Kenaga, E. E.; Goring, C. A. I. Proceedings of the 3rd Symposium on Aquatic Toxicology, ASTM, Philadelphia, 1979.
Lambert, S. M. *J. Agric. Food Chem.* **1967**, *15*, 572–576.
Lambert, S. M. *J. Agric. Food Chem.* **1968**, *16*, 340–343.
Leo, A. J.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525–616.
Lord, K. A.; Briggs, G. G.; Neale, M. C.; Manlove, R. *Pestic. Sci.* **1980**, *11*, 401–408.
Lord, K. A.; Helene, C. G.; de Andrea, M. M.; Ruegg, E. F. *Arq. Inst. Biol., Sao Paulo* **1978**, *45*, 47–52.
Martin, H.; Worthing, C. R. "Pesticide Manual", 5th ed.; British Crop Protection Council: London, 1977.
May, W. E.; Wasik, S. P.; Freeman, D. H. *Anal. Chem.* **1978**, *50*, 997–1000.
McGowan, J. C. *J. Appl. Chem.* **1954**, *4*, 41–47.
McGowan, J. C. *Nature (London)* **1963**, *200*, 1317.
Moriguchi, I. *Chem. Pharm. Bull.* **1975**, *23*, 247–257.
Neely, W. B.; Branson, D. R.; Blau, G. E. *Environ. Sci. Technol.* **1974**, *8*, 1113–1115.
Nys, G. G. Rekker, R. F. *Chim. Ther.* **1973**, *8*, 521–535.
Quayle, O. R. *Chem. Rev.* **1953**, *53*, 439–589.
Riley, D. In "The Persistence of Insecticide and Herbicides"; Beynon, K. I., Ed.; British Crop Protection Council: London, 1976; pp 109–115.
Seidell, A. "Solubilities of Inorganic and Organic Substances", 2nd ed.; Van Nostrand: New York, 1920.
Sharom, M. S.; Miles, J. R. W.; Harris, C. R.; McEwen, F. L. *Water Res.* **1980**, *14*, 1095–1100.
Tulp, M.; Hutzinger, O. *Chemosphere* **1978**, *7*, 849–860.
Veith, G. D.; DeFoe, D. L.; Bergstedt, B. V. *J. Fish. Res. Board Can.* **1979**, *36*, 1040–1048.
Vreeland, V. *Environ. Pollut.* **1974**, *6*, 135–140.
Yalkowsky, S. In "Design of Biopharmaceutical Properties through Prodrugs and Analogs"; Roche, E. F., Ed.; American Pharmaceutical Association: Washington, DC, 1977; pp 392–408.

Received for review December 4, 1980. Accepted June 9, 1981.

Determination of Ivermectin in Cattle and Sheep Tissues Using High-Performance Liquid Chromatography with Fluorescence Detection

Patricia C. Tway,* James S. Wood, Jr., and George V. Downing

An analytical procedure has been developed for the isolation and determination of the antiparasitic agent ivermectin in sheep and cattle tissues. The method is based upon the detection of a fluorescent derivative of this compound following high-performance liquid chromatography. The assay has a lowest limit of reliable measurement of 10 ppb and a limit of detection of 1–2 ppb. Recoveries of ivermectin spikes average 83% for liver, kidney, muscle, and fat. In residue studies liver appears to retain the residue the longest; a negligible residue of 11 ppb was found in cattle liver at the proposed withdrawal time of 28 days. In sheep liver no residue was detected at the proposed withdrawal time of 14 days.

The avermectins, which are isolated from the mycelia of *Streptomyces avermitilis*, are a new family of antiparasitic compounds which are potent broad-spectrum agents at very low dosage levels. For example, avermectin B_{1a} is effective against a wide range of helminths in sheep and cattle in single oral or parental doses of 0.1 mg/kg (Egerton et al., 1979). Ivermectin is a mixture of homologues, not less than 80% 22,23-dihydroavermectin B_{1a} and not more than 20% 22,23-dihydroavermectin B_{1b}. The structures of these compounds are shown in Figure 1.

So that the tissue distribution of ivermectin residues in cattle and sheep could be studied and the requirements of governmental regulatory agencies could be satisfied, a sensitive, specific, and reliable chemical assay for the parent drug and any major drug-related metabolites was needed. Radioactive metabolism studies have shown that dihydroavermectin B_{1a} is the major residue found in all tissues prior to the proposed withdrawal times of 28 days for cattle and 14 days for sheep. Dihydroavermectin B_{1b} is dosed at lower levels than dihydroavermectin B_{1a} and is generally metabolized more rapidly than the B_{1a} component (Jacob, 1979), so that the dihydroavermectin B_{1b} residues are always less than the B_{1a} residues. A tissue assay was, therefore, developed for dihydroavermectin B_{1a}.

The avermectins are not amenable to gas chromatography because of the complexity of the molecules. A reversed-phase high-performance liquid chromatographic (HPLC) assay with UV detection has been previously developed to determine the avermectins in fermentation broth and formulations (Miller et al., 1979), but the assay did not have sufficient sensitivity for tissue residue analysis. Ivermectin is such a potent drug and is dosed at such low levels that the tissue assay needed a sensitivity of ~10 ppb (10^{-9} g/g) to ensure negligible residues. Tolan et al. (1980) developed an HPLC method using fluorescence detection for the avermectins in plasma. A fluorescent product was formed through a chemical reaction of the drug with acetic anhydride in pyridine which resulted in formation of a conjugated dehydration product. This method, however, was found to be too long (reaction time, 24 h), and the chemical yields through the reaction were too variable to meet the requirements for a tissue residue assay. Modification of these reaction conditions, however, has resulted in a new, shorter, more reproducible assay.

The present paper describes a procedure for the isolation, derivatization, and fluorescence HPLC determination of dihydroavermectin B_{1a} from cattle and sheep tissues. The same method has also been applied to swine and horse tissues. The drug is extracted into isooctane, carried through a series of liquid–liquid distributions, and then derivatized in a 1-h reaction to form the same fluorescent product as formed under the previously published reaction conditions (Tolan et al., 1980). The sample is cleaned up by column chromatography before quantitation by HPLC fluorescence detection. The fluorescent derivatives of dihydroavermectins B_{1b} and B_{1a} are resolved by the HPLC column. The assay has a lowest reliable measurement level (*Fed. Regist.*, 1979) of ~10 ppb and a limit of detection of 1–2 ppb. Recoveries of dihydroavermectin B_{1a} spiked into cattle and sheep liver, kidney, muscle, and fat averaged 79–84%.

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.

0021-8561/81/1429-1059$01.25/0

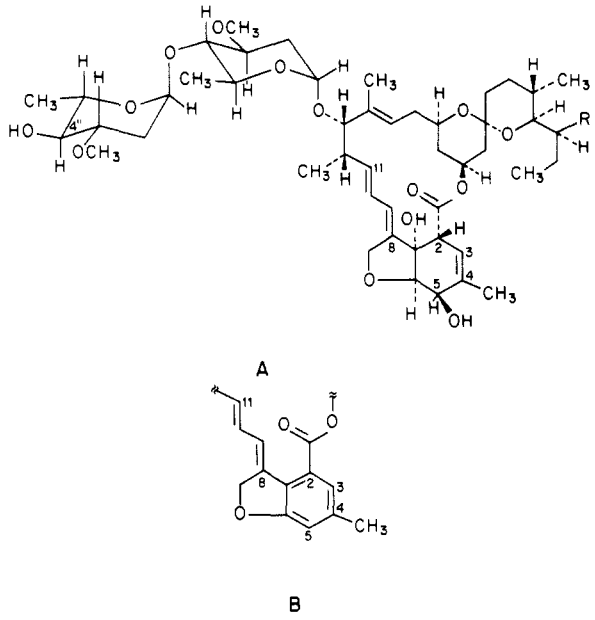

Figure 1. Structure of the avermectins (A) and the fluorescent derivative used for quantitation of the avermectins (B); R = C_2H_5 for dihydroavermectin B_{1a} and R = CH_3 for dihydroavermectin B_{1b}.

EXPERIMENTAL SECTION

Reagents. All organic solvents were either Burdick and Jackson "distilled in glass" or nanograde quality. All other reagents were of analytical-grade purity. The acetic anhydride was Baker reagent grade, and the 1-methylimidazole (99% pure) was obtained from Aldrich Chemical Co. The derivatizing reagent was 1-methylimidazole–acetic anhydride–DMF (2:3:9 by volume) and was made up fresh each time it was used. All aqueous solutions were prepared with water which had been doubly distilled. Water which was used in the LC mobile phase was filtered through a Teflon 5-μm Millipore filter.

The dihydroavermectin B_{1a} reference standard was 95% pure by reversed-phase HPLC. A stock solution of the standard (500 ng/mL) was prepared in methanol and stored in the freezer when not in use. Aliquots of this standard (25–125 μL) were carried through the derivatization reaction and the subsequent cleanup and were dissolved in 0.5 mL of methanol for injection onto the LC to give a standard curve of fluorescence response vs. concentration of dihydroavermectin B_{1a} (25–125 ng/mL). A standard curve is run with each set of samples, since the derivative is not stable for long-term storage.

Apparatus. The derivatization reaction is carried out in silylated 15-mL centrifuge tubes. These tubes are cleaned by hand and soaked for several hours in methylene chloride and then in a detergent solution (Thomas Cleaning Compound No. 3298). They are then rinsed with hot water, distilled water, and acetone and dried. The tubes are silylated with Sylon-CT (Supelco Inc.) for 30 min, rinsed with toluene and methanol, and then soaked in methanol for 30 min. The tubes are then rinsed with acetone and dried in an oven. The same tubes are used for 2 months before it is necessary to repeat the silylation procedure. It is extremely important that all glassware be completely free of all acidic and alkaline residues, since the drug is unstable in both acidic and alkaline media. After the derivatization reaction the sample is cleaned up by column chromatography using a silica cartridge Sep-Pak (Waters Associates, No. 51900).

A Du Pont Model 830 liquid chromatograph with a Du Pont Model 833 constant-flow accessory, a Rheodyne

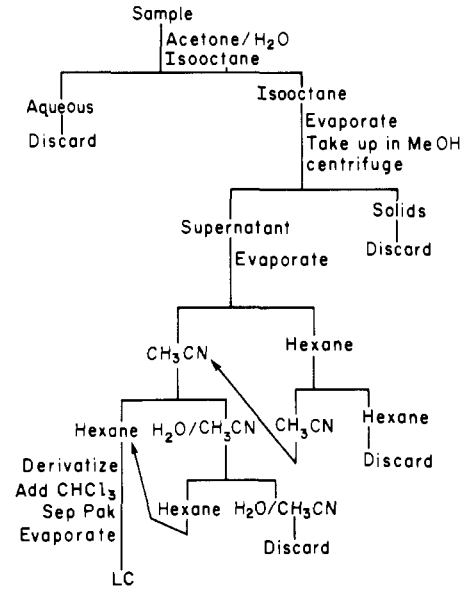

Figure 2. Flow diagram for the cleanup of ivermectin tissue residue samples.

sample valve with a syringe loading sample loop, and a Schoeffel Instruments Model FS970 fluorescence detector comprised the basic chromatographic instrumentation. The column was a Du Pont Zorbax ODS (4.6 mm × 15 cm) operated at ambient temperature. A short (3.5-cm) precolumn packed with Co:Pell ODS (Reeve Angel) was used to prevent contamination of the analytical column; the precolumn was changed whenever a significant pump pressure increase occurs. The chromatographic conditions are as follows: mobile phase, methanol–water (95:5); flow rate, 1.8 mL/min.; retention time of the fluorescent product, 14 min. The detector configuration used the following: excitation wavelength, 364 nm; excitation filter, Corning 7-54; emission filter, 440 nm cutoff filter; voltage to the photomultiplier tube, 1080 V.

Procedure. A flow diagram for the assay procedure is shown in Figure 2. Twelve samples can be completed in 2 man-days. Five grams of tissue is homogenized with 15 mL of 50:50 acetone–water and transferred to a 50-mL centrifuge tube. The homogenizer cup is washed out with 15-mL of isooctane which is added to the sample. The sample is shaken for 1 min and centrifuged for 10 min. For fat samples 20 mL of acetone–water and 10 mL of isooctane are used to homogenize the sample, and the cup is rinsed with 10 mL of isooctane. One gram of sodium chloride is added to the fat samples to prevent emulsions. After centrifugation the isooctane layer is transferred to a clean 50-mL centrifuge tube, and the sample is reextracted 3 times more with 15 mL of isooctane for each extraction, resuspending the tissue solids prior to each extraction. The isooctane extracts are combined and evaporated in a hot water bath (65–70 °C) with a stream of nitrogen as far as possible. The samples are redissolved in 6 mL of methanol and stored in a refrigerator (40 °C) until completely cooled to precipitate some extraneous tissue materials. The cold tubes are centrifuged, and the clear supernatant is decanted to a clean 15-mL centrifuge tube and evaporated to dryness.

Four milliliters of acetonitrile and 3 mL of hexane are added to the centrifuge tube; the tube is ultrasonicated, shaken, and centrifuged. The acetonitrile layer is transferred to a clean 15-mL centrifuge tube. The hexane layer is reextracted with an additional 4 mL of acetonitrile, and the acetonitrile extracts are combined and evaporated to less than 1.0 mL. The sample is made up to a volume of

1.0 mL with acetonitrile, and 4 mL of water and 5 mL of hexane are added to the centrifuge tube. The tube is shaken and centrifuged, and the hexane layer is transferred to a clean 15-mL centrifuge tube. The sample is extracted twice more with hexane (5 mL; 4 mL), and the hexane is evaporated to dryness.

The sample is then dissolved in exactly 1.0 mL of methanol and centrifuged; 0.5 mL of the sample is transferred to a silylated 15-mL centrifuge tube and is evaporated to dryness. One-tenth of a milliliter of the derivatizing reagent, freshly prepared acetic anhydride–methylimidazole–DMF, is added to each sample and to a series of standards in 15-mL centrifuge tubes. The tubes are stoppered, the stoppers are taped in place, and the tubes are placed in a 95 °C oil bath for 1 h. The tubes are allowed to cool, and 1 mL of chloroform is added. A Sep-Pak cartridge is washed with 3–4 mL of chloroform by using a syringe to force the liquid through the cartridge. The sample is put into the syringe with a disposable pipet and forced onto the cartridge. The centrifuge tube is washed with 3 mL of chloroform, and the wash is put into the syringe and forced onto the column. The cartridge is eluted with an additional 8–9 mL of chloroform; the total chloroform eluant is collected in a 15-mL centrifuge tube and evaporated to dryness. The sample is dissolved in 0.5 mL of methanol, and 50 μL is injected onto the LC. Samples containing high drug residues will need further dilution to fit on the standard curve.

The samples and standards are quantitated by peak height measurements. A standard curve of peak height vs. nanograms per milliliter dihydroavermectin B_{1a} is obtained from dihydroavermectin B_{1a} standards (25–125 ng/mL) which were carried through the derivatization and the Sep-Pak procedures and injected into the LC with each set of samples. A new set of standards is included with each set because the standards are not stable for long-term storage, and there are slight differences from day to day in detector response, column conditions, and recovery of the standard through the derivatization procedure. The nanograms per milliliter for each unknown sample is read from the standard curve, and the residue in ppb is calculated from

$$\text{residue (ppb)} = 2 \times (\text{ng/mL}) \times V_1 \times D/G$$

where V_1 = volume (milliliters) in which sample is dissolved for LC, D = dilution of the sample at the end of the assay if the sample in V_1 is too concentrated; 1 if no dilution is made, and G = grams of sample used in the assay, generally 5.

RESULTS AND DISCUSSION

Liquid chromatography with fluorescence detection was chosen as the analytical method, combining a high degree of specificity due to the combination of chromogenic and chromatographic selectivities as well as the sensitivity arising from the strong fluorescence of the derivative. The work by Tolan et al. (1980) demonstrated that the acetylation reaction with the avermectins results in the 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.

This acetylation–dehydration reaction using acetic anhydride–pyridine went to only 60% completion in 24 h, so other stronger acetylating reagents and conditions were tried. The most reproducible reaction conditions were found to be 9:3:2 DMF–acetic anhydride–1-methylimidazole. The reaction is essentially complete in 45 min, but the product is stable under reaction conditions for at least 2.5 h so that the reaction is run for 1 h to ensure that

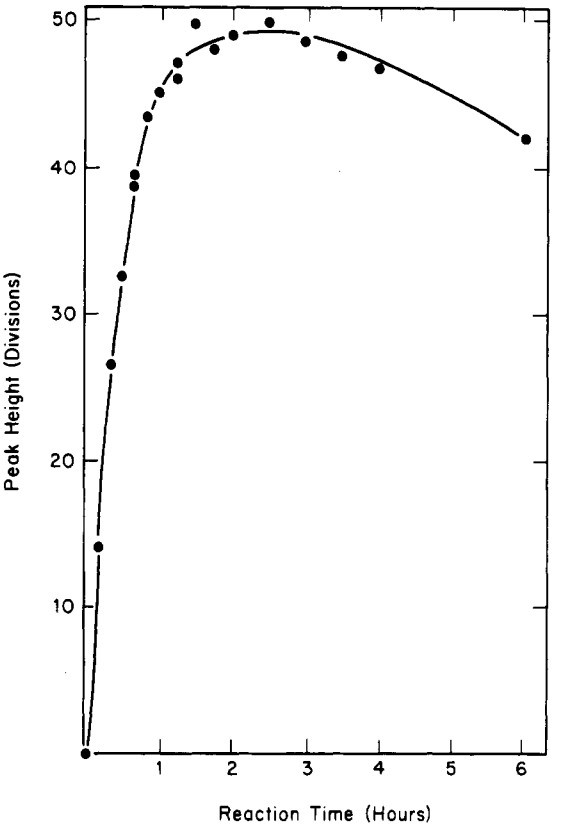

Figure 3. Effect of reaction time on the yield of the fluorescent derivative measured by fluorescence HPLC.

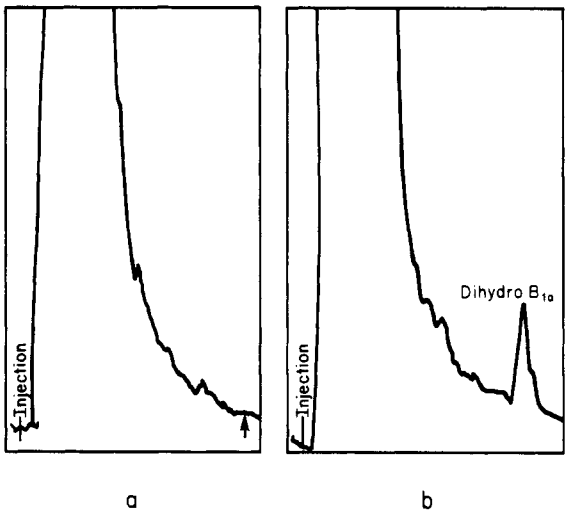

Figure 4. Typical chromatograms: (a) control cattle liver sample; (b) cattle liver containing 8 ppb of dihydroavermectin B_{1a}.

it has gone to completion. A plot of the reaction yield (as detector response) vs. reaction time is shown in Figure 3. Under these rigorous reaction conditions, the fluorescent product is obtained in essentially quantitative yield in 1 h, but the samples are too dirty for direct injection onto a liquid chromatographic column. The column chromatography using a Sep-Pak cartridge is a quick, easy, and effective method of cleanup which yields a homogeneous sample ready for direct injection into the liquid chromatographic column.

Typical chromatograms are shown in Figure 4. Figure 4a shows a blank liver and 4b shows a liver containing 8 ppb of dihydroavermectin B_{1a}. The method has a lower limit of reliable measurement of ~10 ppb. At this level an LC peak of ~5 cm was generally observed, and reproducible quantitation could readily be achieved. The

Table I. Comparison of Chemical and RID Assay Results

	days postdose	dihydroavermectin B_{1a}, ppb: chemical assay	dihydroavermectin B_{1a}, ppb: RIDA[a]	% recovery, chemical assay
cattle liver	7	352	423	83
	7	168	221	77
	21	21	24	86
	21	12	14	86
sheep liver	1	72	87	83
	3	54	74	73
	5	20	21	95

[a] RIDA results are from the Animal Drug Metabolism and Radiochemistry group, Merck Sharp & Dohme Research Laboratories, Rahway, NJ.

limit of detection was 1–2 ppb since discernible peaks were observed at these levels. At levels below 10 ppb, assay results were not reproducible enough to be totally quantifiable.

Recovery Studies. Nonmedicated cattle tissues (muscle, liver, kidney, and fat) were spiked with dihydroavermectin B_{1a} at levels of 9.7–97 ppb and immediately carried through the assay procedure. The recoveries, expressed as the average ± one standard deviation were 84 ± 5% for muscle, 81 ± 8% for liver, 84 ± 10% for kidney, and 82 ± 9% for fat. The overall recovery for all fortified tissues was 83%. Similar recovery studies were run on sheep tissues. The recoveries averaged 83 ± 10% for muscle, 79 ± 6% for liver, 85 ± 12% for kidney, and 81 ± 7% for fat with an overall recovery of 82% for all tissues. The assay works equally well for all four tissues in both animal species. Similar results were also obtained with swine and horse recovery studies.

Cattle and sheep livers from animals which had been dosed with radioactive ivermectin were assayed by the chemical assay to demonstrate that the chemical assay method is capable of recovering dihydroavermectin B_{1a} from tissues when it is naturally present, as contrasted to spiking experiments. Some of the results are presented in Table I. The chemical assay for the cattle livers accounted for 85% of the dihydroavermectin B_{1a} found by RIDA experiments, which is in excellent agreement with the 81% recovery from spiked liver samples. The recovery from the sheep livers averaged 82% of the RIDA values, which is in good agreement with the recovery from spiked sheep liver samples of 79%. From these data one can have confidence that the chemical assay is quantitating accurately dihydroavermectin B_{1a} residues in both animal species at all withdrawal periods.

Tissue Residue Studies. A residue study was carried out on cattle dosed at 0.3 mg/kg with ivermectin by subcutaneous injection. For this study liver, muscle, kidney, and fat from seven groups of five animals were assayed. One group served to furnish nonmedicated control tissues, and the remaining groups were sacrificed at 2, 7, 14, 21, 28, and 42 days postdose, respectively.

The liver which is the tissue in which the drug is most persistent had average residues ranging from 454 ppb at 2 days postdose to 11 ppb at 28 days postdose. The other tissues (fat, muscle, and kidney) had lower residues than liver at all withdrawal times in the order fat > kidney > muscle. All control and 42-day tissues had undetectable residues.

The average liver residues from 2, 7, 14, 21, and 28 days gave an excellent linear regression curve with a correlation coefficient of 0.998. The half-life of dihydroavermectin B_{1a} in cattle liver was 4.9 days. A graph of drug residue

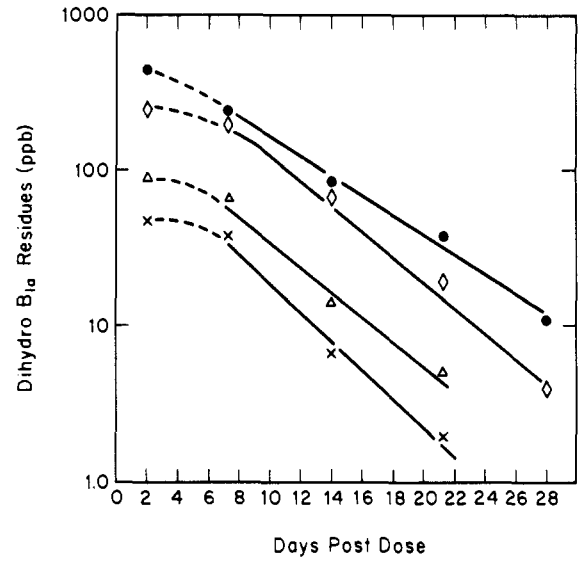

Figure 5. Regression curve for the depletion of dihydroavermectin B_{1a} in cattle tissues: (●) liver; (×) muscle; (△) kidney; (◇) fat.

Table II. Average Ivermectin Residue in Sheep Tissue

days postdose	drug residue, ppb: liver	fat	muscle	kidney
1	72	145	20	30
3	12	32	4	5
5	11	11	2	2
7	8	9	2	1
10	0	0	0	0
14	0	0	0	0

in all tissues as a function of withdrawal time is shown in Figure 5. In muscle, kidney, and fat, peak drug levels were reached between 2 and 7 days postdose. At withdrawal times of 7 days and beyond, the drug residue in these tissues decreased linearly as a logarithmic function. The drug half-lives in muscle, kidney, and fat were 3.3, 3.9, and 3.7 days, respectively. Not only are the highest residue levels found in liver but also the drug has the longest half-life in liver. Liver is the last tissue to clear drug-related residues, but all tissues have no detectable residues by 42 days postdose.

A residue study was also done on sheep dosed at 0.3 mg/kg with ivermectin orally. For this study liver, kidney, muscle, and fat from seven groups of five animals each were assayed. One group served to provide nonmedicated control tissues, and the other groups were sacrificed 1, 3, 5, 7, 10, and 14 days postdose. The data are presented in Table II. Peak drug levels were found in all tissues at 1 day postdose. At 1 and 3 days postdose, fat had higher residues than liver, but by 5 days postdose, liver and fat had equivalent residues. At 5 and 7 days postdose, the drug residue in both fat and liver were at the assay's limit of reliable measurement, so that analytically there is no difference between residue values of 8 and 9 ppb. At 10 and 14 days postdose, no tissue contained any detectable residue. All the control tissues had no detectable residue.

ACKNOWLEDGMENT

We thank George Olson and Paul Baylis for conducting the animal studies and Eugenia Greff for technical assistance.

LITERATURE CITED

Egerton, J. R.; Ostlind, D. A.; Blair, L. S.; Eary, C. H.; Suhayda, D.; Cifelli, S.; Riek, R. F.; Campbell, W. C. *Antimicrob. Agents Chemother.* **1979**, *15*, 372–378.

Fed. Regist. **1979**, *44* (55).
Jacob, T., Merck & Co., Inc., Rahway, NJ, personal communication, 1979.
Miller, T. W.; Chaiet, C.; Cole, D. J.; Cole, L. J.; Flor, J. E.; Goegelman, R. T.; Gullo, V. P.; Kempf, A. J.; Krellwitz, W. R.; Monaghan, R. L.; Ormond, R. E.; Wilson, K. E.; Putter, I. *Antimicrob. Agents Chemother.* **1979**, *15*, 368–371.
Tolan, J. W.; Eskola, P.; Fink, D. W.; Mrozik, H.; Zimmerman, L. A. *J. Chromatogr.* **1980**, *190*, 367–376.

Received for review February 27, 1981. Accepted June 15, 1981.

Protein Quality of Vegetable Proteins As Determined by Traditional Biological Methods and Rapid Chemical Assays

Arlene Wolzak, Luiz G. Elías, and Ricardo Bressani*

Traditional biological assays were compared with chemical estimates of protein quality by using different vegetable proteins. The comparability and reproducibility of protein efficiency ratio (PER), net protein ratio (NPR), and in vivo protein digestibility were tested in two experiments at different times. A highly significant correlation was found between PER and NPR in both experiments, although a higher correlation was observed in the second, in which a smaller and more homogeneous group of samples was tested. The PER showed the best reproducibility. Amino acid scores, essential amino acid indexes, and C-PER values were calculated. PER correlated better with chemical parameters than with NPR. The amino acid score, though an imperfect indicator, still seems to be the best of the chemical parameters studied. C-PER values showed a highly significant correlation with PER for the complete group of samples ($r = 0.871$; $n = 33$), although they overestimated the protein quality of leguminous seeds and processed samples and underestimated that of mixtures supplemented with animal protein.

The nutritional quality of a protein is determined by the quantity, availability, and proportions of the essential amino acids comprising it and the presence, for optimum utilization, of sufficient nonessential amino acids. Bioassays measure the efficiency of the biological utilization of dietary proteins as sources of the essential amino acids under a set of standardized conditions (Lachance et al., 1977).

Many biological methods based on the effects of the quality and amount of dietary protein on growth performance in young animals have been proposed for evaluating protein quality. Among these methods, the protein efficiency ratio (PER), based on weight changes of growing rats, is perhaps the most widely used. This method has been severely criticized by several authors (Pellett, 1978; Steinke, 1977). One of its shortcomings is that no consideration is given to the requirements of protein for maintenance. To overcome this objection, the inclusion of a group of animals consuming a nonprotein diet for a similar period of time was proposed and the procedure is called net protein ratio (NPR; Bender and Doell, 1957).

Biological assays are expensive and time-consuming and require considerable amounts of samples which are not always available. As a result, chemical methods based on amino acid composition of the proteins and enzymatic assays for the measurement of protein quality and digestibility have been devised. Important examples of such assays are the amino acid score (Mitchell and Block, 1946) and the essential amino acid index (Oser, 1951). Recently, the C-PER method was developed (Satterlee et al., 1977). This method corresponds to a PER value derived from the essential amino acid profile and the protein digestibility as determined by a multienzyme in vitro assay (Hsu et al., 1977; Satterlee et al., (1979). These assays require small amounts of samples and provide results on protein quality in shorter periods of time than biological assays.

The purpose of this study was to compare the results of two traditional biological assays, the PER and the NPR, with chemical estimates of protein quality such as amino acid score, essential amino acid index, and C-PER using a group of vegetable proteins widely consumed in developing countries.

MATERIALS AND METHODS

Samples and Sample Preparation. Protein samples were selected to include a set of vegetable proteins covering a wide range of protein quality. The samples used were commercial and laboratory-prepared plant proteins such as cereal grains, leguminous seeds, oilseeds and byproducts, and mixtures of cereal grains and leguminous seeds alone and supplemented with powdered skim milk or meat meal. ANRC casein was used as reference protein.

Leguminous seeds were prepared according to the technique previously described (Elías et al., 1976). Immature corn kernels were dried ($T = 40$ °C) and ground. Sesame seeds (*Sesamum indicum*) were pressed in a disk mill, extracted with hexane, and ground in a hammer mill. Commercial samples included soybean meal, cottonseed meal, white wheat flour, and the corn and bean flours used in the mixtures. The rest of the samples were ground in a hammer mill to pass a 60-mesh screen.

Chemical Assays. The nitrogen content of all the samples was determined by the macro-Kjeldahl method (AOAC, 1970). The crude protein was calculated by using the appropriate factors (FAO/WHO, 1973).

In Vitro Digestibility Experiments. The in vitro digestibility of the samples was assessed by measuring the extent to which the pH of the protein suspension dropped

Center for Higher Studies in Nutrition and Food Sciences (CESNA), University of San Carlos de Guatemala/Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Central America (A.W.), and Division of Agricultural and Food Sciences, Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala, Central America (L.G.E. and R.B.).

0021-8561/81/1429-1063$01.25/0