simultaneously with the derivative produced directly from its 4"-acetate (Fig. 7B, C) is confirmation that the derivatization reaction proceeds via 4"-acetylation. Dihydro-avermectin-B_{1a} and B_{1b}, which differ only in a sec.-butyl vs. an isopropyl group at the 25 position, are resolved in this chromatographic system (Fig. 7D, E).
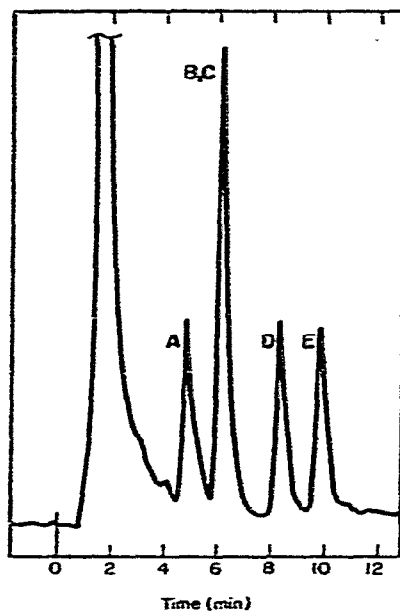
Fig. 6. Fluorescence–HPLC chromatogram for 5-ml cattle plasma sample containing 1.4 ng/ml dihydro-avermectin-B_{1a}.

Fig. 7. Fluorescence–HPLC chromatogram of derivatives. A = avermectin B_{2a}; B = B_{1a}; C = B_{1a}-4″-acetate; D = dihydro-B_{1b}; E = dihydro-B_{1a}.

The avermectins undergo hydrolysis of the disaccaride group under acidic conditions. Fig. 8 shows the separation of dihydro-B_{1a} from its monosaccharide and aglycone forms by fluorescence–HPLC.

Extraction and isolation

Because of the binding of the avermectins to cattle plasma components, quantitative extraction of the drug is difficult to achieve. Of several solvents and extraction systems examined, ethyl acetate proved to be the most efficient for this application. Liquid scintillation counts of radioactive dihydro-avermectin-B_{1a} (680 ng) added in the extraction solvent to a drug-free plasma sample demonstrated that 92–96% of the radioactivity was recovered in this extraction procedure.

Although it is an efficient extraction solvent, ethyl acetate also extracts large amounts of lipid material and other interferences from the plasma, requiring further isolation of the analyte prior to derivatization. Florisil (magnesia–silica gel) has been used as a selective adsorbent in the isolation of drugs from plasma. For example, Bayliss and Steinbeck[7], using Florisil columns, removed interferences with chloroform followed by the use of an ethanol–chloroform eluent, to separate hydroxysteroids from extraneous plasma components. The ethyl acetate–chloroform elution system in the present method provides sufficient isolation for the subsequent derivatization reaction. The silica gel chromatography isolates the derivative from other interfering products and yields a well-filtered solution for the fluorescence–HPLC determination.
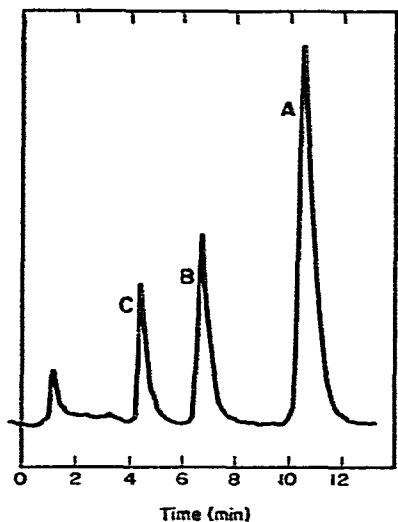
Fig. 8. Fluorescence-HPLC chromatogram of derivatives of dihydro-avermectins; A = B$_{1a}$; B = B$_{1a}$-monosaccharide; C = B$_{1a}$-aglycone.
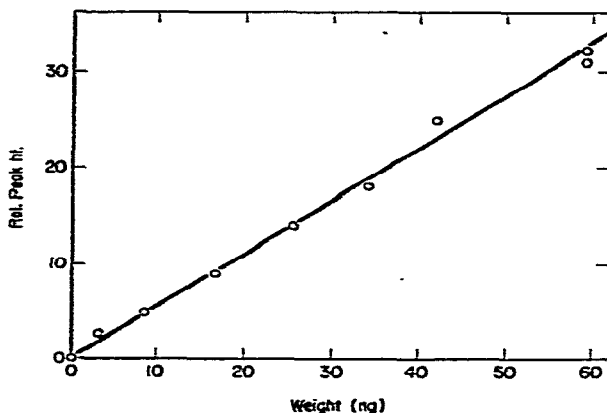
Fig. 9. Calibration line of fluorescence-HPLC peak height *vs.* amount of dihydro-avermectin-B$_{1a}$ taken (pure solutions).

Accuracy and precision

This analytical method is linear and reproducible over the range 1–500 ng analyte. A calibration line obtained from pure solutions of the drug in the 5–60 ng segment is shown in Fig. 9. The small scatter of the data points around this line is a good measure of the precision of the method. A fit of these data to a straight line using a least-squares routine yielded an r^2 value of 0.992. When applied to cattle plasma samples, endogenous interferences restrict the method to samples containing at least 1 ng drug. Using 5-ml plasma samples, therefore, a lower limit of detection of about 0.2 ng/ml is realized.

Ten "spiked" plasma samples were prepared by evaporating to dryness (in centrifuge tubes) aliquots of pure solutions of known dihydro-avermectin-B$_{1a}$ concentration followed by addition of a measured volume of drug-free plasma. These samples were analyzed using the present procedure, and the results are shown in Table I. An average 95% recovery from plasma was obtained *versus* analytical standards processed through the procedure beginning with the Florisil chromatography step. An overall recovery from plasma of 81% *versus* a standard introduced at the derivatization step reflects losses of drug throughout the procedure which are effectively cancelled by using a standard which is processed through the entire procedure.

The reproducibility of the method was examined by performing analyses on eight 200-ng samples, which were prepared by adding pure drug to drug-free plasma. A relative standard deviation of 8% was obtained for these replicates.

This method has been applied to plasma obtained from cattle dosed intravenously with 5[^{3}H]-dihydro-avermetcin-B$_{1a}$. That the samples collected during the

TABLE I

ANALYTICAL RESULTS FOR 1.0-ml PLASMA SAMPLES SUPPLEMENTED WITH DI-
HYDRO-AVERMECTIN B_{1a}

Analytical standard introduced at adsorption column step.

Amount taken (ng)	Amount found	
	ng	% of amount taken
66	66	100%
66	55	83%
132	132	100%
132	127	96%
264	214	81%
264	253	96%
396	391	99%
396	412	104%
528	534	101%
528	480	91%
Mean recovery	$95 \pm 7.8\%$ S.D.	

first few days post dosing yielded nearly identical total radioactivity counts and HPLC analytical results is confirmation of the extraction efficiency and accuracy of this analytical method.

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