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Simultaneous Determination of Febantel, Fenbendazole, Oxfendazole and Oxfendazole Sulfone in Livestock by Matrix Solid Phase Dispersion Extraction Technique and HPLC

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ABSTRACT

Febantel, fenbendazole, oxfendazole and oxfendazole sulfone in livestock products were simultaneously extracted by traditional and matrix solid phase dispersion (MSPD) method, and then analyzed by high performance liquid chromatography (HPLC). In the traditional method, four benzimidazoles were extracted with acetonitrile under basic conditions, partitioned with *n*-hexane, and cleaned by a Sep-Pak C18 cartridge. In the MSPD method, samples were blended with Bondesil-C18, and the mixture was packed in a filtration column. This column was connected in tandem with an alumina N cartridge and eluted with acetonitrile. These benzimidazoles were determined by HPLC with a photodiode array detector. Recovery tests of benzimidazoles by these two methods were performed at three spike levels. In the traditional method, febantel was found to be unstable during the sample preparation. Average recoveries of febantel ranged from 72.3 to 81.6%. Average recoveries at low concentration (0.04 ppm) of fenbendazole ranged from 75.0 to 88.3%, while the remaining benzimidazoles ranged from 80.3 to 108.7%. The coefficients of variation of intra-day and inter-day assays were lower than 6.95 and 12.12%, respectively. Average recoveries of the MSPD method ranged from 80.2 to 109.6%. The coefficients of variation of intra-day and inter-day assays were lower than 4.47 and 7.89%, respectively. The detection limits for benzimidazoles were 0.010~0.020 ppm for the traditional method and 0.025~0.050 ppm for the MSPD method. The MSPD method minimized sample preparation time and solvents, and the analyte was stable during the analytical procedure with high recoveries. The MSPD method is suggested for use in routine analysis of benzimidazoles in livestock products.

Key words: febantel, fenbendazole, oxfendazole, matrix solid phase dispersion extraction (MSPD), high performance liquid chromatography (HPLC)

INTRODUCTION

Febantel [*N*-2(*N*'-*N*"-*bis*-methoxycarbonyl-guanidino) 3-methoxy-aceta-mido, 5-phenylthio, benzene], a broad-spectrum anthelmintic, is active against gastrointestinal roundworms in ruminants[1]. In sheep, metabolism of febantel leads to S-oxidation, S-reduction, and/or cyclization products[2]. Febantel is a pro-drug, which is known to convert into an active compound soon after administration[3]. The metabolic pathway of febantel is converted directly to either fenbendazole or oxfendazole, which is achieved *via* febantel sulfoxide as an intermediate[4]. Of all the 11 identified metabolites, those of toxicologic interest include: fenbendazole, febantel sulfoxide, oxfendazole, and oxfendazole sulfone[5]. Fenbendazole and oxfendazole are also licensed veterinary products[4]. The study of the embryotoxic effect of individual metabolites of oxfendazole indicated that the unchanged compound appears to be responsible for the observed teratogenic effect[6]. Oxfendazole is also the ultimate embryotoxin of febantel in the rat[2].

Febantel is categorized as an antiparasitic agent in Taiwan. This drug is used for animals against gastrointestinal nematodes[7]. Detrimental effect on human health could occur when people consume products containing veterinary drug residues. To prevent the abuse of veterinary drugs, the Department of Health announced the revised "Tolerances for Residues of Veterinary Drugs" in January 2001[8], and the maximum residue limits for febantel, fenbendazole and oxfendazole were set. According to the regulation of the Codex, the marker residue of febantel/fenbendazole/oxfendazole has been defined as the sum of fenbendazole, oxfendazole and oxfendazole sulfone, and expressed as oxfendazole sulfone equivalents[9]. So far, there is no analytical method promulgated by the Department of Health. Therefore, it is an important issue to establish a standard analytical method for monitoring residual benzimidazoles in livestock.

Methods for analyzing benzimidazoles include high performance liquid chromatography (HPLC) with ultraviolet (UV)[10-17] or photodiode array detector (PDAD)[18-21], HPLC/mass spectrometry (HPLC/MS)[22,23], and gas chromatography/mass spectrometry (GC/MS)[23]. A method for the determination of fenbendazole, oxfendazole, thiabenda-

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zole and 5-hydroxythiabendazole in milk was described[12]. This procedure was based on partition between organic and aqueous phases with pH adjustment followed by solid phase extraction and clean up on a silica gel cartridge. Rose[4] developed a method for the determination of nine compounds closely related to oxfendazole in cattle liver. Drug residues in Rose's samples were extracted with acetonitrile followed by strong cation exchange solid phase extraction for sample clean up. Long *et al*.[18-20] developed a matrix solid phase dispersion (MSPD) technique to analyze thiabendazole, oxfendazole, mebendazole, albendazole and fenbendazole. In his study, animal tissue was dispersed onto the hydrophobic C18 support, and the tissue/C18 blend was packed in a column and then washed with *n*-hexane. Afterwards, the benzimidazoles were eluted with acetonitrile.

Our laboratory has developed a method for the determination of benzimidazoles (albendazole, thiabendazole, mebendazole and their metabolites) in livestock[24]. The aim of this study was to develop an HPLC analytical method to simultaneously determine other benzimidazoles (febantel, fenbendazole, oxfendazole and oxfendazole sulfone) in livestock using a photodiode array detector. Traditional and MSPD extraction methods were studied and compared in terms of specificity, sensitivity and accuracy. The method developed is suitable for monitoring residues of febantel, fenbendazole, and oxfendazole in livestock.

MATERIALS AND METHODS

I. *Samples*

Forty-eight samples including pork, swine liver, beef, lamb, bovine milk, and goat milk were purchased from local markets in Taipei from October to December, 2003. All samples were stored at -20˚C before analysis.

II. *Chemicals*

(I) *Standards*

Fenbendazole (FEN, 100%) was purchased from Sigma Chem. Co. (St. Louis, MO, USA). Febantel (FBT, 99.9%) was provided by Institute of Animal Drug Inspection (Chunan Branch, Taiwan). Oxfendazole (OXF, (99%) and oxfendazole sulfone (OXF-S, 99%) were provided by National Institute of Public Health and the Environment (Bilthoven, the Netherlands). Benzthiazuron [BEN, 1-(benzothiazol-2-yl)-3-methylurea, 99.9%] (internal standard, I.S.) was purchased from Riedel-de Haen (Seelze, Germany). The chemical structures of four benzimidazoles and BEN are shown in Figure 1.

(II) *Solvents and other reagents*

LC grade acetonitrile, ethyl acetate, methanol, *n*-hexane and dichloromethane were purchased from Labscan Co., Ltd. (Bankok, Thailand). GR grade 1-propanol, ammonium dihydrogen phosphate, sodium bicarbonate, sodium carbonate and triethylamine were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Filtration column (6 mL) with 2 frits (porous polyethylene, 0.5 inch diameter, 20 μm) was purchased from Supelco Chroma Enterprise Ltd. (Bellefonte, PA, USA). Sep-Pak C18 cartridge (500 mg, 3 mL), alumina N cartridge (500 mg, 6 mL) and florisil cartridge (500 mg, 6 mL) were

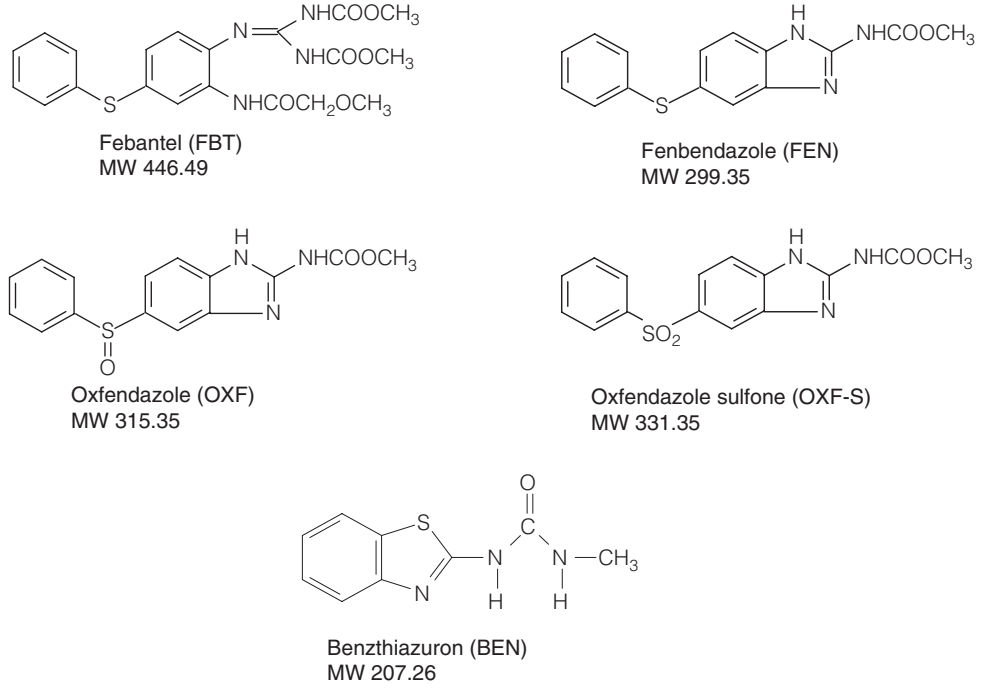

Figure 1. Chemical structures of compounds examined in this study.

purchased from Waters Corporation (Milford, MA, USA). Bondesil-C18 was purchased from Varian (CA, USA), and packed 20 g in a column and washed with 40 mL each of *n*-hexane, dichloromethane and methanol separately before dried for use.

III. *Equipment*

Samples were minced and ground with a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). Rotary evaporator (Buchi, Labortechnic AG, Flawil, Switzerland), centrifuge (Labofuge 400, Heraeus Instruments GmbH, Hanau, Germany), vortex mixer (Type 37600 Mixer, Thermolyne Corporation, Iowa, USA) and glass mortar were used during the sample preparation. A high performance liquid chromatographic system consisted of a Shimadzu LC-10AT pump, a CBM-10A interface controller, a SIL-10A auto injector, and an SPD-M6A photodiode array (PDA) detector (Shimadzu Corporation, Kyoto, Japan) was used to analyze the samples.

IV. *Preparation of Buffer Solution for Extraction*

Saturated sodium bicarbonate solution was adjusted to pH 10 with saturated sodium carbonate solution.

V. *Standard Curve*

FBT, FEN, OXF, OXF-S and internal standard BEN (5 mg of each) were separately weighed into a 100-mL volumetric flask, dissolved and made up to volume with acetonitrile. The stock solutions were mixed and diluted with acetonitril/0.01M ammonium dihydrogen phosphate (1/1, v/v) solution to a series of concentrations. Appropriate amounts of the internal standard solutions were added to make standard solutions containing 0.1, 0.5, 1.0, 2.5 and 5.0 μg/mL of each benzimidazole and 1.0 μg/mL of BEN as an internal standard. These standard solutions were analyzed three times by HPLC/PDAD. Response factors were calculated by dividing the peak areas of each benzimidazole by those of the internal standard, and plotted against the concentration of each benzimidazole in the standard solutions.

VI. *Analytical Methods*

(I) *Traditional procedure*

The previously described extraction and cleaning procedure[24] was slightly modified. To 5 g of ground sample, 20 μL of internal standard solution (50.0 μg/mL), 1 mL of the buffer solution and 30 mL of acetonitrile were added and the mixture was homogenized for 2 min. After filtration, the filtrate was partitioned with 50 mL of *n*-hexane saturated with acetonitrile. The acetonitrile layer was evaporated to dryness. The extract resulting from this procedure was purified by using a Sep-Pak C18 cartridge. The benzimidazoles were eluted with acetonitrile, and the eluate was evaporated to dryness. The residue was reconstituted with 1 mL of acetonitrile/0.01M ammonium dihydrogen phosphate (1/1, v/v) solution, mixed on a vortex mixer to dissolve the residue, and then filtered through a 0.2 μm membrane (nylon, Micron Separations Inc., West Borough, MA, USA). Fifty microliter of the sample solution was injected for HPLC analysis.

(II) *MSPD extraction procedure*

Test samples of pork, swine liver, beef and lamb were ground. One gram of ground sample (milk was 1 mL) was weighed into a glass mortar. Ten microliter of internal standard solution (50.0 μg/mL) and 2 g of Bondesil-C18 were added, and gently blended using a glass pestle for a few minutes until the mixture was homogeneous in appearance. The mixture was loaded into a filtration column plugged with a frit. Column contents were pressed to a final height of 4 cm with a glass rod. The resulting column was washed with 8 mL of *n*-hexane. When flow ceased, excess *n*-hexane was removed from the column with positive pressure. The column was connected in tandem with an alumina N cartridge, which was preconditioned with 4 mL of acetonitrile. These two cartridges were connected in the order of Bondesil-C18 to alumina N, and 8 mL of acetonitrile passed through each of these two cartridges. The eluate was collected and evaporated to dryness at 40˚C using a rotary evaporator. The residue was reconstituted with 0.5 mL of acetonitrile/0.01M ammonium dihydrogen phosphate (1/1, v/v) solution, mixed on a vortex mixer to dissolve the residue, and then filtered through a 0.2 μm membrane prior to HPLC analysis. The MSPD procedure for four benzimidazoles is illustrated in Figure 2.

VII. *HPLC Analysis*

(I) *Analytical conditions*

The column for separating benzimidazoles was

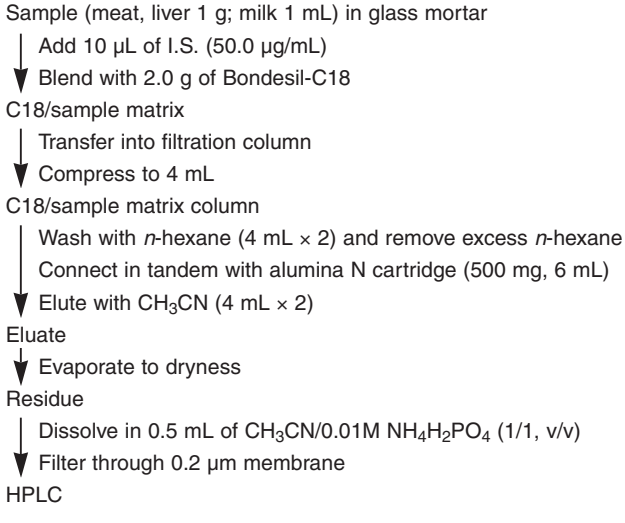

Figure 2. MSPD procedure for four benzimidazole residues.

Cosmosil 5C18 MS-II (5 μm, 4.6 mm i.d. × 250 mm; Nacali Tesque Inc., Kyoto, Japan). The detector was a PDA detector. The scan range of the PDA detector was 200 ~ 400 nm, and UV detector was set at 298 nm. Acetonitrile and 0.01M ammonium dihydrogen phosphate were used as the mobile phase, and the gradient profile is shown in Table 1. The flow rate was set at 1 mL/min. The injection volume of samples was 50 μL.

(II) *Identification and quantification*

Benzimidazoles were identified by: (1) comparing the peak retention times of samples with those of standards from HPLC, (2) comparing the spectra of analytes with those of the standards. The following formula was used to calculate the contents of veterinary drugs in test samples:

Content of veterinary drug (ppm) = C × V / W

Where C is the drug concentration (μg/mL) calculated from standard curve, V is the volume of sample solution (mL), and W is the weight/volume of sample (g or mL).

VIII. *Recovery Test*

Recovery studies of the traditional method was performed in triplicate by spiking standards at 0.04, 0.10, and 0.20 ppm (0.04, 0.50 and 1.00 ppm for liver samples) and 20 μL of internal standard solution (50.0 μg/mL) into samples. The MSPD method was performed in triplicate by spiking standards at 0.10, 0.50, and 1.00 ppm and 10 μL of internal standard solution (50.0 μg/mL) into samples. The spiked samples and blank samples without standard solution were then analyzed by HPLC. Recoveries were calculated by comparing the amount of benzimidazole added with that found by HPLC.

IX. *Detection Limit Test*

Samples, spiked with various concentrations of standard solutions, were analyzed by traditional and MSPD methods. Limits of detection were based on a signal to noise (S/N) ratio of 3 as the minimum.

RESULTS AND DISCUSSION

I. *Study on the HPLC Conditions*

(I) *The optimal wavelength for detection*

HPLC with a UV or PDA detector has been used for benzimidazoles analysis in many cases. The maximum absorption of benzimidazoles occurred at 298 nm, which eliminates most of the interferences from conjugated and aromatic compounds[23]. After spectrum scanning of benzimidazoles with a PDA detector, a maximum absorption wavelength at 230 nm (or 256 nm) and an absorption peak at 298 nm were found. In order to reduce interference peaks in HPLC chromatogram, a higher absorbance at 298 nm was selected to detect the four benzimidazoles.

(II) *Mobile phase selection*

Although some chemical similarity exists among the four benzimidazoles, there is a significant difference in polarity between the first and the last eluted compounds. Figure 3 (A) shows the HPLC chromatogram of the benzimidazoles with BEN internal standard using acetonitrile/0.01M ammonium dihydrogen phosphate (1/1, v/v) solution as the mobile phase for isocratic elution. It was shown that OXF and OXF-S (capacity factor, K' = 0.5 and 1.0, respectively) were eluted near the solvent front. The last-eluted FBT (K' = 15.2), which is the most hydrophobic among the four benzimidazoles, appeared as a broad and badly tailed peak, resulting in more difficult quantification. It is therefore inappropriate to determine all benzimidazoles with isocratic elution because of the difference in hydrophobic character. Therefore, a gradient elution was applied. A mobile phase consisted of acetonitrile and 0.01M ammonium dihydrogen phosphate was selected and the gradient elution was performed as described in Table 1. Figure 3 (B) shows the HPLC chromatogram of a mixed

Table 1. Gradient elution profile for HPLC analysis

Time (min)	A (%)[a]	B (%)[a]
0	30	70
9	70	30
25	30	70

[a]Mobile phase A: acetonitrile; mobile phase B: 0.01M ammonium dihydrogen phosphate.

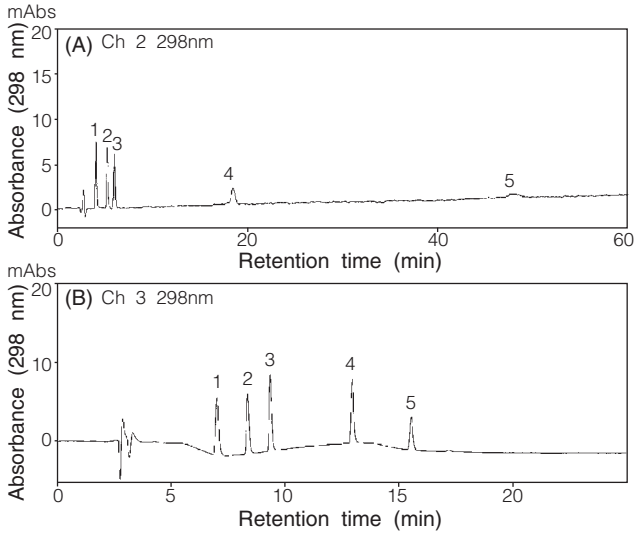

Figure 3. HPLC chromatograms of four benzimidazole standards after (A) isocratic elution with acetonitrile/0.01M ammonium dihydrogen phosphate (1/1, v/v), and (B) gradient elution with acetonitrile and 0.01M ammonium dihydrogen phosphate according to the profile described in Table 1.

Injected amounts: 25ng for each substance, except 50 ng for BEN (I.S.). Peak identification: 1, OXF: 2, BEN; 3. OXF-S; 4. FEN; 5. FBT.

Table 2. Linear regression equations and correlation coefficients of standard curves for benzimidazoles

Benzimidazole[a]	Linear equation[b]	Correlation coefficient
OXF	Y = 1.6452 X + 0.0035	0.9999
OXF-S	Y = 1.9925 X – 0.0385	0.9999
FEN	Y = 1.8376 X – 0.0702	0.9997
FBT	Y = 1.3127 X + 0.0233	0.9995

[a]Concentration ranges from 0.1 to 5.0 μg/mL.
[b]Y = AX + B, where Y is peak area ratio of analyte and internal standard, X is the concentration of the analyte.

standard solution of four benzimidazoles containing BEN as an internal standard, using a gradient elution and detection by a PDAD/UV 298 nm. All four benzimidazoles and internal standard were well separated within 16 min with good peak resolution, sharpness and symmetry. We employed BEN as internal standard for the following reason: BEN exhibits marked absorbance in the UV region of 250 to 300 nm. The HPLC chromatogram presented a good separation between benzimidazoles and BEN, and the recovery of BEN was acceptable.

II. *Standard Curve*

Linear regression equations of the standard curves for the benzimidazoles in the range of 0.1 ~ 5.0 μg/mL are shown in Table 2. Satisfactory linearity with correlation coefficients greater than 0.999 was achieved.

III. *Sample Preparation*

(I) *Traditional method*

It was found that FBT was unstable during the extraction procedure by the traditional extraction method. The HPLC chromatogram of FBT spiked into bovine milk at 0.20 ppm is shown in Figure 4, in which the peak with an arrow was derived from FBT. The results were consistent with those reported by Rose[4]. FBT and FBT sulfoxide were found to be unstable as they were converted to FEN during the extraction and cleanup procedure.

(II) *MSPD method*

The theoretical aspects of MSPD, including the disruption, unfolding and dispersion of the biological matrix onto the solid support, greatly increase the surface area of the matrix that will be exposed to the eluting solvent[20]. This simple procedure used a small amount of sample and a minimal amount of solvents requiring no chemical manipulation, such as pH adjustment. The advantages of saving time and less solvent consumption made this procedure an alternative to the traditional extraction method.

MSPD extraction and cleanup procedures described previously by Long *et al.*[20] were slightly modified. In order to increase the sensitivity, 1 g instead of 0.5 g[20] of ground liver sample was weighed. Ten microliter of internal standard solution (50.0 μg/mL), 0.5 ppm of benzimidazoles and 2 g of Bondesil-C18 were added and blended well. The mixture was loaded into a filtration column and washed with *n*-hexane. The lipid materials and some neutral chromophores which might interfere with subsequent benzimidazole analysis were removed by the wash with *n*-hexane. Therefore, excess *n*-hexane must be removed completely from the filtration column. If the benzimidazoles were eluted directly from the filtration column with acetonitrile, the eluate may contain interfering chromophores, making it difficult to quantify benzimidazoles. Therefore further cleanup by cartridges was tested consequently.

Alumina N, Sep-Pak C18 and florisil cartridges were examined for their cleanup efficiency. The acetonitrile eluate was passed through an alumina N or a Sep-Pak C18 cartridge directly. For florisil cartridge, on the other hand, acetonitrile eluate was concentrated, and the residue was dissolved in dichloromethane/methanol/triethylamine

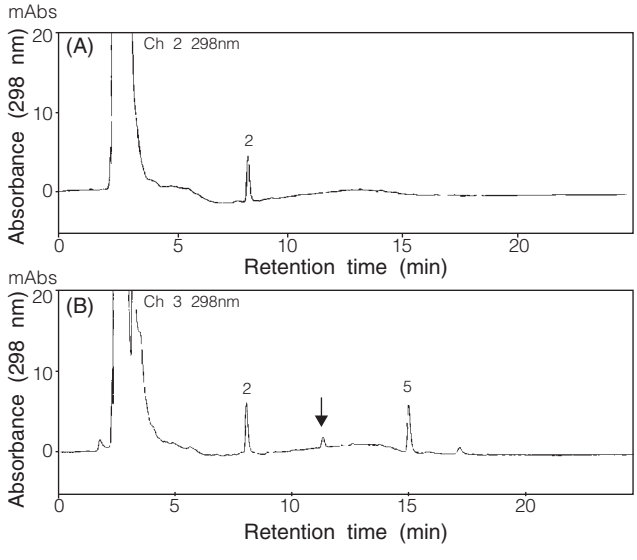

Figure 4. HPLC chromatograms of FBT spiked into bovine milk sample at 0.20 ppm using traditional method of extraction. (A) Blank bovine milk, (B) spiked bovine milk, the peak with an arrow was derived from FBT (peak 5). Peak identification is shown in Figure 3.

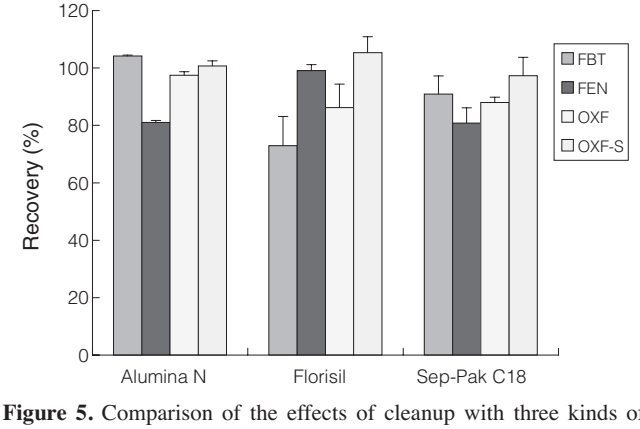

Figure 5. Comparison of the effects of cleanup with three kinds of cartridges on the recovery of four benzimidazoles by MSPD extraction method.

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Table 3. Recoveries of four benzimidazoles fortified into pork, swine liver, beef, lamb, bovine milk and goat milk at various spike levels using traditional extraction method

Tissue source	Spiked level (ppm)	Recovery[a] (%)			
		OXF	OXF-S	FEN	FBT
Pork					
	0.04	105.3 (9.9)[b]	91.7 (1.5)	77.6 (4.5)	72.3 (5.3)
	0.10	106.2 (4.9)	95.8 (1.9)	88.4 (3.1)	80.7 (1.1)
	0.20	98.3 (0.4)	106.5 (2.2)	81.5 (6.2)	78.1 (6.9)
Swine liver					
	0.04	99.8 (1.9)	88.3 (1.7)	81.1 (9.7)	79.7 (2.2)
	0.50	101.0 (0.5)	94.8 (1.2)	93.4 (0.1)	77.4 (9.4)
	1.00	102.7 (3.2)	99.1 (4.0)	96.8 (2.2)	75.4 (2.7)
Beef					
	0.04	108.7 (2.2)	100.1 (0.5)	75.0 (0.1)	74.9 (0.8)
	0.10	100.1 (0.5)	107.6 (2.1)	80.3 (6.2)	76.3 (9.8)
	0.20	98.3 (0.6)	103.2 (0.3)	81.6 (2.1)	73.9 (4.1)
Lamb					
	0.04	95.3 (4.7)	86.4 (2.9)	80.1 (7.9)	75.7 (0.5)
	0.10	92.9 (3.2)	100.0 (0.4)	80.3 (2.9)	73.9 (7.8)
	0.20	95.4 (4.8)	101.6 (1.4)	81.3 (3.0)	74.7 (8.8)
Bovine milk					
	0.04	90.9 (9.2)	107.3 (2.0)	80.5 (3.6)	81.6 (4.5)
	0.10	92.3 (2.4)	108.6 (0.5)	89.3 (3.4)	76.4 (2.6)
	0.20	93.7 (0.2)	98.7 (3.5)	85.5 (1.1)	79.1 (8.0)
Goat milk					
	0.04	107.9 (0.7)	89.0 (6.3)	88.3 (2.9)	81.3 (5.7)
	0.10	106.5 (0.6)	97.1 (3.8)	84.9 (1.3)	80.7 (5.4)
	0.20	102.6 (2.9)	102.9 (5.0)	82.5 (0.9)	79.5 (9.3)

[a]Average of triplicate analyses.
[b]Value in parenthesis is the coefficient of variation (CV, %).

(90/10/1, v/v/v) and passed through the cartridge for HPLC analysis. Figure 5 shows that both alumina N and Sep-Pak C18 cartridges were acceptable in respects to cleanup efficiency and recoveries. The elutate which passed through a Sep-Pak C18 cartridge appeared a little turbid, thus alumina N cartridge was selected for use. In conclusion, the diagram of sample preparation columns for the extraction and cleanup of benzimidazoles in MSPD method is shown in Figure 6. The animal tissue sample was dispersed onto Bondsil-C18, the mixture was packed in a column and washed with *n*-hexane. The washed mixture was connected in tandem with an alumina N cartridge and then eluted with acetonitrile for HPLC analysis.

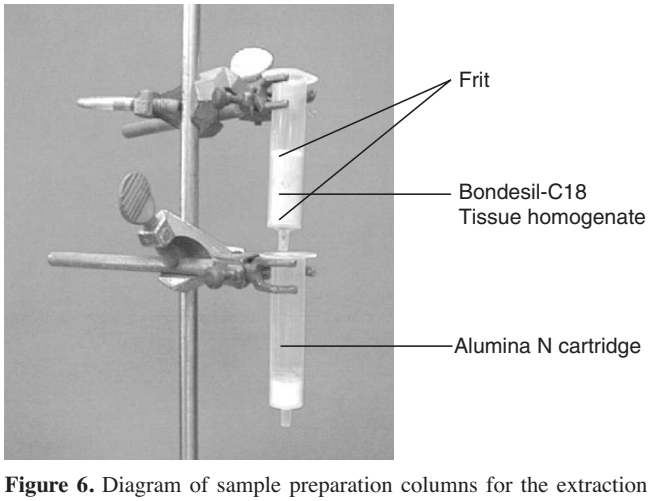

Figure 6. Diagram of sample preparation columns for the extraction and cleanup of four benzimidazoles in MSPD method.

IV. *Recovery and Detection Limit*

Accuracy is generally expressed as the percent recovery of the analyte of interest[25]. Recovery test of the traditional method was performed in triplicate by spiking 0 (as blank), 0.04, 0.10, and 0.20 ppm (0.04, 0.50 and 1.00 ppm for swine liver) of the four benzimidazoles to 5 g of ground samples (pork, beef and lamb) or 5 mL of milk samples (bovine milk and goat milk). The results of recovery are shown in Table 3. Since FBT was partially converted to metabolites during the sample preparation, recoveries of FBT were in the low range of 72.3 ~ 81.6%. Average recoveries of low concentration at 0.04 ppm of FEN ranged from 75.0 to 88.3%, and the remaining benzimidazoles ranged from 80.3 to 108.7% with coefficients of variation less than 9.9%. The recovery test of the MSPD extraction method was performed in triplicate by spiking 0, 0.10, 0.50, and 1.00 ppm of the four benzimidazoles to 1 g of ground samples or 1 mL of milk samples. The results of recovery are shown in Table 4. The average recoveries ranged from 80.2 to 109.6% with coefficients of variation less than 8.7%.

According to the Codex guidelines for the attributes of analytical methods for residues of veterinary drugs in foods, average recoveries of 80 to 110% should be achieved when the spike level is 100 μg/kg or higher and the coeffi-

Table 4. Recoveries of four benzimidazoles fortified into pork, swine liver, beef, lamb, bovine milk and goat milk at various spike levels using MSPD extraction method

Tissue source	Spiked level (ppm)	Recovery[a] (%)			
		OXF	OXF-S	FEN	FBT
Pork					
	0.10	99.4 (7.0)[b]	107.7 (2.3)	87.0 (4.8)	106.1 (2.1)
	0.50	102.2 (4.2)	106.9 (0.3)	82.2 (3.0)	109.2 (4.9)
	1.00	91.8 (0.4)	103.1 (0.1)	80.2 (3.7)	101.3 (0.3)
Swine liver					
	0.10	102.4 (7.0)	93.9 (8.7)	87.7 (6.4)	97.8 (7.7)
	0.50	97.5 (1.1)	100.7 (1.8)	81.4 (3.4)	107.2 (0.1)
	1.00	87.8 (0.2)	95.0 (1.0)	83.2 (1.9)	97.6 (1.5)
Beef					
	0.10	98.1 (0.7)	95.3 (8.5)	89.6 (5.1)	96.9 (5.8)
	0.50	99.7 (0.4)	101.7 (1.5)	101.3 (6.7)	109.0 (6.5)
	1.00	91.3 (1.8)	105.0 (4.3)	82.5 (1.5)	107.9 (0.2)
Lamb					
	0.10	97.3 (3.2)	108.7 (0.7)	80.5 (1.2)	108.4 (0.8)
	0.50	90.9 (3.7)	100.3 (2.8)	84.5 (1.9)	105.1 (1.0)
	1.00	89.6 (0.2)	99.7 (1.3)	82.6 (3.2)	108.2 (0.1)
Bovine milk					
	0.10	100.6 (8.7)	105.7 (6.2)	95.5 (5.6)	103.8 (5.3)
	0.50	99.4 (1.5)	104.7 (4.1)	83.2 (3.4)	109.6 (0.9)
	1.00	90.4 (2.7)	99.6 (1.7)	84.1 (3.1)	107.2 (0.3)
Goat milk					
	0.10	95.1 (6.7)	102.4 (3.6)	94.0 (4.8)	96.1 (5.2)
	0.50	100.9 (5.4)	106.5 (1.5)	89.8 (1.9)	97.4 (6.4)
	1.00	83.3 (5.9)	97.4 (3.1)	81.7 (7.6)	100.4 (5.7)

[a]Average of triplicate analyses.
[b]Value in parenthesis is the coefficient of variation (CV, %).

Table 5. Detection limits of four benzimidazoles by traditional and MSPD extraction methods

Benzimidazole	Detection limit (ppm)	
	Traditional method	MSPD method
OXF	0.010	0.025
OXF-S	0.010	0.025
FEN	0.020	0.025
FBT	0.020	0.050

cients of variation should be less than 15%. The recommended acceptable recoveries were 70 to 110% when the spike level is 10 to 100 μg/kg and the coefficients of variation is less than 20%[25]. Some of the average recoveries for FBT using the traditional extraction method did not comply with the criterion of the Codex due to the partial conversion of FBT. On the contrary, the average recoveries of MSPD extraction method were in compliance with the Codex guidelines.

The detection limits by the traditional extraction method were 0.010 ppm for OXF and OXF-S and 0.020 ppm for FEN and FBT, while those by MSPD extraction method were 0.025 ppm for OXF, OXF-S and FEN and 0.050 ppm for FBT, respectively (Table 5). Although the detection limits by MSPD extraction method were slightly higher than those by the traditional extraction method, they were still lower than those by the method of Long *et al*. (0.1 ppm). In addition, both values were lower than the residue limits of veterinary drugs set by the Department of Health[8]. HPLC chromatograms of swine liver for the detection limits by the traditional and MSPD extraction methods are displayed in Figure 7. The MSPD method has higher clean up efficiency and much less background interferences.

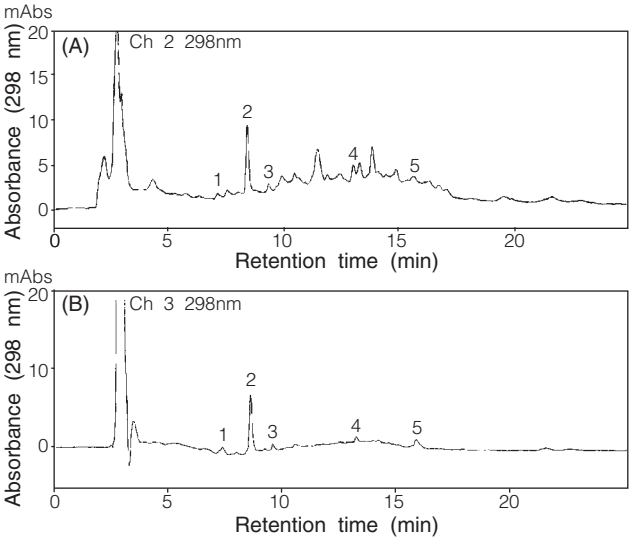

Figure 7. HPLC detection limits of four benzimidazoles in swine liver by (A) traditional extraction method, and (B) MSPD extraction method.

V. *Intra-day and Inter-day Repeatability*

Four benzimidazoles were fortified into swine liver

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Table 6. Intra-day and inter-day repeatability of the analysis of four benzimidazoles in swine liver samples using traditional extraction method

Benzimidazole	Spiked level (ppm)	Intra-day		Inter-day	
		Recovery	CV (%) (n = 3)	Recovery	CV (%) (n = 9)
OXF	0.04	93.6	6.95	92.1	5.93
	0.50	98.4	2.01	98.7	2.38
	1.00	101.1	2.10	102.7	1.94
OXF-S	0.04	96.3	3.08	95.1	5.95
	0.50	97.4	3.27	91.9	7.23
	1.00	97.7	1.52	97.6	2.15
FEN	0.04	104.3	4.47	98.6	4.57
	0.50	98.1	1.08	96.6	5.02
	1.00	92.7	1.37	94.3	2.43
FBT	0.04	79.6	1.60	73.7	12.12
	0.50	77.4	5.42	71.0	11.10
	1.00	78.7	0.81	78.3	8.31

Table 7. Intra-day and inter-day repeatability of the analysis of four benzimidazoles in swine liver samples using MSPD extraction method

Benzimidazole	Spiked level (ppm)	Intra-day		Inter-day	
		Recovery	CV (%) (n = 3)	Recovery	CV (%) (n = 9)
OXF	0.10	95.2	2.38	92.5	5.69
	0.50	97.5	1.16	92.8	3.23
	1.00	87.8	0.16	89.7	7.89
OXF-S	0.10	107.2	0.92	101.8	3.79
	0.50	92.8	2.05	95.1	2.09
	1.00	98.1	1.04	99.6	5.15
FEN	0.10	87.8	4.47	86.3	4.35
	0.50	80.7	0.70	80.1	0.70
	1.00	91.3	2.40	88.1	5.10
FBT	0.10	99.2	3.77	101.3	7.04
	0.50	100.9	3.54	101.3	2.57
	1.00	101.9	2.01	102.2	4.29

samples at three spiked levels and analyzed by both methods for examination of the intra-day and inter-day repeatability. Each spike level was analyzed in triplicate samples for intra-day repeatability. For inter-day repeatability, sample of each spiked level was analyzed three times for three days using daily prepared standard solutions and mobile phase. In other words, sample of each spiked level was analyzed nine times. The coefficients of variation of intra-day and inter-day assays by the traditional extraction method were lower than 6.95 and 12.12%, respectively (Table 6), and those by MSPD extraction method were lower than 4.47 and 7.89%, respectively (Table 7). These results indicated that both methods presented an acceptable precision.

VI. *Comparison of Traditional and MSPD Extraction Methods*

Comparing traditional and MSPD extraction methods for the analysis of benzimidazoles, the following characteristics were observed from the data in Table 8. (1) The detection limits for benzimidazoles were 0.010 ~ 0.020 ppm using the traditional method and 0.025 ~ 0.050 ppm using the MSPD method. (2) Traditional method required sample homogenization in large volumes of extracting solvents, more solvent partitioning steps and evaporation of large volumes of extracting solvents. On the other hand, MSPD method needs only a few steps, and the filtration column was washed with *n*-hexane instead of solvent partitioning step. (3) Traditional procedure required pH adjustment, while MSPD extraction did not. (4) MSPD method minimized sample preparation time (1 hr/sample) in comparison to traditional method (2.5 hr/sample). (5) MSPD method used minimal amount of solvents (16 mL/sample), while traditional method used larger volumes of extracting solvents (130 mL/sample). (6) FBT was partially converted to metabolites during sample preparation in the traditional method. (7) Cleanup efficiency of MSPD method was higher than that of the traditional method.

Table 8. Comparison of traditional extraction and MSPD extraction methods for the analysis of benzimidazoles

	Traditional method	MSPD method
Detection limit	0.010~0.020 ppm	0.025~0.050 ppm
Sample preparation step	Complicated	Simple
Preparation time	2.5 hr/sample	1.0 hr/sample
Organic solvent	130 mL/sample	16 mL/sample
FBT	Unstable	Stable
Background interferences	A little	None

VII. *Inspection of Benzimidazoles in Commercial Livestock Products*

Traditional and MSPD extraction methods were used to inspect four benzimidazoles in eight types of samples,

including pork, swine liver, beef, lamb, bovine milk, and goat milk (48 samples in total), purchased from local markets in Taipei. The inspection results showed that no benzimidazole residues were detected.

CONCLUSIONS

Traditional and MSPD extraction techniques, in combination with HPLC with a PDA detector, were developed and evaluated for simultaneous determination of four benzimidazoles in livestock. Compared to the traditional method, MSPD method is faster, less laborious and consumes less solvents. In addition, the proposed method developed in this study combined MSPD extraction technique with solid phase extraction cleanup, and performed thorough extraction and cleanup in a single elution step. A PDA detector was used to confirm the compounds of interest by comparing the spectra of analytes with those of standards. The MSPD method developed is suggested to be used in the routine analysis of the four benzimidazoles in livestock products.

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