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Liquid chromatographic determination of ivermectin in animal plasma with trifluoroacetic anhydride and *N*-methylimidazole as the derivatization reagent

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Abstract: Ivermectin is a potent anthelmintic agent which was detected at low concentrations in cattle plasma by LC after conversion to a fluorescent derivative. This was accomplished by reaction with acetic anhydride (AA) and pyridine for 24 h at 100°C or with AA and *N*-methylimidazole (NMIM) for 1 h at 95°C. Substituting trifluoroacetic anhydride (TFAA) for AA reduced the reaction time to <30 s at 25°C, yielding an intensely fluorescent derivative with substantially fewer reagent by-products. The need for further sample preparation after derivatization with TFAA–NMIM was thereby eliminated, and detection limits of <20 pg ml^{-1} ivermectin could be achieved with 1 ml of plasma by a considerably simpler analytical procedure.

Keywords: *Ivermectin; plasma; pre-column derivatization; trifluoroacetic anhydride; N-methylimidazole; high-performance liquid chromatography; fluorescence detection.*

Introduction

Ivermectin, the 22,23-dihydro-derivative of the fermentation product avermectin B_1, is highly effective for the treatment of both endo- and ectoparasites [1, 2] in numerous animal species. In addition, it has recently been found very effective in interrupting the transmission of *Onchocerca volvulus*, the cause of "river blindness" in man [3]. Because of the high potency of this agent, which is a mixture of >80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and <20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}), there is a need for the determination of ivermectin in plasma at concentrations significantly below 1 ng ml^{-1}.

Analytical procedures for the determination of low concentrations of ivermectin by LC are numerous. The conjugated-diene chromophore of ivermectin, which has an ultraviolet absorbance maximum at 245 nm and a molar absorptivity >30,000 l/mole-cm has been used as the basis for several methods with photometric detection. In general such procedures give detection limits of 1–2 ng ml^{-1} [4–7]. Lower detection limits have been achieved [8] by dehydrative derivatization of ivermectin with acetic anhydride (AA) to produce an intensely fluorescent product. With pyridine as the solvent and catalyst the reaction is carried out in 24 h at 100°C. More recently, the derivatization conditions were modified to include the use of *N*-methylimidazole (NMIM) in dimethylformamide (DMF) [9–11]. This catalyst allows the formation of the fluorophore in 1 h at 95°C. Both reaction conditions, however, require extensive sample preparation before and after the derivatization.

This report describes an improved fluorogenic reagent system using trifluoroacetic anhydride (TFAA) and *N*-methylimidazole, the catalyst, in acetonitrile for the determination of ivermectin in animal plasma. With TFAA–NMIM, in lieu of AA–NMIM, no further sample preparation is required following the derivatization of ivermectin, and significantly lower detection limits can be achieved.

Experimental

Chemicals and reagents

Trifluoroacetic anhydride (>99%) and *N*-methylimidazole (99%) were obtained from Aldrich and used as received. Water used throughout was deionized and filtered. HPLC

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grade tetrahydrofuran (stabilized with BHT), chloroform and acetonitrile were obtained from Fisher. Sep–Pak C18 cartridges, (500 mg) were supplied by Waters. Ivermectin and avermectin B_1 reference standards and cattle plasma were obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA).

Apparatus

Isocratic liquid chromatography was performed on a modular system consisting of an ABI Analytical Spectroflow 400 pump, a Waters model 712 autosampler and a Shimadzu RF-535 fluorometer. Integration was accomplished using a Spectra-Physics SP-4290 integrator. Fluorescence spectra were obtained using a Shimadzu RF-5000U spectrofluorometer. A Waters 990+ photodiode array HPLC detector was used to obtain the absorption spectrum of the "*in situ*" fluorescent derivative of ivermectin.

Reagent solutions

The reagent solutions were prepared by mixing separately trifluoroacetic anhydride in acetonitrile (1:2, v/v) and *N*-methylimidazole also in acetonitrile (1:1, v/v). Both solutions were freshly prepared on a weekly basis.

Standard ivermectin solution

The reference standard stock solution was prepared by dissolving ivermectin (84.8% H_2B_{1a}) in acetonitrile (1.0 mg ml^{-1} H_2B_{1a}). Working solutions were prepared by diluting the stock reference standard solution to concentrations of 4.0, 2.0, 1.0, 0.4 and 0.2 ng ml^{-1} with acetonitrile. The internal reference standard avermectin stock solution was prepared in acetonitrile (1.0 mg ml^{-1} B_{1a}). The working internal reference solution was diluted with acetonitrile to a concentration of 1.0 ng ml^{-1}.

Sample preparation

Drug-free cattle plasma samples (1.0 ml) were fortified with ivermectin (500 μl of 0.2, 0.4, 1.0, 2.0 and 4.0 ng ml^{-1}) in acetonitrile and with the internal standard avermectin (500 μl of 1.0 ng ml^{-1}) in acetonitrile. After mixing for 15 s the solutions were centrifuged at 2500 rpm for 10 min, and 1.0 ml of water was added to the supernatant. After mixing again and centrifugation (2500 rpm for 10 min), the supernatant was applied to a Sep–Pak C18 cartridge preconditioned with acetonitrile (4 ml), chloroform (5 ml), acetonitrile (4 ml) and water (4 ml), respectively. The Sep–Pak sorbent was carefully dried by drawing air through with vacuum for 15 min. Elution of the avermectins was achieved with 5 ml of chloroform. This eluate was evaporated to dryness under a gentle stream of dry nitrogen at elevated temperature (<50°C) in a water bath.

Derivatization procedure

The dry residue was dissolved in 100 μl of the *N*-methylimidazole solution in acetonitrile (1:1, v/v). To initiate the derivatization, 150 μl of the trifluoroacetic anhydride solution in acetonitrile (1:2, v/v) was added. After completion of the reaction (<30 s) an aliquot (100 μl) of this solution was injected directly into the chromatograph.

Chromatographic conditions

The mobile phase consisted of THF–acetonitrile–water (40:40:20, v/v/v) pumped at a flow rate of 1.0 ml min^{-1} through a Zorbax C8 column (Dupont, 5 μm, 4.6 mm i.d. × 250 mm), thermostatted at 30°C, with fluorescence detection at an excitation wavelength of 365 nm and an emission wavelength of 475 nm.

Results and Discussion

Fluorogenic derivatization of ivermectin

Ivermectin contains a tertiary hydroxyl group at C7 and two secondary hydroxyl groups at C4″ and C5 (Fig. 1). When ivermectin is reacted with acetic anhydride in the presence of a base catalyst (pyridine or *N*-methylimidazole) all three hydroxyl groups are acetylated [12]. Subsequently, this acetylated derivative undergoes dehydration at the C2–C7 and C5–C6 positions to form a fluorescent derivative having a six-membered aromatic ring conjugated to a butadiene unit (Scheme 1). With AA–NMIM in dimethylformamide, this conversion requires 1 h at 95°C.

In this study, an alternative anhydride was identified which significantly increased the rate of formation of the fluorophore. With trifluoroacetic anhydride (TFAA), substituted for AA, the derivatization of ivermectin and avermectin (internal standard) in acetonitrile was complete in <30 s at 25°C. The presence of the strong electron withdrawing substituent (trifluoro) appeared to facilitate the dehydration of the acylated intermediate.

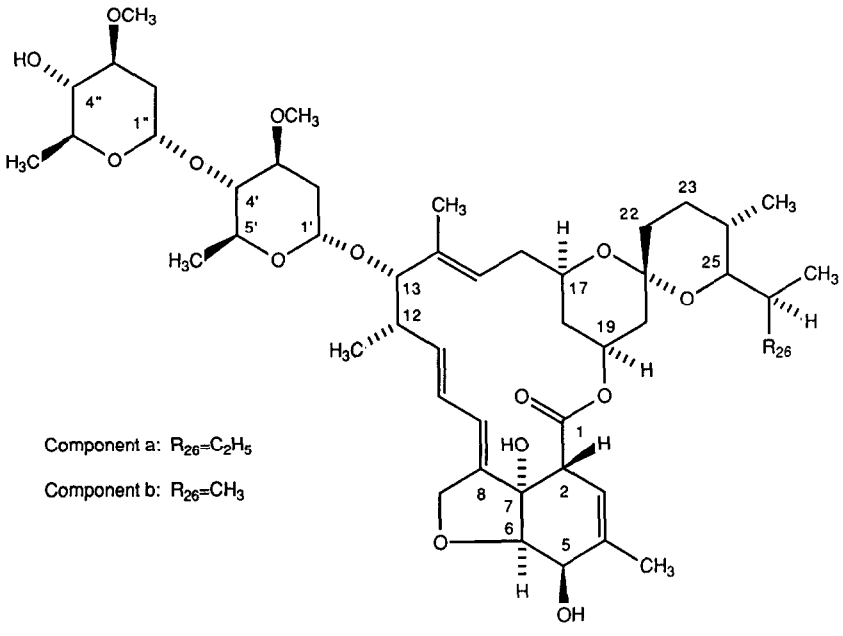
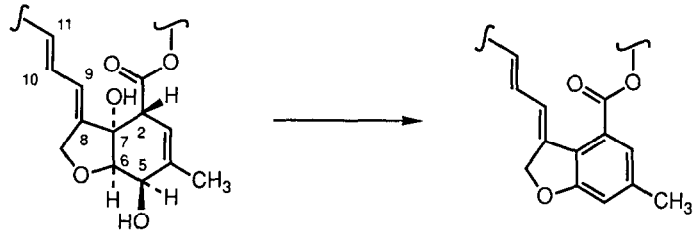


Figure 1
Chemical structure of ivermectin (H_2B_{1a}, H_2B_{1b}).



Scheme 1
Reaction scheme of the formation of the fluorescent derivative of ivermectin following dehydration.

No sign of significant instability was observed for either fluorescent derivative in the reaction mixture. Repeated LC analyses of a solution of ivermectin (4 ng ml^{-1}) and the internal standard (4 ng ml^{-1}) in TFAA–NMIM–ACN (1:1:3, v/v/v) at hourly intervals showed <15% decrease in peak areas after 18 h and constant (±0.7%) peak area ratios (H_2B_{1a}/B_{1a}) throughout this time.

The absorption and fluorescence spectra of the reaction product formed with TFAA–NMIM in ACN are shown in Figs 2 and 3. In a mixed solvent of ACN–water the excitation and emission maxima were 365 and 475 nm, respectively. The fluorescence detector used in this study employed a 150 W xenon lamp with intensity and spectral characteristics well suited for the detection of low concentrations of the ivermectin fluorophore [11].

Application to plasma analysis

Plasma samples were first mixed with acetonitrile to precipitate the unwanted proteins and then subjected to solid-phase extraction, to remove the other interfering plasma constituents prior to the derivatization of ivermectin. The optimum selectivity was achieved by eluting the avermectins from the reversed-phase sorbent with chloroform. Following the rapid evaporation of chloroform, TFAA and NMIM were added for the derivatization of the avermectins. Because substantially fewer reagent by-products were formed when the avermectins were reacted with TFAA–NMIM, no

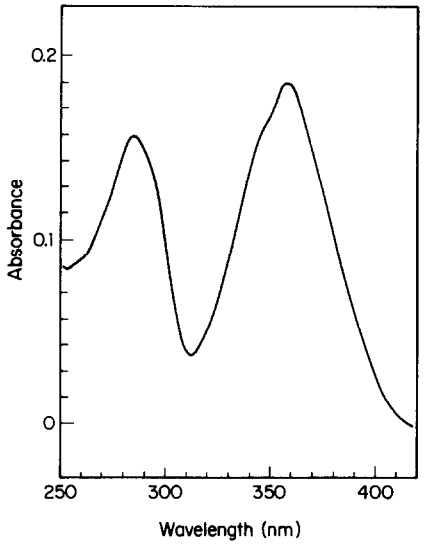


Figure 2
Absorption spectrum of the ivermectin fluorescent derivative determined by HPLC with UV photodiode array detection.

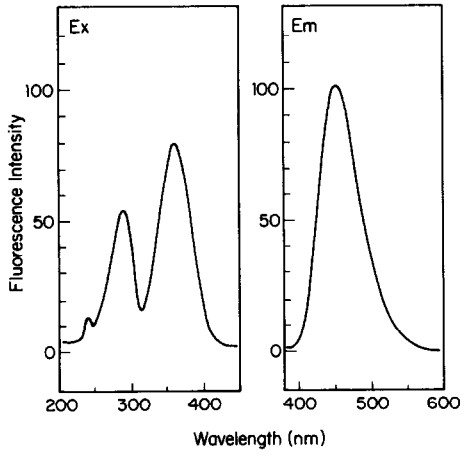


Figure 3
Excitation and emission spectra of the fluorescent derivative of ivermectin formed from the reaction with TFAA and NMIM.

further purification of the reaction mixture was necessary, unlike the previous analytical procedure with AA–NMIM that requires further solid-phase extraction and evaporation steps for LC analysis.

Analyses were carried out by reversed-phase chromatography with a mobile phase consisting of ACN–THF–water. A chromatogram depicting a plasma sample (1.0 ml) obtained from a medicated animal is shown in Fig. 4(A). This plasma sample was fortified with 0.5 ng ml^{-1} internal standard, and the concentration of ivermectin was determined to be 0.13 ng ml^{-1}. The avermectins are completely resolved from each other and display excellent peak symmetry. Figure 4(B) shows the chromatogram of a corresponding drug-free plasma sample without the internal standard. The lack of interferences offered by this method suggests that the detection is limited by instrumental noise.

Assay validation

The linearity and reproducibility of the analytical procedure were tested at 0.1, 0.2, 0.5, 1.0 and 2.0 ng ml^{-1} in quadruplicate at each concentration ($n = 20$) by comparing the peak areas obtained from the cattle plasma samples fortified with ivermectin and internal standard (avermectin 0.5 ng ml^{-1}) with the peak areas of the drug in solution at the same concentrations. The linear expression obtained for the plasma samples was best described by the equation $Y = 0.943$ [±0.010 (SD)] X ng ml^{-1} H_2H_{1a} + 0.021 ng ml^{-1} H_2B_{1a} [±0.010 ng ml^{-1} H_2B_{1a} (SD)] with a correlation coefficient (r) of 0.999. These values demonstrated that the recovery for ivermectin was 94.3% with minimal background at these very low concentrations. A detection limit of approximately 20 pg ml^{-1} ($S/N = 2$) was achieved with 1 ml of plasma, which represents a 10-fold increase in sensitivity compared with the analytical method that requires AA–NMIM as the derivatization reagent system.

This method was also applicable for the determination of avermectin B_{1a} (abamectin) in plasma by selecting ivermectin (H_2B_{1a}) as the internal standard. No modifications are required to achieve the same selectivity and detection limits, unlike the analytical method with acetic anhydride as the derivatization reagent.

Conclusions

An improved derivatization procedure for the determination of ivermectin has been developed. This method allowed the rapid conversion of ivermectin to a fluorescent derivative with TFAA–NMIM in ACN. Furthermore, fewer reagent by-products were formed with TFAA–NMIM than with AA–NMIM, thereby eliminating the need for further sample preparation after derivatization. The TFAA–NMIM reaction conditions moreover, should be applicable to low-level

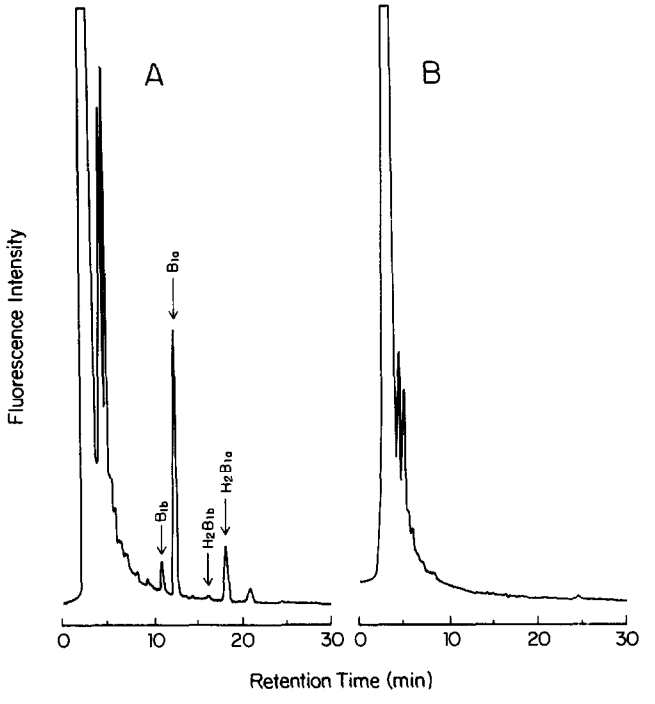


Figure 4
Chromatograms of plasma with (A) and without (B) the internal standard (0.5 ng ml^{-1}, B_{1a}) and ivermectin (0.13 ng ml^{-1} H_2B_{1a}).

determinations of a variety of avermectin analogues in biological fluids.

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