Determination of Ivermectin Residue in Animal Tissues by High-Performance Liquid Chromatography–Reverse Isotope Dilution Assay

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A microgram-scale reverse isotope dilution assay (RIDA) method has been developed for the determination of tritium-labeled ivermectin residue in animal tissues. The method consists of (1) extraction of tissue radioactivity by solvent partition in the presence of unlabeled ivermectin carrier, (2) high-performance liquid chromatography (HPLC) of tissue extract to resolve the two components of ivermectin, i.e., dihydroavermectin B_{1a} and dihydroavermectin B_{1b}, and (3) determination of the specific activities of the chromatographically recovered ivermectin components by UV absorbance. This HPLC–RIDA method has been used in assays of the unaltered ivermectin residues in the edible tissues of steers, sheep, rats, and swine mostly at residue levels above 60 ppb. This method should be generally applicable in assays of radioactive residues at submicrogram levels.

The reverse isotope dilution assay (RIDA) is a useful technique for both qualitative and quantitative identification of radioactive compounds in complex mixtures. The conventional method that is widely used in all areas of organic analyses is by the addition of a known weight in large excess of an unlabeled compound to the labeled counterpart of known specific activity, usually in a mixture. The diluted radioactive material is then isolated, purified by multiple recrystallization, and radioassayed. The criterion of purity is a constant specific activity after consecutive recrystallization (Tolgyessy et al., 1972; Marshall, 1976). RIDA is widely applied in drug metabolism studies for the characterization and quantitation of metabolites or drug residues because of the accuracy of the method. However, the recrystallization procedure is impractical and is impossible in some cases when the compound of interest is present at extremely low levels or when the purification of the compound cannot be achieved by crystallization. One particular example is the assay of drug residues in animal tissues where the radioactive residue to be assayed is often present at ppb levels.

A new antiparasitic agent, ivermectin (Figure 1) is one of such compounds that presents problems in conventional tissue residue assay methods because of its low levels. Ivermectin is a synthetic derivative of the naturally occurring avermectin B_{1a} and B_{1b} and consists of two components, the 22,23-dihydroavermectin B_{1a} (H_2B_{1a}, ≥80%) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b}, ≤20%) (Chabala et al., 1980). In the tissue residue study by conventional RIDA, the addition of milligrams of the "cold" ivermectin carrier would dilute the residue so extensively that accurate determination of the specific activity would be extremely difficult. On the other hand, the use of large tissue samples that will provide more radioactive residues is also impractical because of the extensive purification required in the extraction and isolation of the drug residues. Because of the similar chemical properties of H_2B_{1a} and H_2B_{1b}, it would be also impossible to separate and purify these residue components by the conventional crystallization procedure. In this paper we describe a modified microgram-scale RIDA method in which high-performance liquid chromatography (HPLC) was employed to resolve and purify the microgram quantities of the diluted radioactive ivermectin residues isolated from 5–20 g of animal tissues. Purification of the unaltered drug to constant specific activity was achieved by repetitive reverse-phase (RP) HPLC or by consecutive two-stage HPLC with both the reversed-phase and normal-phase (NP) modes of chromatography. Comparison of the RIDA results with assay by an alternative method using fluorescence detection (Tolan et al., 1980; Tway et al., 1981) is also presented.

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EXPERIMENTAL SECTION

Reagents. All organic solvents were HPLC grade from either Fisher or MCB Co. All other reagents were of analytical-grade purity. Aqueous solutions were prepared with water that had been doubly distilled and filtered through a 0.45-μM filter (Millipore). The dihydroavermectin B_{1a} and B_{1b} reference standards were at least 97% pure by reversed-phase HPLC. A stock standard solution of ivermectin was prepared in absolute ethanol at about 1 mg/mL. The concentration was determined from the absorbance at 247 nm of a 1:50 diluted solution of the stock and calculated based on the absorption coefficient of ivermectin ($A^{1\%}_{1cm}$ = 365).

HPLC Apparatus. Liquid chromatographs from Spectra Physics (Model 8700) and Lab Data Control (Constametric I) were used in all studies described in this paper. These chromatographs were equipped with a sample valve (Rheodyne, Model 7120), a 100-μL sample loop, and an ultraviolet detector (Schoeffel Instruments, Model 770). A recorder–integrator (Hewlett Packard, Model 3280) and a fraction collector (LKB, Model 2111) were connected to the SP 8700. The LDC unit was equipped with a fraction collector (LKB, Model 2211). Fractions on HPLC charts were marked by an event marking device installed between the detector and the fraction collector. All chromatograms were monitored by UV detection at 245 nm except for the fluorescence derivatives, which were monitored with a fluorometric detector (Schoeffel, Model FS-970) at an excitation wavelength of 364 nm and an emission wavelength of 440 nm.

HPLC Conditions. *Reversed-Phase HPLC (RP-HPLC).* A Zorbax ODS analytical column (Du Pont, 4.6 mm × 25 cm) was used, with a CO:Pell ODS packed guard column (Whatman Co.). Mobile phases were mixtures of acetonitrile–methanol–water, (A) 49.2:32.8:18 v/v/v and (B) 56.4:37.6:6 v/v/v, at flow rate 1.0 mL/min.

Normal-Phase HPLC (NP-HPLC). A Zorbax Sil analytical column (Du Pont, 4.6 mm × 25 cm) was used with HC Pellosil packed guard column (Whatman Co.). The mobile phase used was ethanol–isooctane (10:90 v/v) at a flow rate of 1.0 mL/min.

Scintillation Counting. Aliquots of extraction and HPLC recovered solution were scintillation counted in

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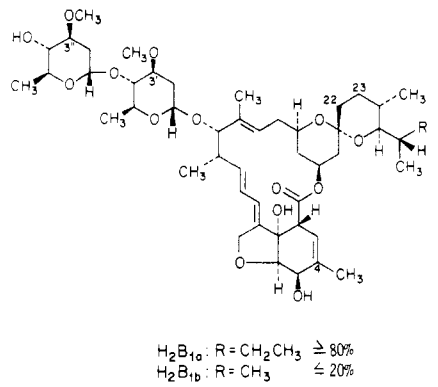

Figure 1. Structure of ivermectin.

Insta-Gel cocktail (Packard) with a TriCarb Model 3310 scintillation spectrometer (Packard). The external standard ratio method was used in all scintillation counting. Efficiency of 36–40% was generally obtained with tritium radioactivity.

UV Spectroscopy. UV absorption spectra of the HPLC recovered H_2B_{1a} and H_2B_{1b} were recorded with a UV–vis spectrometer (Perkin-Elmer, Model 559) in ethanol in 1.0-cm cuvettes.

Assay of Fluorescence Derivatives of H_2B_{1a} and H_2B_{1b}. The derivatization method reported by Tway et al. was followed with modifications. 22,23-Dihydroavermectin H_2B_{1a} monosaccharide (H_2B_{1a}-MS) was added as internal standard in all tissue samples. Tissue residue derivatives were chromatographed by RP-HPLC using solvent condition B. Standard curves of H_2B_{1a} and H_2B_{1b} were established with the HPLC peak height ratio of H_2B_{1a}:H_2B_{1a}-MS and H_2B_{1b}:H_2B_{1a}-MS, respectively. All compounds were spiked into tissues from control animals and were extracted with the same procedure as that used for the experimental tissue (Chiu et al., 1980).

Animal Tissue Samples. Steer kidney samples were from Angus steers dosed subcutaneously with 22,23-[^{3}H]ivermectin at 0.3 mg/kg (specific activity of 0.1 μCi/μg) and slaughtered 7 days postdose. Rat muscle samples were composited from 24 male CRCD rats (Charles River Laboratories, Massachusetts) dosed by gavage with 22,23-[^{3}H]ivermectin at 0.3 mg/kg in sesame oil (specific activity of 0.5 μCi/μg) and killed 1 day after dosing. Radioactive residue in the tissue was determined by combustion assay (Jacob et al., 1979; Campbell et al., 1983).

RIDA Procedure. The procedure for the extraction of radioactive ivermectin residue from animal tissues is shown in the flow diagram (Figure 2). For residue levels between 50 and 200 ppb, 5–20 g of tissue samples was used so that a minimum of 50 000 dpm of radioactivity was available for HPLC analysis. Unlabeled ivermectin (750 μg) was added to the tissue and homogenized in 100 mL of acetone–water (50:50 v/v). After the homogenate was transferred to a 250-mL centrifuge bottle, the contents were extracted with an equal volume of methylene chloride and centrifuged to separate the phases. The methylene chloride layer was evaporated to dryness, redissolved in 20 mL of ethanol–water (90:10 v/v), and washed with about 20 mL of isooctane. To the ethanol solution 1.5 volumes of sodium phosphate buffer (0.1 M, pH 7) was added and the mixture was extracted with cyclohexane. The cyclohexane extract was evaporated to dryness, redissolved in about 2 mL of methylene chloride, and passed through a Silica Sep-Pak cartridge (Waters Associates) with a syringe. The cartridge was then eluted with an additional 10 mL of methylene chloride and about 20 mL

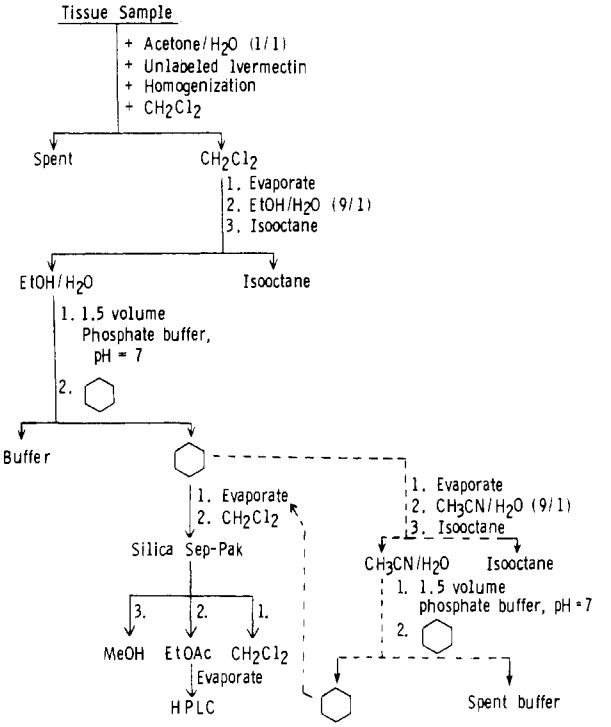

Figure 2. Extraction procedure for the isolation of unaltered 22,23-[^{3}H]ivermectin from liver, kidney, muscle, and fat tissues. Dashed lines indicate additional purification steps required in some samples.

of ethyl acetate. The ethyl acetate eluate was concentrated by evaporation and redissolved in 200 μL of acetonitrile–methanol (6:4 v/v) for RP-HPLC analysis in solvent system A. The column effluent fractions corresponding to the peaks of H_2B_{1a} and H_2B_{1b} were collected and pooled separately. The solution was evaporated to dryness and redissolved in 2–4 mL of ethanol for UV measurements. The concentration of the solution was determined from the absorbance at 247 nm with reference to the absorbance coefficient of ivermectin. Aliquots of the same solution were removed for scintillation counting, and the specific activity in dpm/μg was calculated.

The proportion of the unaltered ivermectin components in the total radioactive residue was calculated as

$$\% \text{ of } H_2B_{1a} = \text{total radioactivity in } H_2B_{1a} (R_{H_2B_{1a}})/\text{total radioactivity in tissue sample } (R_{TS})$$

where $R_{H_2B_{1a}} = SA_{H_2B_{1a}} \times W_{H_2B_{1a}}$.

$SA_{H_2B_{1a}}$ designates the specific activity (dpm/μg) of H_2B_{1a} recovered from HPLC purification and $W_{H_2B_{1a}}$ is the weight of H_2B_{1a} in cold carrier. The percent of H_2B_{1b} in the total radioactive residue is calculated similarly by using the above equation with data from the analysis of H_2B_{1b}.

Purification of Ivermectin Residue from Rat Tissue to Constant Specific Activity. A 2.5-g (121 000-dpm) sample of composite rat muscle was extracted and analyzed as described, with the addition of 500 μL (560.4 μg) of unlabeled ivermectin. The final muscle extract was analyzed by RP-HPLC (solvent condition A) as described. Column effluent was collected at 1-min invervals, and those corresponding to the same peak on the UV tracing were pooled and the specific activity was measured. The two samples thus recovered from the H_2B_{1a} and H_2B_{1b} peaks were then combined, repurified by methylene chloride extraction, and rechromatographed with the same HPLC condition. Peak fractions from the second HPLC were analyzed similarly. Each of the resulting H_2B_{1a} and H_2B_{1b} samples was then chromatographed separately twice, and

Table I. Study of Specific Activities of 22,23-[^{3}H]H_2B_{1a} and 22,23-[^{3}H]H_2B_{1b} in Rat Muscle Extract by Repetitive HPLC–RIDA

successive HPLC runs	H_2B_{1a} peak fractions	H_2B_{1a} sp act., dpm/μg	H_2B_{1a} % of ^{3}H residue	H_2B_{1b} peak fractions	H_2B_{1b} sp act., dpm/μg	H_2B_{1b} % of ^{3}H residue
tissue extract I[a]						
first	28–32	139.5	54.4	20–24	309.6	22.3[b]
second	33–37	136.3	53.2	25–27	134.5	9.7
third	28–32	131.6	51.5	21–24	127.8	9.3
fourth	36–39	133.6	52.3	25–27[c]		
tissue extract II						
first	31–36	160.5	52.3	23–26	157.3	9.5

[a] Extracts I and II were from two muscle tissue samples and carried out by two individuals. [b] The UV peak corresponding to these fractions showed a distinct shoulder. The fractions corresponding to the shoulder were removed and the remainder was chromatographed a second and third time. [c] Not enough mass for UV determination.

the specific activities were determined.

RESULTS AND DISCUSSION

The RIDA of 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b}) in the animal tissues described in this paper consists of essentially three steps: (1) the multistep extraction of the unchanged drug from tissues by solvent partition in the presence of several hundred micrograms of the cold carrier, (2) the resolution of H_2B_{1a} and H_2B_{1b} in the final tissue extract by reverse-phase HPLC and further purification by normal-phase HPLC, if necessary, and (3) determination of specific activity of the HPLC recovered H_2B_{1a} and H_2B_{1b}. Comparison of the specific activities before and after the dilution by cold carrier gives the proportion of each compound in the total radioactive residue.

The described method was applied to a number of control plasma and tissue samples to which [^{3}H]H_2B_{1a} was added. Recoveries and accountabilities for the spiked-in radioactivity in plasma, liver, and fat were between 90 and 96%, and the specific activities of the final HPLC isolates were within about 5% of the initial value.

With most tissue samples extracted by the described procedures, the final extract was clean enough that only one reverse-phase chromatography was needed to obtain pure H_2B_{1a} and H_2B_{1b}. In samples where contamination was present (e.g., asymmetrical HPLC peaks), the column effluent corresponding to the drug peak on the UV tracing was recovered, repurified either by solvent extraction or more efficiently by a second HPLC using the reversed-phase or normal phase HPLC system. The combination of the two phases of HPLC has given very satisfactory purities of the recovered drug components. In most experiments, the UV absorption profile of each column effluent fraction under a peak was determined separately (Figure 3) and the individual specific activity calculated. Fractions that did not show an identical UV profile as the standard ivermectin were discarded and not included in subsequent purification and analysis. Results showed that with samples that gave symmetrical HPLC peaks, the pooled effluent fractions under the peak usually give identical specific activity to that obtained from individual effluent fractions.

In order to substantiate the methodology and prove that the HPLC–RIDA method does provide compounds with constant specific activity, the tissue extract of a composite rat muscle sample was chromatographed as described under Experimental Section to isolate the radioactive ivermectin components. The compounds were then chromatographed repetitively for purification and the specific activities of H_2B_{1a} and H_2B_{1b} determined at each stage of chromatographic isolation. The results are shown in Table I. Results from an independent RIDA experiment carried out on another sample of the same tissue is included in the table for comparison. As shown in Table I, the RIDA results agree well within the repetitive HPLC runs of the first experiment and are consistent also with those of the second experiment. The difference of the specific activities of the same compound between the two experiments was due to the different amount of cold carrier used. Examination of results in Table I shows that the specific activities of H_2B_{1a} remain constant in the four consecutive HPLC runs. Constant specific activity in H_2B_{1b} was also achieved after the removal of a contaminant by the second HPLC.

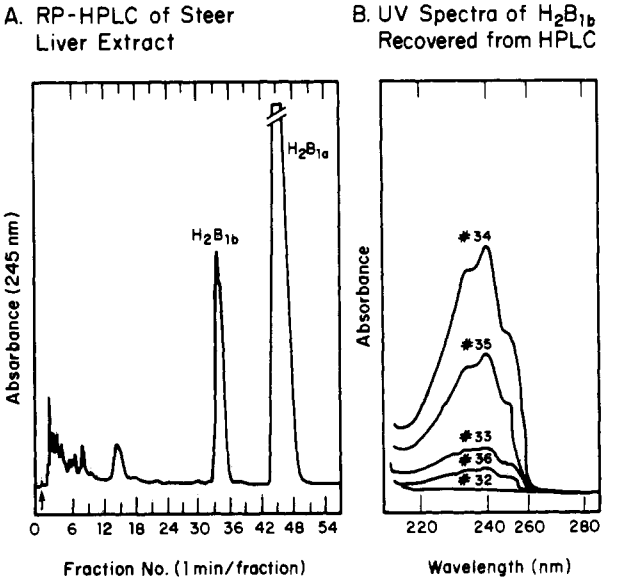

Figure 3. HPLC–RIDA of unaltered 22,23-[^{3}H]ivermectin in steer liver. Panel A shows the RP-HPLC of steer liver extract in solvent condition A. The UV tracing shows the peaks of H_2B_{1a} and H_2B_{1b} from unlabeled ("cold") carrier of ivermectin added prior to the extraction. Panel B shows the UV absorption profiles of the HPLC column effluent fractions of the H_2B_{1b} component recovered from the chromatogram.

In addition to the repetitive HPLC assays described above, the accuracy of the assay was further validated by charging the reversed-phase HPLC isolated components to a normal-phase column. The new column eluate was then run again on the reversed-phase column. No change in the assay values were observed for duplicate samples from the first reversed-phase chromatography.

In the tissue RIDA experiments discussed above, the masses of the unaltered radioactive H_2B_{1a} and H_2B_{1b} in the analytical samples were usually between 0.1 and 1 μg. The dilution of the cold carrier by the residue mass would be minimal (~0.05–0.2%) and is not a concern in the specific activity calculation.

The ivermectin components H_2B_{1a} and H_2B_{1b} can be converted to fluorescent compounds by chemical reaction

Table II. Comparison of RIDA and Fluorescence Assay of 22,23-[^{3}H]Ivermectin Residue in Steer Kidney Tissue

unaltered drug	1981,[a] 63 ppb[b] % of ^{3}H residue	ppb	1994,[a] 68 ppb[b] % of ^{3}H residue	ppb
by RIDA[c]				
H_2B_{1a}	47	30	53	36
H_2B_{1b}	9.2	5.8	12	8.1
by fluorescence assay[d]				
H_2B_{1a}	49	31	59	40
H_2B_{1b}	7.3	4.6	12	8.2

[a] Animal number. [b] Total residue. [c] Concentration (ppb) of unaltered drug calculated from percent of ^{3}H residue by RIDA. [d] Percent of drug in ^{3}H residue calculated from concentration (ppb) of drug by fluorescence assay.

with acetic anhydride in the presence of a base, which leads to the formation of conjugated dehydration products (Tolan et al., 1980). An assay method that utilizes fluorescence detection of these conjugated derivatives of the unlabeled tissue residue was developed by Tway et al. The assay was useful because of its sensitivity and application in assay of tissue residues where isotope labeling is not available. By using the published procedure with the addition of standard 22,23-dihydroavermectin B_{1a} monosaccharide (H_2B_{1a}-MS) as the internal standard, we have also assayed some of the tissues with residue contents below 60 ppb. Comparison of the HPLC–RIDA results obtained from the steer kidney tissue and those by the fluorescence detection method is shown in Table II. These assays were carried out on kidney tissues with residue levels at about 65 ppb. Agreement within 5% is usually observed with H_2B_{1a} at the 30–40-ppb level. Deviation of about 10% exists between the values of the minor component H_2B_{1b}, which was present at 4–8 ppb. At this level, it is approaching the sensitivity limit of the fluorescence assay, and agreement about 10% is nearly the best one can obtain. Since the fluorescence assay measures directly the amount of H_2B_{1a} and H_2B_{1b} residue content independent of the total tissue residue, comparable results of the RIDA and the fluorescence assay further substantiates the validity of the HPLC–RIDA method. The advantage of the RIDA of course is that the accuracy of the method is independent of the total recovery of the compound from the tissue extraction procedure. In cases where the extraction of the drug residue or metabolite is difficult, the RIDA method is probably the only reliable means for accurate quantitation.

The HPLC–RIDA method described in this paper has been successfully applied in assays of ivermectin residues in edible tissues from steers, sheep, swine, and rats. Assays of the tissue residue of another animal health drug, Clorsulon [4-amino-6-(trichlorovinyl)-1,3-benzenedisulfonamide], are also satisfactory (Chiu et al., 1983). On the basis of our results, this method should be generally applicable as long as the concentration of the compound of interest can be determined accurately.

ACKNOWLEDGMENT

We thank Ruta Sestokas for technical assistance and Terry Rafferty for typing the manuscript.

Registry No. H_2B_{1a}, 71827-03-7; H_2B_{1b}, 70209-81-3; ivermectin, 70288-86-7.

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Received for review April 2, 1984. Accepted August 28, 1984.