Determination of Flubendazole and Its Metabolites in Eggs and Poultry Muscle with Liquid Chromatography–Tandem Mass Spectrometry

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The optimization of a quantitative and sensitive LC-MS/MS method to determine flubendazole and its hydrolyzed and reduced metabolites in eggs and poultry muscle is described. The benzimidazole components were extracted from the two matrices with ethyl acetate after the sample mixtures had been made alkaline. The HPLC separation was performed on an RP C-18 column with gradient elution, using ammonium acetate and acetonitrile as mobile phase. The analytes were detected after atmospheric pressure electrospray ionization on a tandem quadrupole mass spectrometer in MS/MS mode. The components were measured by the MS/MS transition of the molecular ion to the most abundant daughter ion. The overall extraction recovery values for flubendazole, the hydrolyzed metabolite, and the reduced metabolite in eggs (fortification levels of 200, 400, and 800 μg kg^{-1}) and muscle (fortification levels of 25, 50, and 100 μg kg^{-1}) were, respectively, 77, 78, and 80% and 92, 95, and 90%. The trueness (fortification levels of 400 and 50 μg kg^{-1}, respectively, for eggs and muscle), expressed as a percentage of the added values for these analytes, was, respectively, 89, 100, and 86 and 110, 110, and 98%. The proposed MS detection method operating in the MS/MS mode is very selective and very sensitive. The limits of detection for flubendazole and its hydrolyzed and reduced metabolites in egg and muscle were, respectively, 0.19, 0.29, and 1.14 μg kg^{-1} and 0.14, 0.75, and 0.31 μg kg^{-1}. The limits of quantification were, respectively, 1, 1, and 2 μg kg^{-1} and 1, 1, and 1 μg kg^{-1}. The discussed method was applied to a pharmacokinetic study with turkeys. Residue concentrations in breast and thigh muscle of turkeys orally treated with flubendazole were quantified. Medicated feed containing 19.9 and 29.6 mg kg^{-1} flubendazole was provided to the turkeys for seven consecutive days. For the trial with the recommended dose of 19.9 mg kg^{-1}, one day after the end of the treatment, the mean sum of the flubendazole plus hydrolyzed metabolite residue values in thigh and breast muscle declined to below the maximum residue limit (50 μg kg^{-1}) and were, respectively, 36.6 and 54.1 μg kg^{-1}. The corresponding values with the higher dose of 29.6 mg kg^{-1} were, respectively, 101.7 and 119.7 μg kg^{-1}.

Keywords: *Flubendazole; metabolites; residues; poultry; LC-MS/MS*

INTRODUCTION

Flubendazole (FLUB) is a broad-spectrum benzimidazole anthelmintic, effective against endoparasites such as gastrointestinal roundworms, gapeworms, and tapeworms (*1*). This drug is widely used in the veterinary medicine of poultry. An administration of FLUB can result in the presence of residues of the parent compound FLUB and its hydrolyzed metabolite (HMET) and reduced metabolite (RMET) in eggs and meat. By Council Regulation (*2*) 2377/90 and updates, the European Union sets the maximum residue limits (MRLs) for FLUB in eggs at 400 μg kg^{-1} and for FLUB plus HMET in poultry muscle at 50 μg kg^{-1}.

Only a few chromatographic methods have been published to determine flubendazole in eggs and/or

muscle (*3*–*6*). Some excretion data in eggs of laying hens are available (*3*, *4*). However, no data on residue formation in poultry muscle after administration are published.

First, this paper reports the optimization and validation of a quantitative LC-MS/MS analytical method for the determination of FLUB, HMET, and RMET in eggs and poultry muscle. In addition, the excretion of flubendazole and its metabolites in turkey muscle after oral administration of flubendazole at two concentration levels was studied. Some liver samples were also analyzed.

MATERIALS AND METHODS

Reagents. Analytical standards of FLUB, HMET, RMET, and internal standard [IS = methyl [5-(4-chlorobenzoyl)-1*H*-benzimidazol-2-y1]carbamate] were kindly provided by the manufacturer Janssen Animal Health (Beerse, Belgium). The chemical structures of these analytes are shown in Figure 1. Acetonitrile (HPLC grade), methanol (HPLC grade), and acetic acid, 100% (analytical grade), were from Merck (Darmstadt,

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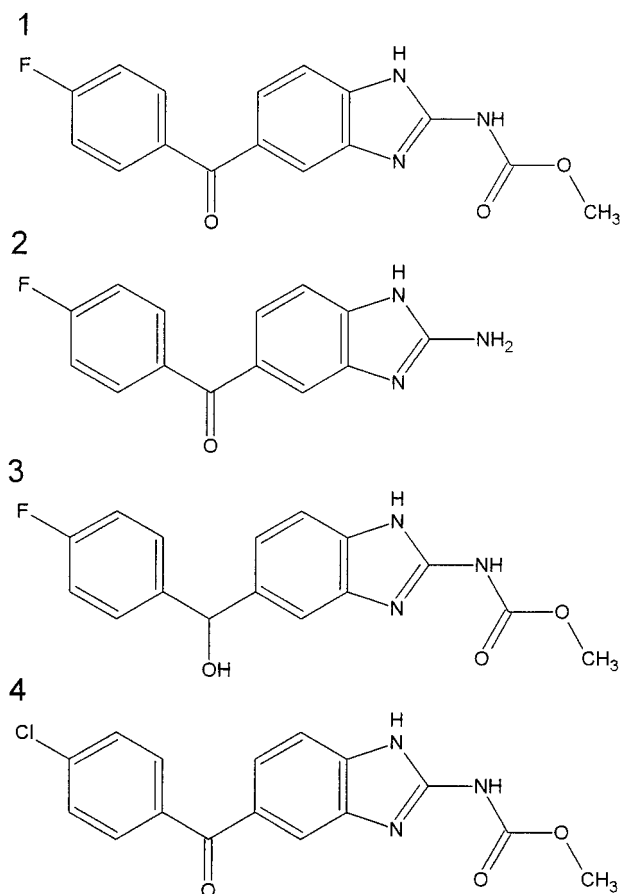
Figure 1. Molecular structures of FLUB (**1**), HMET (**2**), RMET (**3**), and IS (**4**).

Germany). Dimethyl sulfoxide (HPLC grade), ethyl acetate (HPLC grade), ammonium acetate (analytical grade), and 0.1 N sodium hydroxide (analytical grade) were purchased from Panreac (Barcelona, Spain). *n*-Hexane (analytical grade) was from BDH (Poole, U.K.). All water used was of HPLC grade (Maxima Ultrapure LC 113 water purification system, Elga, Bucks, U.K.).

Standard Solutions. The analytical standards were dissolved in dimethyl sulfoxide at a concentration of 0.3125 mg mL^{-1}. These solutions were diluted with methanol to stock standard solutions of 0.1 mg mL^{-1}. The working standard solutions were made by dilution with methanol and prepared immediately before use. The stock standard solutions were stored refrigerated at 5 °C for up to 2 months. Standard solutions of 1 μg mL^{-1} in 30:70 (v/v) ammonium acetate and acetonitrile were applied for tuning the mass spectrometer and for acquisition of the analyte identification spectra.

Instrumentation. The muscle tissue samples were minced and ground with a Knifetec sample mill (Tecator, Hoganas, Sweden). The ground muscle tissue samples and the egg samples were homogenized with a Unimix homogenizer (Haagen and Rinau, Bremen, Germany). An RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE) and a vortex mixer (Scientific Industries, Bohemia, NY) were used during the sample preparation. The LC-MS/MS system comprised an LC system (Kontron, Biotech Instruments, Milan, Italy) with a 325 ternary pump system, a vacuum degasser, a 465 autosampler, and a 433 UV detector, coupled with a quattro LCZ tandem quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, U.K.) with z-spray electrode and electrospray ion interface. The mass spectrometer was fully controlled by Masslynx software version 3.3. A syringe pump (Harvard Apparatus model 11, Holliston, MA) was connected to the interface for tuning purposes. The LC separations were carried out on a reversed-phase column Alltima C18 (Alltech, Deerfield, IL), 5 μm, 150 × 2.1 mm i.d., in combination with an Alltima C18 guard cartridge of 7.5 × 2.1 mm i.d..

Sample Preparation. *Muscle and Liver Samples.* One gram of ground sample was weighed into a 50 mL beaker. If necessary, FLUB, HMET, RMET, and/or IS solutions were added. The IS was spiked with a working standard solution of 1 μg mL^{-1}.

After 30 min, the mixture was homogenized in 10 mL of water for 30 s. The mixture was made alkaline with 5 mL of 0.1 N sodium hydroxide. The benzimidazole compounds were extracted with 20 mL of ethyl acetate in centrifuge tubes on a shaker for 10 min. After centrifugation (5 min, 5000 rpm), the supernatant was transferred into a sidearm flask. The extraction procedure was repeated once with 10 mL of ethyl acetate. The collected organic fractions were evaporated to 4–5 mL on a water bath at 60 °C using vacuum. The residual fraction was transferred into a graduated tube and was evaporated to dryness under a stream of nitrogen. The residue was dissolved with 600 μL of methanol. After mixing, the mixture was heated at 60 °C on a water bath during 5 min. The mixture was defatted with 2 mL of *n*-hexane. After standing for 5 min, the upper hexane layer was removed. If necessary, for recovery experiments, the IS was added with a working standard solution of 1 μg mL^{-1}. The solution was made up to 1 mL with methanol and heated at 60 °C on a water bath for 2 min. After mixing, the mixture was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter into an autosampler vial.

Egg Samples. One gram of mixed whole egg was weighed into a centrifuge tube. If necessary, FLUB, HMET, RMET, and/or IS solutions were added. The IS was spiked with a working standard solution of 1 μg mL^{-1}. After 30 min, the mixture was made alkaline with 1 mL of 0.1 N sodium hydroxide. The following steps were identical to those of the extraction and cleanup for muscle tissue and liver samples.

LC-MS/MS Method. The HPLC separation of flubendazole and its metabolites was carried out on a reversed-phase column with a buffer solution. The mobile phase consisted of a buffer solution containing 0.04 M ammonium acetate adjusted to pH 5.2 (A) with a few microliters of acetic acid 100% and acetonitrile (B). Gradient elution was applied, and the program consisted of 50A:50B (0 min), 50A:50B to 25A:75B (0–3 min), 25A:75B (4–5 min), 25A:75B to 50A:50B (6–7 min) and 50A:50B (8–15 min). The flow rate was 0.25 mL min^{-1}, and the injection volume was 10 μL.

The analytes were detected and identified with a tandem quadrupole mass spectrometer. An incorporated split device was set to a split ratio of 1:1. Atmospheric pressure electrospray ionization in the positive mode (ESI$^+$) was applied. The parent ions were fragmented in the collision cel with argon. FLUB, HMET, RMET, and the IS were determined with MS/MS by the multiple reaction monitoring (MRM) function of one transition. Cone voltage and collision energy were tuned to optimize the transition of the molecular, parent ion to the most abundant daughter ion.

Method of Calculation. The measured data of the samples by MRM of one transition are evaluated by an internal standard procedure based on the calibration curves. Quantification is conducted by internal calibration following the formula

$$C_S \ (\mu g \ kg^{-1}) =$$
$$[(area_S \times C_{IS} \ (\mu g \ kg^{-1})/area_{IS} - intercept)/slope] \times 1/CF$$

where C is concentration, S is sample, IS is internal standard, and CF is concentration factor.

The results were calculated automatically by MassLynx software version 3.3 of the mass spectrometer.

Validation of the Analytical Method. During the validation procedure, the EU rules of Commission Decision 93/256/EC (*7*) for the determination of drug residues were followed as much as possible. The validation procedure was carried out completely for the matrices egg and chicken muscle tissue.

The linearity of the LC-MS/MS response was checked by analyzing three series standard solutions of a mixture of FLUB and its metabolites with concentration levels of 0.5, 1, 5, 25, 100, 500, 600, and 800 μg L^{-1}.

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The calibration curves were obtained by analyzing three series of spiked egg and muscle samples with a mixture of FLUB and its metabolites at concentration ranges of 0.5, 1, 5, 25, 100, 500, 600, and 800 μg kg^{-1}. The curves were calculated using the linear least-squares regression analyses of the peak area to concentration ratios. The accuracy of the linearity of the whole analytical procedure is expressed as the relative standard deviation (s_r) of the slope of the curves.

The extraction efficiencies for FLUB, HMET, and RMET were determined with 10 blank samples fortified at three concentration levels, namely, half-MRL, MRL and 2 MRL. For this validation parameter only, the IS was not extracted but added just before injection on the HPLC column. The recovery values were calculated with calibration curves especially made during the evaluation of these validation parameter with concentration levels for muscle and eggs, respectively, of 10, 25, 50, 100, and 200 μg kg^{-1} and 100, 200, 400, 800, and 1000 μg kg^{-1}.

The limits of detection (LOD) were calculated as the apparent content corresponding to the value of the mean plus three times the standard deviation (s) obtained for at least 20 representative blank sample determinations. If only noise was obtained, a technical LOD was calculated as those concentrations that yield a signal to noise ratio (S/N) of 3:1.

The limits of quantification (LOQ) were defined as the lowest concentration of the analytes for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EC (*7*).

The trueness was calculated as the closeness between the mean measured value and the true value. This validation parameter was determined by analyzing 10 blank samples fortified with the analytes at MRL level. To calculate the trueness values, calibration curves especially made at this time were used with concentration levels for muscle and eggs, respectively, of 25, 50, and 100 μg kg^{-1} and 200, 400, and 800 μg kg^{-1}. The trueness is expressed as the percentual agreement between the mean measured value and the added concentration level. This validation parameter gives the measured concentrations after compensation for recovery which is incorporated in the calibration curves. The repeatability or within-day precision of the assay for eggs was studied by repetitively analyzing a blank egg sample, spiked at 100 μg kg^{-1} with a mixed spiking solution. This validation parameter for muscle was evaluated by repetitively analyzing a turkey breast sample with an incurred residue taken during the administration period of the depletion study. The repeatability was expressed as s_r value.

The IS was spiked or added with a working standard solution of 1 μg mL^{-1}. For the validation parameters linearity of response, calibration curves, LODs, LOQs, and repeatability, the IS was spiked to a concentration, respectively, of 100, 100, 10, 10, and 100 μg mL^{-1} or μg kg^{-1}. IS concentrations for recovery and trueness experiments are summarized in next section. For the real turkey muscle samples, the IS was spiked to a concentration of 50 μg kg^{-1}.

Sample Fortification Procedure for Recovery and Trueness. *Recovery.* For the recovery experiments, the standard solutions of target analytes and IS were added after the hexane removal during the sample preparation of fortified blank egg samples and blank muscle samples.

To fortify egg samples at 200, 400, and 800 μg kg^{-1} (IS at 400 μg kg^{-1}), respectively, 40, 80, and 160 μL of a standard solution of 5000 μg L^{-1} of a mixture of compounds was added. For the calibration curves (concentration levels of 100, 200, 400, 800, and 1000 μg kg^{-1}), respectively, 100 μL of a standard solution of 1000 μg L^{-1} and 40, 80, 160, and 200 μL of a solution of 5000 μg L^{-1} were used.

To fortify muscle samples at 25, 50, and 100 μg kg^{-1} (IS at 100 μg kg^{-1}), respectively, 25, 50, and 100 μL of a standard solution of 1000 μg L^{-1} were added. The calibration curves (10, 25, 50, 100, and 200 μg kg^{-1}) for this matrix were made, respectively, with 100 μL of a standard solution of 100 μg L^{-1} and 25, 50, 100, and 200 μL of a solution of 1000 μg L^{-1}.

Trueness. For this validation parameter the same volumes and concentrations of standard solutions as for recovery were

used. The standard solutions were added to the blank samples into the centrifuge tube at the beginning of the extraction step.

The egg samples were spiked at 400 μg kg^{-1} for target analytes and IS. Calibration were done with curves of a concentration range of 200, 400, and 800 μg kg^{-1}. The muscle samples were fortified at 50 μg kg^{-1} for the target analytes and at 100 μg kg^{-1} for the IS. The concentration levels for calibration were 25, 50, and 100 μg kg^{-1}.

Animal Treatment. Day-old turkeys of the BUT 9 heavy medium strain (136 of each sex) were obtained from a commercial hatchery. They were reared separately until 8 weeks of age. Afterwards, males and females were allocated to three pens. The feeding program consisted of a phase-feeding system. Metabolizable energy, nitrogen correction = 0 (ME$_N$), in megajoules per kilogram and crude protein (CP) in percent in the feed for the day-old animals were 11.5 and 28.0, respectively. At 4, 8, and 12 weeks of age these composition parameters were, respectively, 12.0 and 25.0, 12.2 and 22.0, and 12.5 and 19.0. Feed (in mash for the first period and in pellets for the subsequent periods) and water were available ad libitum. To prevent coccidiosis and histomoniasis, 1 mg kg^{-1} Diclazuril (Janssen-Cilag, Beerse, Belgium) and 75 mg kg^{-1} Nifursol (Duphar, Weesp, The Netherlands) were supplemented into the diets for periods 1−3. The turkeys were vaccinated against Newcastle disease with La Sota (Intervet, Boxmeer, The Netherlands) at days 1, 21, and 72. In two pens, the turkeys were fed medicated feed containing 20 and 30 mg kg^{-1} flubendazole (Flubenol 5%, Janssen-Cilag) during weeks 13 and 15 of age for females and males, respectively. The measured FLUB concentrations in the feed were 19.9 ± 1.4 mg kg^{-1} (mean ± s) and 29.6 ± 1.4 mg kg^{-1}, respectively (State Analysis Laboratory, Tervuren, Belgium). Feed intake and feed conversion rate were calculated for the respective periods. Three male and three female turkeys were weighed and slaughtered at different ages according to the FLUB feeding schedule: just before the start, daily during the administration, and 2, 4, and 6 h and 1, 2, 5, and 7 days postadministration. At each time the same muscle group of breast and thigh and the liver were removed, frozen, and stored at −18 °C until investigation.

RESULTS AND DISCUSSION

Analytical Method. A relatively simple, fast, and reliable method was developed to determine and to quantify residues of FLUB and its metabolites.

Because of good results in former research work on benzimidazole drugs in milk (*8*), ethyl acetate was used as extraction agent in this study. Previous work and also the experience of Issar et al. (*9*) indicated a great influence of the alkaline conditions of the sample mixture on the extraction recovery of these weak basic compounds. Sodium hydroxide was chosen to attain alkaline conditions. Interfering residual fat particles were removed with *n*-hexane. This sample preparation and cleanup is comparable with those reported by Kan et al. (*4*) and Balizs (*3*).

Identification and determination of FLUB, its metabolites, and the IS were carried out by MRM. The mass spectrometer was previously tuned and optimized for maximum ion response of each analyte by infusion of a standard solution of 1 μg mL^{-1} with the syringe pump. A summary of the ions obtained with the examined compounds and the optimized MS parameters are given in Table 1. The indicated ions are protonated. The detection of the analytes in standard solutions and spiked and real known samples was performed by the transition of the parent ion $(M + H)^+$ to the most abundant daughter ion (italicized in Table 1). Preliminary tests indicated that the ratio of the intensity of the different fragment ions was constant. For unknown samples of monitoring programs, for example, more

Table 1. Summary of the Ions and MS Parameters

analyte	parent ion (m/z)	daughter ions (m/z)	cone voltage (V)	collision energy (eV)
FLUB	314.0^{+a}	282.0^{+a}, 122.8^{+}	40	25
HMET	255.9^{+a}	122.9^{+a}	50	30
RMET	316.2^{+a}	284.1^{+a}, 238.1^{+}, 160.0^{+}	45	25
IS	330.1^{+a}	297.9^{+a}, 138.8^{+}	50	25

[a] Ions used for MRM detection.
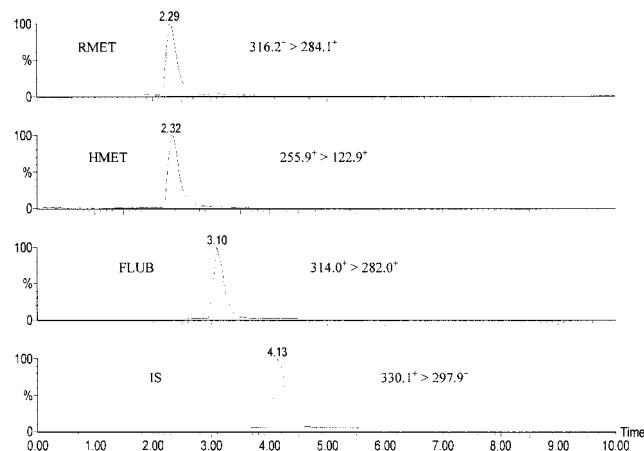
Figure 2. Chromatogram of a spiked egg sample at 10 μg kg^{-1} with a mixture of FLUB, HMET, RMET, and IS.

than one transition in the MRM mode has to be followed to confirm a positive sample.

The HPLC separation of the analytes was obtained with a gradient of 0.04 M ammonium acetate, pH 5.2, and acetonitrile. Several publications report separation methods with a mobile phase consisting of ammonium acetate (*3, 4, 10*). In previous work, methanol did not sufficiently separate the analytes. First of all, a gradient program was optimized for the separation of FLUB, HMET, and the IS. Increasing the flow rate from 0.2 to 0.25 mL min^{-1} limits the total HPLC run time to 15 min. The preliminary detection was done by UV absorption at 254 nm. Afterwards, RMET was also involved in the study and was determined with the same HLPC

procedure, and only MS detection was applied. As illustrated in Figure 2, the two metabolites are not chromatographically separated. HPLC separation, however, is not necessary because of the powerful separation capacity of the mass spectrometer in the MRM mode. Figure 2 shows a chromatogram of a spiked egg sample at 10 μg kg^{-1} with a mixture of the analyte compounds.

Validation of the Analytical Method. *Linearity of Response.* The linearity of the LC-MS/MS response was proven with eight calibration points in the concentration ranges of 0.5, 1, 5, 25, 100, 500, 600, and 800 μg L^{-1}. Three series of standard solutions were made. The s_r values of the slope of the standard curves for FLUB, HMET, and RMET were, respectively, 3.6, 1.8, and 1.0%.

Matrix Calibration Curves. The results of the regression analysis of the data obtained by analyzing the spiked egg and muscle samples are summarized in Table 2. The linearity of the eight point matrix calibration curves was proven in the concentration ranges of 0.5, 1, 5, 25, 100, 500, 600, and 800 μg kg^{-1}. The s_r values of the slope of the three different curves were very low and below 6%.

Extraction Recovery Efficiency. Table 3 reports the mean extraction recovery values obtained for egg and muscle samples spiked with a mixture of the analytes. The values ranged from 77 to 95% and were higher for the muscle samples. The overall extraction recovery values for FLUB, HMET, and RMET in eggs and muscle were, respectively, 77, 78, and 80% and 92, 95, and 90%. The values for eggs are comparable with those obtained by Kan et al. (*4*). Balizs (*3*) reported a somewhat lower recovery for FLUB in eggs. Our recovery for FLUB in muscle is comparable with the data obtained by Takeba et al. (*6*) (86%). However, Marti et al. (*5*) and Balizs (*3*) found, respectively, only 73 and 50%.

LOD. In contrast with the preliminary results described in our earlier short publication (*11*), the final LOD values were determined by analyzing series of 24 and 23 blank samples, respectively, for eggs and muscle tissues. The calculated LOD values are summarized in Table 4. For the HMET compound in each matrix, only white noise was measured. Therefore, a technical LOD

Table 2. Regression Analysis of Calibration Curves of Flubendazole and Its Metabolites

analyte		egg				muscle		
	curve	slope	intercept	R^2	curve	slope	intercept	R^2
FLUB	1	2.81	2.37	0.9926	1	2.18	1.80	0.9643
	2	2.95	1.39	0.9977	2	2.35	0.98	0.9829
	3	2.87	0.77	0.9952	3	2.29	9.55	0.9831
		$s_r = 2.4\%$				$s_r = 3.8\%$		
HMET	1	3.64	2.63	0.9820	1	2.71	0.41	0.9215
	2	4.04	1.45	0.9844	2	2.80	0.28	0.9711
	3	3.99	0.97	0.9946	3	2.60	8.12	0.9927
		$s_r = 5.6\%$				$s_r = 3.7\%$		
RMET	1	3.98	8.59	0.9825	1	1.84	3.28	0.9563
	2	4.34	6.22	0.9749	2	2.04	1.35	0.9774
	3	4.02	5.13	0.9754	3	1.99	8.17	0.9984
		$s_r = 4.8\%$				$s_r = 5.3\%$		

Table 3. Extraction Recovery Efficiencies for Fortified Blank Egg and Muscle Samples at 0.5, 1.0, and 2 MRL (Micrograms per Kilogram) Concentrations with Flubendazole and Metabolites [Mean Value (%) (s_r (%)), $n = 10$]

analyte		egg				muscle		
	200	400	800	overall	25	50	100	overall
FLUB	70.2 (7.7)	76.8 (9.0)	83.3 (7.0)	76.7 (10.4)	92.4 (6.8)	91.0 (6.8)	92.8 (6.1)	92.1 (6.4)
HMET	69.9 (11.8)	91.4 (9.4)	74.0 (7.9)	78.4 (15.4)	104.4 (6.4)	88.9 (9.4)	90.7 (7.4)	94.7 (10.5)
RMET	71.6 (9.3)	88.1 (8.2)	79.8 (5.9)	79.8 (11.5)	90.9 (6.8)	90.6 (8.4)	87.6 (6.5)	89.7 (7.3)

Table 4. LOD (Micrograms per Kilogram) of Flubendazole and Metabolites Determinations

compd	egg	muscle
FLUB	0.19	0.14
HMET	0.29[a]	0.75[a]
RMET	1.14	0.31

[a] Technical LOD with S/N = 3.

Table 5. LOQ of Flubendazole and Metabolites Determinations

	egg (n = 6)			muscle (n = 7)		
compd	LOQ (μg kg^{-1})	measd concn (μg kg^{-1})	s_r (%)	LOQ (μg kg^{-1})	measd concn (μg kg^{-1})	s_r (%)
FLUB	1	1.13 ± 0.13[a]	11.5	1	1.05 ± 0.09[a]	8.4
HMET	1	1.14 ± 0.18	15.6	1	1.01 ± 0.14	13.6
RMET	2	1.62 ± 0.14	8.5	1	1.15 ± 0.11	9.3

[a] Mean ± standard deviation.

Table 6. Repeatability of Benzimidazole Determinations [Mean Value (Micrograms per Kilogram) (s_r (%)] at 100 μg kg^{-1} for Eggs and for a Turkey Breast Muscle Sample with Incurred Residue

analyte	egg[a]	muscle[a]
FLUB	83.6 (7.0)	61.6 (8.7)
HMET	86.8 (10.5)	243.1 (13.4)
RMET	95.5 (7.2)	nd[b]

[a] n = 10. [b] Not detected.

with fortified samples was calculated as that concentration which yields an S/N of 3:1. These results illustrate the extreme sensitivity of the proposed LC-MS/MS method. With the exception of RMET in egg, all analytes can be detected at a residue level lower than 1 μg kg^{-1}. Our LOD values are lower than those reported by Balizs (*3*) and Kan et al. (*4*) and much lower than those of Marti et al. (*5*) and Takeba et al. (*6*).

LOQ. An overview of the LOQ values is given in Table 5. With the exception of RMET in egg (2 μg kg^{-1}), for all of the analytes in egg and muscle, the method was validated with an accuracy and precision that fall within the ranges recommended by the EC (*7*) for spiked samples at a concentration of 1 μg kg^{-1}.

Trueness. The trueness values for muscle samples are expressed as the percentual agreement between the mean measured concentration (n = 10) and 50 μg kg^{-1} (MRL). These values [s_r (%) in parentheses] for FLUB, HMET, and RMET were, respectively, 110 (10.1), 110 (4.9), and 98 (7.1). The corresponding figures for egg obtained with fortified samples at 400 μg kg^{-1} (MRL, n = 10) were, respectively, 89 (5.6), 100 (8.7), and 86 (6.9).

Repeatability. A summary of the repeatability or within-day precision is given in Table 6. For most of the analytes, the repeatability, expressed as s_r value, was not higher than 10%. This validation parameter was least favorable for the HMET compound and was 13.4% in the real turkey breast muscle sample at a concentration level of 243 μg kg^{-1}.

Depletion Study in Turkeys. During the animal trials, all turkeys were in good general health as indicated by the absence of any clinical sign. The growth results were in line with the target of BUT, the 12-week body weights were 12.7 and 7.6 kg for males and females, respectively. The corresponding feed conversion rates were about 1.84 and 1.91 for males and females, respectively. The administration of FLUB did not influence daily feed intake, and average values of 530 and

290 g day^{-1} were obtained for males and females, respectively. The results of the depletion study are shown in Figures 3 and 4. In some breast and thigh muscle samples, residues of RMET could be detected in concentrations around the LOD. These values were not shown in the figures. Some liver samples were also analyzed for residues with the same extraction method as for muscle. The determination method was not completely validated for liver tissues. However, the residue values were calculated with calibration curves obtained with spiked blank liver samples.

Figure 3 represents the mean FLUB and HMET residue values in breast and thigh muscles of turkeys during and after the administration of FLUB medicated feed at the recommended dose of 19.9 mg kg^{-1}. In the blank muscle samples, no drug residues could be detected. The LC-MS/MS analytical technique is very specific. No interference of the supplemented veterinary products Diclazuril and Nifursol was obtained. The mean body weights just before slaughtering in this trial were 8.1 and 12.9 kg for females and males, respectively. During the treatment period, no relationship was found between body weight and residue concentration. Probably, the effect of increasing of body mass on residue level was compensated for the higher feed intake. The shown residue values are mean values of six turkeys, three males and three females. The variability of the residue values of the six turkeys taken for one sampling time was high and is probably caused by physiological differences. The most important differences between the individual turkeys are the biodisponibility and the rates and routes of metabolism and excretion of the parent drug and the metabolites. The variability of the residues in thigh muscle for one sampling time expressed as s_r for FLUB and HMET ranged (mean values in parentheses) from 15.3 to 81.5% (36.5%) and from 12.1 to 74.3% (40.4)%, respectively. These ranges for breast muscle were, respectively, 20.0−50.1% (32.8%) and 18.9−72.7% (38.7%). The residue values of HMET were much higher than these of FLUB in both the thigh and breast muscles. Figure 3 shows a clear trend of a higher residue level in thigh muscle. To verify whether these differences were statistically significant, a four-parameter Weibull function was fitted to both data series of the two trials with different administration doses. Comparing the confidence intervals of the estimated Weibull parameters showed clear overlap and thus no significant difference for all cases. Nevertheless, when individual points of the breast−thigh series were compared with a *t* test assuming homogeneous variances, as validated by a Browne−Forsyth test, some measurement points proved to be significantly different. The majority of the individual points were not significantly different, which can be attributed to the substantial variance on the individual residue values of the six animals.

The higher fat content of thigh muscle can be an explanation for the higher residue level. Residue levels are also higher in yolk than in egg white (*3, 4*). Another possible explanation could be the higher blood flow in the thigh, which is necessary for the mobility. Figure 3 shows a somewhat more rapid depletion of both analytes in thigh muscle, probably also due to the higher blood flow. After 1 day of treatment, the residue level of FLUB plus HMET was already above the MRL value (50 μg kg^{-1}). The concentrations in thigh and breast muscles were, respectively, 304.8 and 266.6 μg kg^{-1}. The level

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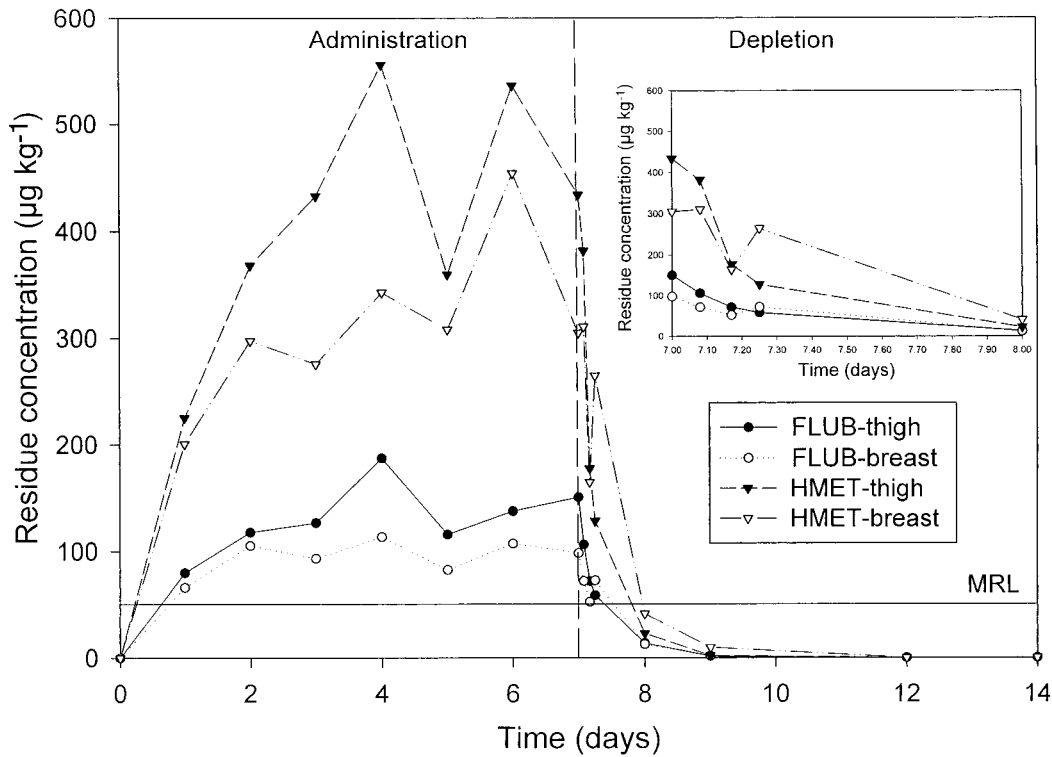
Figure 3. FLUB and HMET residues in turkey breast and thigh muscles during and after oral FLUB administration of 19.9 mg kg⁻¹ of feed for 7 days.
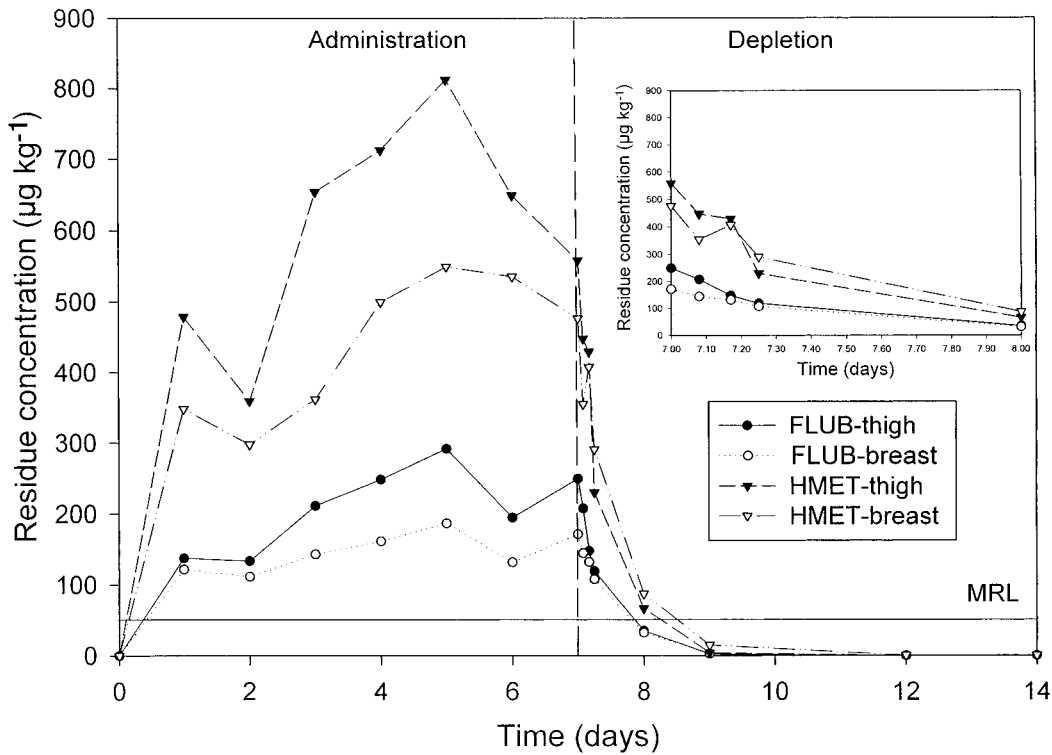
Figure 4. FLUB and HMET residues in turkey breast and thigh muscles during and after oral FLUB administration of 29.6 mg kg⁻¹ of feed for 7 days.

in the liver tissue was very high and reached 8304.6 μg kg⁻¹. A mainly steady state situation in residue level was obtained after 4 days of administration. The maximum FLUB plus HMET concentrations in the thigh and breast muscles were, respectively, 742.8 and 561.1 μg kg⁻¹ and were obtained after, respectively, 4 and 6 days of treatment. The liver concentration after 4 days of administration was 10604.2 μg kg⁻¹. On day

7, just before the treatment was stopped, this value was 10275.7 μg kg⁻¹. The decline in residue levels after cessation of the FLUB administration is very rapid, and after 1 day the residue concentration already decreased to a level around the MRL. To show clearly the decline of the residue level on the first day after administration, a detailed inset is given in Figure 3. As already mentioned, the drug residue excretion in breast muscle

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was somewhat slower. The residue levels of FLUB plus HMET in thigh and breast muscles were, respectively, 36.6 and 54.1 μg kg^{-1}. In the liver tissue, a residue concentration of 916.9 μg kg^{-1} was found. Five days after the administration was stopped, the drug residue depletion was complete and no residues could be detected in the muscle samples anymore. The mean calculated percentual ratios of HMET in thigh and breast meat to the total of FLUB and HMET were, respectively, 73 and 78%. This value in liver tissue was 93%.

The distribution of these benzimidazoles in muscle is higher than in eggs. Kan et al. (*4*) found a mean residue value for FLUB plus HMET of ~250 μg kg^{-1} in whole egg during a treatment with feed medicated near the recommended dose for laying hens.

The results of the depletion study of turkeys treated with the FLUB medicated feed at the highest concentration of 29.6 mg kg^{-1} are shown in Figure 4. Again, the excretion curves are made with the mean residue values for FLUB and HMET in thigh and breast muscles of six turkeys, three females and three males. Again, the fluctuation on the individual residue values was high. The variability of the residues in thigh muscle for one sampling time expressed as s_r respectively for FLUB and HMET ranged (mean values in parenthesess) from 17.9 to 67.6% (39.5%) and from 22.9 to 65.8% (40.2%). These ranges for breast muscle were, respectively, 11.0−56.7% (31.7%) and 9.5−63.8% (35.9%). No residues could be detected in the blank muscle samples. The mean body weights just before slaughtering in this second trial for females and males were, respectively, 8.1 and 12.5 kg. No relationship could be found between body weight and residue concentration. The same conclusions as for the first trial with the recommended FLUB dose can be made. The shapes of the excretion curves of the two trials are very similar. As expected, the residue levels of this trial are higher. The residue concentrations of HMET were much higher than those of FLUB in both the thigh and breast muscles. Again, a clear trend of a higher residue level of both the FLUB and HMET in thigh muscle was observed.

After 1 day of treatment, the residue levels of FLUB plus HMET in thigh and breast muscles were, respectively, 616.6 and 470.2 μg kg^{-1}. In liver, a residue level of 14206.7 μg kg^{-1} was already found. The maximum FLUB plus HMET concentrations in the muscle tissues were, respectively, 1104.3 and 736.5 μg kg^{-1} and were obtained after 5 days of treatment. The residue value in liver was 18127.0 μg kg^{-1}. One day after cessation of the FLUB administration, the residue levels of FLUB plus HMET in thigh and breast muscles were, respectively, 101.7 and 119.7 μg kg^{-1}. The residue value in liver had declined to 2333.9 μg kg^{-1}. Again, a detailed inset is given in Figure 4 to show clearly the drop of the residue level the first day after the drug administration was stopped. Generally, the depletion of the drug residues in breast muscle was somewhat slower. Seven days after the administration, a low mean HMET residue concentration of 0.6 μg kg^{-1} could be detected in breast muscle. The mean calculated percentual conversions for flubendazole to the hydrolyzed metabolite in thigh and breast meat were, respectively, 71 and 75%. This conversion value in liver tissue was 95%.

Conclusions. A relatively fast, sensitive, and very selective LC-MS/MS method for the determination of flubendazole and its hydrolyzed and reduced metabo-

lites is described. Due to the relatively intensive sample preparation and the very classic equipment required, it was not possible to determine more than 16 muscle samples or 32 egg samples per day.

Because of the lack of screening methods for these group of drugs, the method can be proposed for screening purposes in monitoring programs for poultry. With regard to the new rules for residue analysis, more than one transition in the MRM mode has to be followed to confirm a positive sample.

The results of the depletion study with turkeys demonstrated that there is no risk for public health when the veterinary drug is correctly applied, which means that farmers have to cease the Flubenol treatment at least 1 day before slaughtering. One day after cessation of the administration, the drug residue level of the sum of flubendazole and its hydrolyzed metabolite in muscle declined to or below MRL level.

ABBREVIATIONS USED

FLUB, flubendazole; HMET, hydrolyzed metabolite of flubendazole; RMET, reduced metabolite of flubendazole; MRL, maximum residue limit; IS, internal standard; PTFE, polytetrafluoroethylene; ESI$^+$, electrospray ionization in the positive mode; MRM, multiple reaction monitoring; C, concentration; S, sample; CF, concentration factor; s_r, relative standard deviation; LOD, limit of detection; s, standard deviation; S/N, signal to noise ratio; LOQ, limit of quantification; ME$_N$, metabolizable energy, nitrogen correction = 0; CP, crude protein.

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