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Note

Determination of ivermectin in human plasma and milk by high-performance liquid chromatography with fluorescence detection

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The avermectins are a new class of macrocyclic disaccharide antiparisitic agents having anthelmintic and insecticidal properties. They are fermentation products derived from *Streptomyces avermitilis*. Ivermectin is the 22,23-dihydro derivative of avermectin B1 and consists of two compounds differing from each other by one methylene group. The two compounds are designated 22,23-dihydroavermectin B1a (or H_2B1a) and 22,23-dihydroavermectin B1b (or H_2B1b). Ivermectin contains $\geqslant 80\%$ H_2B1a and $\leqslant 20\%$ H_2B1b.

The ivermectins have shown efficacy against various stages of filarial parasites [1] and have been studied in numerous animal species. These species include ferrets, dogs, cattle, horses, sheep, pigs and cats. Efficacy has been observed after oral and parenteral administration of the drug and often at low doses (typically 0.05 mg/kg).

The metabolism of ivermectin has been studied in pig liver microsomes [2] and in cattle, sheep, swine and rats [3] using tritium-labelled ivermectin. Several metabolites were isolated and characterized, but in each case the major component was the unchanged drug. Several analytical procedures have been used to measure ivermectin in animal tissues. A high-performance liquid chromatographic (HPLC) assay in combination with reverse isotope dilution analysis has been reported [4] for the determination of tritium-labelled ivermectin. The method involved solvent extraction of tissue radioactivity in the presence of cold carrier followed by HPLC separation of the two components of ivermectin (H_2B1a and H_2B1b). Another assay for ivermectin in cattle tissue involved solvent extraction, preparative liquid chromatography and positive-ion chemical-ionization mass spectrometry [5].

An assay for the determination of ivermectin in plasma that involved deriva-

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tization to form a fluorescent product and subsequent HPLC separation and quantification has been described [6]. The procedure used 5 ml of plasma for the determination of low ng/ml concentrations of drug. High volumes of ethyl acetate were used as the extraction solvent and the overall derivatization procedure was long (24 h) making it unsuitable for large numbers of clinical samples. Several modifications to this method were made [7] for the determination of ivermectin in cattle and sheep tissues. The sample preparation procedure used multiple solvent extraction steps, and 5 g of tissue were required. Another HPLC method reported [8] used normal-phase HPLC with ultraviolet detection for the determination of ivermectin in cattle blood. The method required 5 ml of blood and had a detection limit of 4–5 ng/ml.

Ivermectin is currently undergoing clinical trials in man for the treatment of river blindness. Preliminary data suggest that ivermectin may be active against one or more stages of development of human filarial parasites as well as gastrointestinal nematodes. It may, therefore, be useful in the prevention of blindness caused by *Onchocerca microfilariae*. An assay for ivermectin in human plasma was, therefore, required.

A method was required for the analysis of human plasma that used either 0.5 or 1 ml of plasma and had a detection limit of ~ 1 ng/ml drug. The present method uses reversed-phase HPLC with fluorescence detection. The sample clean-up involves various solid-phase extraction procedures. The derivatization step is based on the method of Tolan et al. [6] and a further solid-phase extraction step is carried out on the derivatized drug. No solvent extraction steps are used in this procedure. The volume of plasma used was 0.5 ml and the limit of detection was 0.2 ng/ml.

Ivermectin has been detected in cow's milk following an oral dose of drug. A recent clinical study with healthy lactating women who were not breast-feeding their infants was carried out to determine whether ivermectin was secreted in the milk. An assay was, therefore, required that was capable of measuring the drug in human milk. The method used was essentially the same as that for plasma. A larger aliquot of milk (2.5 ml) was used for the assay giving a limit of detection of 0.05 ng/ml.

As stated in the introduction, ivermectin consists of two components, H_2B1a and H_2B1b. These components can be resolved by HPLC before and after derivatization. In the methods described, ivermectin will be used as the drug name. However, it is the major component, H_2B1a, that is being assayed in both plasma and milk. All concentrations and limits of detection, therefore, are based on H_2B1a.

EXPERIMENTAL

Reagents

HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Acetic anhydride (99%) and 1-methylimidazole (99%) were purchased from Aldrich (Milwaukee, WI, U.S.A.) and N,N-dimethylformamide from Mallinckrodt (Paris, KY, U.S.A.). Ethyl acetate and chloroform (reagent grade) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). C_2 (500

TABLE I

HPLC GRADIENT CONDITIONS

Time (min)	Percentage methanol
0	70
4	96
12	96
12.1	100
16.5	100
16.6	70

mg) and diol (500 mg) Bond-Elut cartridges were supplied by Analytichem International (Harbor City, CA, U.S.A.). Human control plasma was obtained from Sera-Tec Biologicals (North Brunswick, NJ, U.S.A.). Ivermectin and a monosaccharide analogue of ivermectin, the internal standard, were supplied by Merck Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.).

Instrumentation

A Varian 5000 liquid chromatograph with a built-in column heater was used for this analysis. The autosampler was a Perkin-Elmer ISS-100 and the fluorescence detector a Perkin-Elmer 650S. A Spectra-Physics SP4270 computing integrator was used to measure peak areas and calculate results.

Standard solutions

In this assay of plasma and milk samples only the H_2B1a component of ivermectin was quantified. The ivermectin reference standard contained 83.4% by weight of H_2B1a and a stock solution was prepared in methanol (1 mg/ml as H_2B1a). A series of working ivermectin standard solutions were prepared in methanol by diluting the stock solution. The concentrations were 400, 200, 100, 50, 25, 10 and 5 ng/ml. This produced equivalent plasma concentrations of 40, 20, 10, 5, 2.5, 1 and 0.5 ng/ml using a 500-μl aliquot of plasma spiked with 50 μl of each working standard solution. The corresponding range for milk was 8, 4, 2, 1, 0.5, 0.2 and 0.1 ng/ml using a 2.5-ml aliquot of milk spiked with 50 μl of each working standard. A stock standard solution of ivermectin monosaccharide, the internal standard, was prepared in methanol (1 mg/ml). A working internal standard solution (1 μg/ml) was prepared from this stock solution.

High-performance liquid chromatography

The mobile-phase used in this analysis consisted of water and methanol. Gradient elution was necessary to obtain satisfactory resolution of ivermectin and the internal standard from endogenous plasma peaks. The gradient profile is shown in Table I. The analytical column was a C_{18} reversed-phase Sepralyte (5 cm$\times$4.6 mm, 3-μm packing) supplied by Analytichem International. A Rainin (Woburn, MA, U.S.A.) 0.2-μm filter was connected in front of the analytical column. The columns were maintained at 50°C and a flow-rate of 1.5 ml/min was

TABLE II

INTER-DAY VARIATION OF IVERMECTIN IN PLASMA AND MILK ANALYSIS

Concentration (ng/ml)	Peak-area ratio drug/internal standard (mean ± S.D.)	R.S.D. (%)
Plasma (n=6)		
1	0.044 ± 0.006	12.7
2.5	0.110 ± 0.011	10.1
5	0.220 ± 0.014	6.1
10	0.442 ± 0.024	5.4
20	0.866 ± 0.037	4.3
40	1.762 ± 0.061	3.5
Milk (n=5)		
0.1	0.058 ± 0.007	12.6
0.2	0.109 ± 0.012	10.6
0.5	0.243 ± 0.034	13.8
1	0.483 ± 0.015	3.1
2	0.900 ± 0.028	3.2
4	1.777 ± 0.204	11.5
8	3.411 ± 0.231	6.8

used. The detector was set at an excitation wavelength of 364 nm and an emission wavelength 470 nm. The injection volume used was 30 μl. The integrator was run at an attenuation of 2 (2 mV f.s.d).

Analysis of plasma samples

Plasma standards were prepared by taking blank plasma (0.5 ml), ivermectin working standard (50 μl), working internal standard (50 μl) and methanol (1.25 ml). The solutions were vortex-mixed (15 s) and then centrifuged (20 min at 2000 *g*). The supernatant was transferred to a clean tube and water (1 ml) added.

A C_2 extraction cartridge (500 mg) was activated with methanol (2×1 ml) and then rinsed with water (2×1 ml). The plasma solutions were loaded into

TABLE III

QUALITY-CONTROL SAMPLES FOR IVERMECTIN IN PLASMA AND MILK

Sample	Target (ng/ml)	*n*	Concentration (mean ± S.D.) (ng/ml)	R.S.D. (%)
Plasma				
Low	2.5	9	2.59 ± 0.27	10.48
High	30	9	30.89 ± 0.93	3.00
Milk				
Low	0.5	13	0.50 ± 0.05	10.32
High	6	13	6.07 ± 0.28	4.68

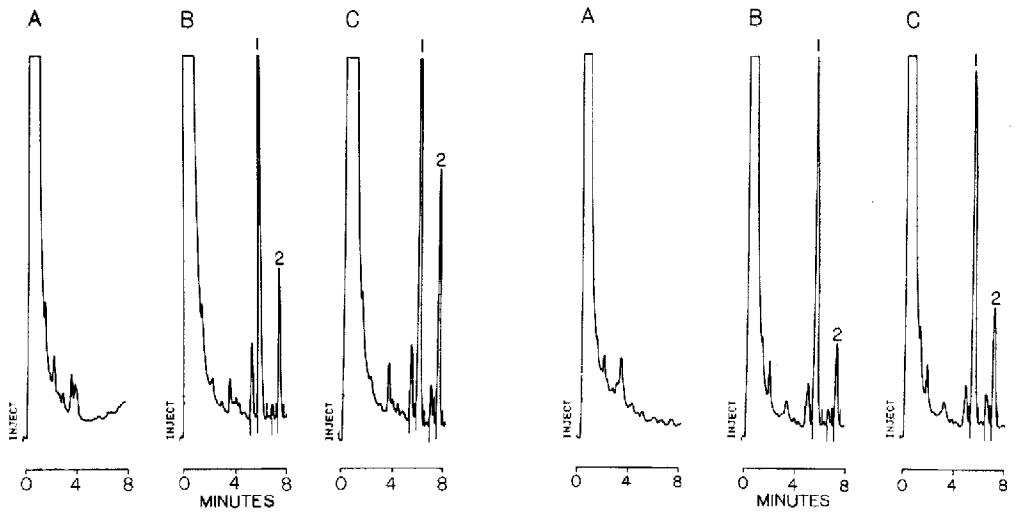

Fig. 1. Typical chromatograms for ivermectin in plasma. (A) Blank plasma; (B) blank plasma containing 100 ng/ml internal standard and 5 ng/ml ivermectin; (C) patient plasma containing 100 ng/ml internal standard and 8.6 ng/ml ivermectin. Peaks: 1 = internal standard; 2 = ivermectin.

Fig. 2. Typical chromatograms for ivermectin in milk. (A) Blank milk; (B) blank milk containing 4 ng/ml internal standard and 0.5 ng/ml ivermectin; (C) patient milk sample containing 4 ng/ml internal standard and 0.73 ng/ml ivermectin. Peaks: 1 = internal standard; 2 = ivermectin.

respective cartridges and a vacuum applied. The eluates were discarded. The cartridges were further washed with water (6×1 ml) and methanol–water (60:40, v/v, 6×1 ml) and the eluates discarded. The cartridges were then eluted with methanol (0.5 ml) and the eluates collected. The collection tubes were transferred to a 60°C water bath where the methanol was evaporated under a stream of dry nitrogen. Ethyl acetate (1 ml) was added to the residue and the tube was then vortex-mixed (15 s) and centrifuged (10 min at 2000 *g*). The supernatant was transferred to a silylated 2-ml autosampler vial and evaporated to dryness with nitrogen as before.

Derivatization reagent consisting of N,N-dimethylformamide, 1-methylimidazole and acetic anhydride (2:3:7, v/v/v) was prepared fresh daily. An aliquot (50 μl) was added to the residue in the autosampler vial and vortex-mixed (15 s). The vial was capped with a PTFE-lined cap and then transferred to a dry heating block (1 h at 95°C). The vials were removed from the heating block, allowed to cool, de-capped, and excess derivatization reagent was removed under nitrogen.

A diol extraction cartridge (500 mg) was conditioned with chloroform (2×1 ml). Chloroform (250 μl) was added to the residue from derivatization and vortex-mixed (15 s). The chloroform solution was transferred to the diol cartridge. The autosampler vial was rinsed with chloroform (250 μl) and this solution also transferred to the cartridge. A vacuum was applied and the eluates were collected. The cartridge was further washed with chloroform (500 μl) and the eluates were combined. The chloroform was evaporated under nitrogen and the residue was

redissolved in methanol (150 μl). An aliquot (50 μl) of this solution was injected for HPLC analysis.

Plasma samples from subjects receiving ivermectin were analyzed by the same scheme substituting subjects' plasma (500 μl) for blank plasma and methanol (50 μl) for working ivermectin standard solution.

Analysis of milk samples

Milk standards were prepared for analysis by taking an aliquot of blank human milk (2.5 ml), ivermectin working standard (50 μl), working internal standard (10 μl) and methanol (6.25 ml). The solutions were vortex-mixed (15 s) and then centrifuged (20 min at 2000 *g*). The supernatant was transferred to a clean tube and water (3.75 ml) added.

This solution was prepared for analysis using the scheme described for plasma. Milk samples were assayed in the same way substituting methanol (50 μl) for the working standard solutions.

RESULTS AND DISCUSSION

This assay was linear over the range of 0.5 – 40 ng/ml using a 500-μl aliquot of plasma. For the analysis of milk samples a standard line of 0.1 – 8 ng/ml was used and a 2.5-ml aliquot of milk was needed to achieve this sensitivity. A plot of drug concentration against the ratio of drug peak area to internal standard peak area confirmed the linearity of both assays. Typical values for the correlation coefficient (r) were $\geqslant 0.9995$. The overall recovery of ivermectin from plasma and milk was $\geqslant 95\%$. This was confirmed by taking direct standards through the derivatization procedure but omitting the cartridge clean-up steps.

The reproducibility of each assay was checked by assessing the inter-day variability of the two standard lines. The results are presented in Table II. For the plasma assays all points on the standard line had relative standard deviation (R.S.D.) of $\leqslant 12.7\%$. The corresponding value for the milk assays was $\leqslant 13.8\%$. The stability of ivermectin in plasma and milk was checked by preparing quality-control samples of known concentration at the beginning of the analysis. These samples were then frozen and stored with the subjects' samples. The quality-control samples were assayed alongside subjects' samples and the results are presented in Table III. The R.S.D. values for the plasma quality-control samples were 10.48 and 3.00% for the low and high controls, respectively. The corresponding values for the milk controls were 10.32 and 4.68%, respectively.

The selectivity of these assays was checked by processing blank plasma and milk samples as well as pre-dose blank samples from the subjects. No significant peaks eluted at the retention times of the drug or internal standard. Typical chromatograms from the analysis are shown in Fig. 1 for plasma and Fig. 2 for milk. The internal standard eluted at 6.0 min and ivermectin (H_2B1a) at 7.6 min. The limit of detection for this assay was approximately 0.2 ng/ml for plasma and 0.05 ng/ml for milk (based on a signal-to-noise ratio of 5:1).

The gradient elution procedure was found to give superior resolution of the

internal standard from endogenous compounds co-eluting at similar retention times. Switching to 100% methanol between injections was necessary to remove several late eluting peaks that would have caused problems with subsequent injections. This procedure has also increased the life of the analytical HPLC column. No deterioration in column performance has been observed following greater than 200 injections of plasma extracts.

The choice of fluorescence detector used in this assay is a key factor in obtaining high sensitivity. Detectors having xenon lamp sources were found to be superior to detectors with deuterium sources. The sample preparation steps and derivatization procedure have been optimized so that minimal endogenous interference is encountered. The wash step with 60% methanol is particularly important in this regard. A higher percentage of methanol gives elution of ivermectin from the C_2 cartridge. The overall recovery of ivermectin using this procedure was $>95\%$ over the entire range of the standard line. Solvent extraction procedures were also tried as a means of sample preparation. They were consistently more tedious and time-consuming than the cartridge method. Additionally, more endogenous interference was encountered. The overall recovery rates were lower, and the reproducibility was worse than the cartridge procedure, particularly at low concentrations on the standard line.

The method described is presently being used for the analysis of ivermectin in human plasma and milk samples and has been successfully used for previous studies. The method is rugged and few problems have been encountered.

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