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Note

Isocratic separation of seven benzimidazole anthelmintics by high-performance liquid chromatography with photodiode array characterization

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The benzimidazole anthelmintics are used in animal production to control internal worm parasites and are of concern since foods derived from treated animals may contain residues of these drugs and their metabolites that exceed maximal (800 ng/g, fenbendazole) legal levels[1]. In this regard the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has included five benzimidazoles in its National Residue Program over the past ten years (albendazole, fenbendazole, mebendazole, oxfendazole and thiabendazole) for monitoring[2]. However, the analytical methodology has varied greatly for each and no single approach has been proven to be adequate for the extraction, separation, detection and quantitation of these compounds as a class in a single analysis.

This laboratory has reported methods for the analysis of the anthelmintic fenbendazole and its major metabolites (oxfendazole, fenbendazole sulfone and *p*-hydroxyfenbendazole) in samples of urine, feces and tissue homogenates (S9) obtained from *in vivo* and *in vitro* metabolism studies[3–8]. This approach utilized a modified solid phase extraction method for isolation of the drugs, a high-performance liquid chromatographic (HPLC) method for their separation and ultraviolet detection (UV, 290 nm) for their quantitation. However, fixed-wavelength UV detection, which has been used to detect and quantitate many of the benzimidazoles[3–8], does not provide sufficient information about a peak. Thus, the analyst must rely on retention times of suspect peaks compared to those of standard benzimidazoles in making decisions for further action. This difficulty may be overcome by the coupling of HPLC with photodiode array UV detection, providing for a means by which one may separate and characterize benzimidazoles based on retention times and UV spectra. The practical application of this approach is that it has the potential of minimizing suspect peaks in sample extracts and could greatly reduce the number of samples requiring further analysis or attempts at confirmation. The use of column heating may also facilitate these separations and has not been utilized in previous work on benzimidazoles[3–8].

We have analyzed seven benzimidazole compounds under various HPLC conditions, varying solvent composition and column temperature, in order to determine the best separation scheme for a particular analysis. An examination of the

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ability of chromatographic retention parameters and characteristic diode array UV spectra to provide a dual criteria by which one can characterize benzimidazoles was also undertaken. We report here a method for the isocratic separation and photodiode array UV characterization of seven benzimidazole anthelmintics that should prove useful for the screening of food extracts for these compounds.

MATERIALS AND METHODS

Chemicals and expendable materials

Solvents were obtained from a commercial source (Fisher Scientific, Pittsburgh, PA, U.S.A.) at the highest purity (HPLC-grade) available and were used without further purification. HPLC-grade water was obtained by passing triple distilled water through a Modulab Water Polisher I (Continental Water Systems Co., San Antonio, TX, U.S.A.) water purification system. Oxfendazole, fenbendazole, fenbendazole sulfone and *p*-hydroxyfenbendazole were obtained from Hoechst (Frankfurt, F.R.G.). Mebendazole was supplied by Janssen Pharmaceutical (Piscataway, NJ, U.S.A.). Thiabendazole was obtained from Merck Sharp & Dohme (Rahway, NJ, U.S.A.) and albendazole from Dr. J. O'Rangers (Center for Veterinary Medicine, Rockville, MD, U.S.A.) Stock solutions (1 mg/ml) were prepared by dissolving standard benzimidazoles with dimethylformamide (DMF). A standard stock mixture was prepared by adding an aliquot of each stock solution (except mebendazole, MBZ) to a single vial and diluting with DMF to the desired concentration. Samples for HPLC analysis were prepared by placing an aliquot (10 μl) of standard stock mixtures (3.12, 6.25, 12.5, 25, 50 and 100 μg/ml; 12.5 μg/ml mebendazole internal standard) into separate vials and removing the DMF by passing a steady flow of dry nitrogen gas over the vial contents maintained at 30°C in a water bath. The residue was solubilized in 0.5 ml of mobile phase [0.017 *M* H_3PO_4– acetonitrile (60:40, v/v)] and an aliquot (20 μl) was analyzed by HPLC.

Chromatographic conditions and measurements

Analyses were performed on a Hewlett Packard Model 1090 HPLC/Chemstation system equipped with a photodiode array detector set at 290 nm with a bandwidth of 20 nm, 100–350 nm reference spectrum and sensitivity of 0.1 ma.u.f.s. The column dimensions were 30 cm × 4 mm I.D., octadecylsilyl derivatized silica, 10-μm ODS Varian MicroPak (Sunnyvale, CA, U.S.A.). The temperature was varied according to experimental design. Peak area ratio curves for the concentrations (62.5–2000 ng/ml) examined were determined by dividing integrated areas of each peak by that of the internal standard (mebendazole).

RESULTS

Fig. 1 shows the structures of the benzimidazoles studied. Fig. 2 is a representative chromatogram showing the separation of the seven benzimidazoles. Fig. 3 shows the UV spectra (photodiode array, 1000 ng/ml and 250 ng/ml, smoothed; 20 μl injection volume) of thiabendazole, oxfendazole, *p*-hydroxyfenbendazole, fenbendazole sulfone, mebendazole, albendazole and fenbendazole. Table I lists the compounds examined, concentrations, column temperatures and standard curve

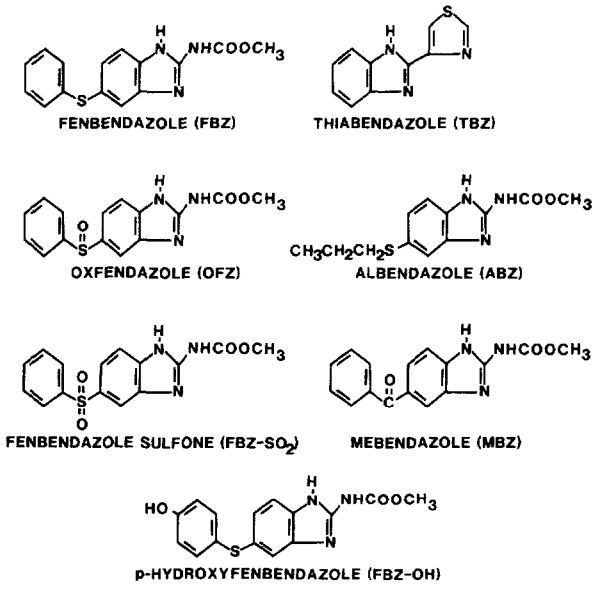

Fig. 1. Structures of the benzimidazoles examined.

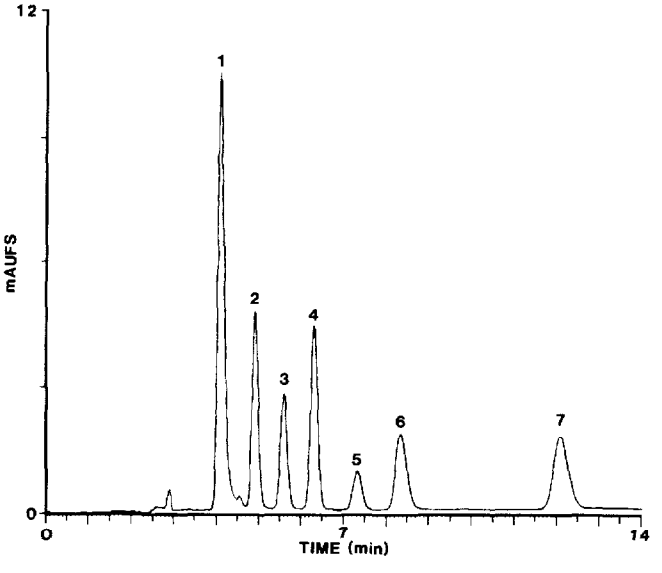

Fig. 2. Representative chromatogram (photodiode array, 290 nm) showing the separation of the seven benzimidazoles examined (1000 ng/ml, 20 μl injection) using 0.017 M H_3PO_4–acetonitrile, (60:40, v/v) as the mobile phase. The column temperature was 45°C. Order of elution: (1) thiabendazole, (2) oxfendazole, (3) *p*-hydroxyfenbendazole, (4) fenbendazole sulfone, (5) mebendazole, (6) albendazole and (7) fenbendazole.

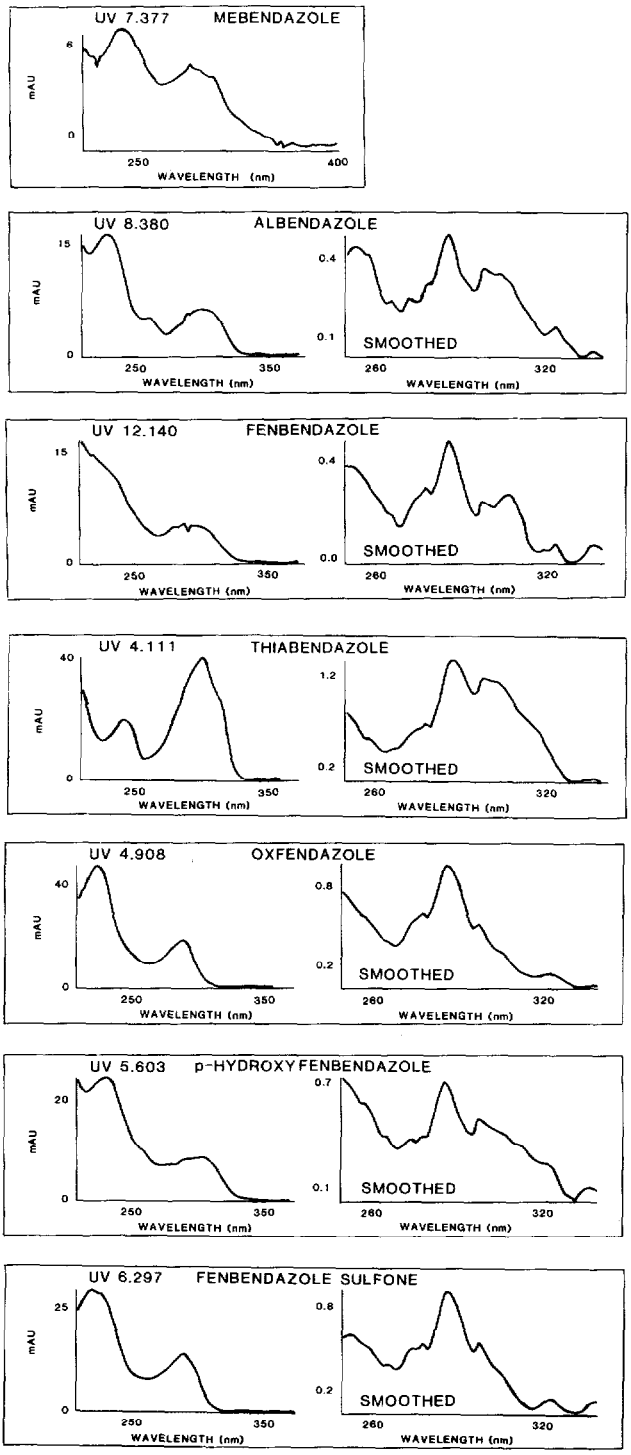

Fig. 3. UV (photodiode array, 290 nm) spectra (1000 ng/ml, 20 μl injection volume) and smoothed spectra (250 ng/ml, 20 μl injection volume) of the seven benzimidazoles examined. The UV numbers represent the retention times where each spectrum was acquired.

TABLE I

PEAK AREA RATIO STANDARD CURVE CORRELATION COEFFICIENTS ACHIEVED FOR COLUMN TEMPERATURES FROM 35 TO 60°C FOR EACH BENZIMIDAZOLE ANALYZED

The concentrations were 62.5, 125, 250, 500, 1000 and 2000 ng/ml. The injection volume was 20 μl. The mobile phase consisted of 0.017 *M* H_3PO_4–acetonitrile (60:40, v/v) at a flow-rate of 1 ml/min. The internal standard was mebendazole (MBZ) at a concentration of 250 ng/ml. Abbreviations: TBZ = thiabendazole; OFZ = oxfendazole; FBZ-OH = *p*-hydroxyfenbendazole; FBZ-SO_2 = fenbendazole sulfone; ABZ = albendazole; FBZ = fenbendazole; I.S. = internal standard.

Column temperature (°C)	*Benzimidazole*						
	TBZ	*OFZ*	*FBZ-OH*	*FBZ-SO_2*	*MBZ*	*ABZ*	*FBZ*
35	0.9998	0.9999	0.9999	0.9999	I.S.	0.9999	0.9999
40	0.9999	0.9999	0.9999	0.9999	I.S.	0.9999	0.9999
45	0.9999	0.9998	0.9999	0.9999	I.S.	0.9998	0.9998
50	0.9997	0.9997	0.9996	0.9995	I.S.	0.9992	0.9994
55	0.9972	0.9975	0.9976	0.9982	I.S.	0.9995	0.9987
60	0.9981	0.9986	0.9989	0.9996	I.S.	0.9995	0.9982

correlation coefficients determined. Table II lists retention data for separations at solvent strengths from 30 to 50 % acetonitrile. Table III lists retention data for separations at column temperatures from 35 to 60°C.

DISCUSSION

Baseline separation of the seven benzimidazoles (Fig. 1) examined was accomplished utilizing an isocratic 0.017 *M* H_3PO_4–acetonitrile (60:40, v/v) mobile

TABLE II

RETENTION DATA (k') FOR BENZIMIDAZOLES SEPARATED AT DIFFERENT SOLVENT STRENGTHS

Benzimidazole concentrations were 500 ng/ml (20 μl injection). The mobile phase consisted of 0.017 *M* H_3PO_4–acetonitrile. Column temperature: 45°C. $k' = (t_R - t_0)/t_0$, where t_R = retention time and t_0 the retention time of the unretained compound (2.89 min). Abbreviations as in Table I.

Compound	*Acetonitrile (%)*				
	30	*35*	*40*	*45*	*50*
TBZ	0.56	0.47	0.43	0.40	0.40
OFZ	1.49	0.99	0.69	0.53	0.40
FBZ-OH	2.11	1.34	0.93	0.70	0.55
FBZ-SO_2	2.95	1.82	1.16	0.80	0.55
MBZ	3.37	2.19	1.52	1.13	0.86
ABZ	3.81	2.57	1.90	1.47	1.20
FBZ	7.66	4.71	3.22	2.30	1.60

TABLE III
RETENTION DATA (k') FOR BENZIMIDAZOLES AT DIFFERENT COLUMN TEMPERATURES
The mobile phase was 0.017 *M* H_3PO_4–acetonitrile (60:40, v/v). k' determined as in Table II. Abbreviations as in Table I.

Compound	*Column temperature (°C)*					
	35	*40*	*45*	*50*	*55*	*60*
TBZ	0.47	0.45	0.43	0.41	0.40	0.38
OFZ	0.74	0.73	0.69	0.69	0.66	0.63
FBZ-OH	1.05	1.00	0.93	0.91	0.85	0.79
FBZ-SO_2	1.33	1.26	1.16	1.14	1.04	0.97
MBZ	1.65	1.60	1.52	1.52	1.43	1.34
ABZ	2.06	1.98	1.90	1.88	1.79	1.69
FBZ	3.51	3.37	3.22	3.16	2.94	2.77

phase, producing complete separation (Fig. 2) without resorting to exotic solvent systems or solvent programming, in 13 min. This solvent system was significantly different from that used in earlier studies to obtain separation of fenbedazole and its metabolites[3–8].

The linearity of standard curves (Table I) and the peak shape of each compound (Fig. 2) were maintained at the column temperatures (35–60°C) examined. Retention data (Table III) for separations at different column temperatures indicate there is no advantage in terms of resolution and/or run times at column temperatures above 45°C. In addition, because of the possibility of solvent outgassing at elevated temperatures, benzimidazole separations should be done at column temperatures below 50°C. Thus, a column temperature of 45°C was optimal for benzimidazole separations.

Retention data (Table II) for different acetonitrile concentrations suggested the optimal separation, with a minimal run time, was achieved utilizing a 0.017 *M* H_3PO_4–acetonitrile (60:40, v/v) mobile phase. Acetonitrile concentrations above 40% resulted in less than optimal separations of thiabendazole and oxfendazole. Baseline separations for the seven benzimidazoles were achieved at acetonitrile concentrations of less than 40% but resulted in lengthy run times. Use of the solvent system water–0.017 *M* H_3PO_4–acetonitrile (5:15:80) as previously reported[3–8] failed to separate albendazole and thiabendazole from the other compounds.

The utilization of photodiode array detection aided in the screening of suspected peaks in terms of UV characteristics. The photodiode array UV spectra of the compounds (1000 ng/ml, 20 μl injection) examined are shown in Fig. 3. By comparing the UV spectra of suspected sample peaks with known UV spectra, at each respective retention time, one could use these criteria as a preconfirmational screening procedure. UV specra were obtained for these benzimidazoles at the action level of 800 ng/g utilizing the separation conditions outlined and photodiode array detection (20 μl injection of a 500 μl final sample volume, 16 ng on column). However, one must be careful not to overextend this technology. The characteristic UV spectra of these compounds at much lower concentrations (5 ng on column) do not correlate well with the spectra obtained at higher concentrations, as evidenced by a comparison of the

spectra (1000 ng/ml, 20 μl injection) to smoothed spectra at a lower concentration (250 ng/ml, 20 μl injection) in Fig. 3. The spectra for the 250 ng/ml concentration were obtained by utilizing a mathematical smoothing function. The smoothing function can be used cumulatively, however this does not result in the correct UV spectrum. Thus, the characterization of benzimidazoles at low concentrations by photodiode array detection is limited to approximately 5 ng on column or, as assayed here, a level of 250 ng/ml. The absolute limit of detection, based only on the presence of a peak at the correct retention time and having a peak height of greater than three times baseline, was approximately 1.25 ng on column for all of the compounds examined. The minimal detectable limit for mebendazole was not determined. However, lower ng/ml sample concentrations may be determined in this manner by dissolving the final residue for analysis in a smaller volume and/or injecting a volume of greater than 20 μl of the sample.

CONCLUSIONS

The peak shape, detector response and linearity of standard curves for the benzimidazoles studied, at concentrations down to 62.5 ng/ml (20 μl injection volume; minimal detectable limit of 1.25 ng on column) and at column temperatures of less than 50°C, were sufficient to allow for quantitative determinations of these compounds. The method described also allows for preconfirmational screening of the benzimidazoles studied at the action level of 800 ppb[a] (20 μl injection volume; 16 ng on column). However, the UV spectra obtained for samples below 250 ng/ml (20 μl injection volume, 5 ng on column) would perhaps not be suitable for preconfirmational screening purposes. The separation protocol as outlined here has been utilized for quantitative determinations of benzimidazoles isolated from meat[9] and milk[10] and should be applicable as a multiresidue screening method for other benzimidazole determinations.

ACKNOWLEDGEMENTS

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[a] The American billion (10^9) is meant.

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