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Sensitive chiral high-performance liquid chromatographic determination of anthelmintic flubendazole and its *phase I* metabolites in blood plasma using UV photodiode-array and fluorescence detection
Application to pharmacokinetic studies in sheep

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Dedicated to the 90th birthday of the late Professor Ivo M. Hais, MD, PhD (b. 5th July 1918, d. 25th October 1996), an excellent researcher in the field of chromatography, biochemistry and xenobiochemistry, an inspiring postgraduate teacher and a long term Editor of the Journal of Chromatography (1959–1996).

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Chiral HPLC with UV photodiode-array and fluorescence detection
Stereospecificity of the carbonyl reduction of flubendazole

ABSTRACT

Although benzimidazole anthelmintic flubendazole, methyl ester of [5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl]carbamic acid, is extensively used in veterinary and human medicine for the treatment of gastrointestinal parasitic helmint infections, reliable data about its pharmacokinetics in various species have not been reported. Our previous work [M. Nobilis, Th. Jira, M. Lísa, M. Holčapek, B. Szotáková, J. Lamka, L.Skálová, J. Chromatogr. A 1149 (2007) 112–120] had described the stereospecificity of carbonyl reduction during *phase I* metabolic experiments *in vitro*. For *in vivo* pharmacokinetic studies, further improvement and optimization of bioanalytical HPLC method in terms of sensitivity and selectivity was necessary. Hence, a modified chiral bioanalytical HPLC method involving both UV photodiode-array and fluorescence detection for the determination of flubendazole, both enantiomers of reduced flubendazole and hydrolyzed flubendazole in the extracts from plasma samples was tested and validated. Albendazole was used as an internal standard. Sample preparation process involved a pH-dependent extraction of the analytes from the blood plasma into *tert*-butylmethyl ether. Chromatographic separations were performed on a Chiralcel OD-R 250 mm × 4.6 mm column with mobile phase methanol-1 M $NaClO_4$ (75:25, v/v) at the flow rate 0.5 ml min^{-1}. In quantitation, selective UV absorption maxima of 290 nm (for reduced flubendazole), 295 nm (for albendazole), 310 nm (for flubendazole) and 330 nm (for hydrolyzed flubendazole) were used in the UV photodiode-array detection, and λ(exc.)/λ(emis.) = 228 nm/310 nm (for reduced flubendazole) and λ(exc.)/λ(emis.) = 236 nm/346 nm (for albendazole) were set on the fluorescence detector. The fluorescence detection was approximately 10-times more sensitive than the UV detection. Each HPLC run lasted 27 min. The validated chiral HPLC-PDA-FL method was employed in the pharmacokinetic studies of flubendazole in sheep. The stereospecificity of the enzymatic carbonyl reduction of flubendazole was also observed *in vivo*. (+)-Reduced flubendazole was found to be the principal metabolite in ovine blood plasma and only low concentrations of hydrolyzed flubendazole, the parent flubendazole and (−)-reduced flubendazole were detected in this biomatrix.

1. Introduction

A survey of flubendazole pharmacology, its physico-chemical properties and the possibilities of its determination in various biomatrices has been presented in our previous paper [1]. In the paper, a combination of both achiral and chiral HPLC methods

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was employed for the bioanalyses of flubendazole and its *phase I* metabolites in the liver and intestinal microsomal and cytosolic fractions prepared from pigs and pheasants. The use of an achiral internal standard method provided information about the concentrations of hydrolyzed flubendazole, total reduced flubendazole (the total of both enantiomers) and flubendazole with albendazole as an internal standard. The chiral method also enabled the separation of both enantiomers of reduced flubendazole and the evaluation of their enantiomeric excess (e.e.). Unfortunately, the coelution of flubendazole and albendazole concentration zones prevented the use of the chiral method in an internal standard mode, which is usually required in bioanalytical HPLC. The use of the chiral external standard method for the analyses of benzimidazole carbamates in simple biomatrices was acceptable for the evaluation of *in vitro* experiments and, in this way, flubendazole biotransformation studies in subcellular fractions of homogenates from the parasitic worm *Haemonchus contortus* [2], mouflon, pheasant and pig tissues [1,3,4] were performed.

On the other hand, chiral HPLC analyses of the extracts from more complicated biomatrices (blood plasma) should be encumbered by losses and errors introduced throughout the sample preparation process. Hence, the use of a suitable internal standard in the chiral HPLC method was desirable.

Consequently, this study is focused on resolving the principal deficiencies and limitations of the previously described method [1]: achieving chromatographic separation of flubendazole and albendazole (I.S.) and increasing the sensitivity and selectivity of the chiral HPLC method using a tandem UV-photodiode-array and fluorescence detection. The modified and validated analytical method should be more suitable for pharmacokinetic and other *in vivo* studies of flubendazole, where low concentrations of benzimidazole carbamates could be expected.

2. Experimental

2.1. Chemicals, preparations, solutions, materials

Flubendazole, [5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl]-carbamic acid methyl ester, $C_{16}H_{12}FN_3O_3$, MW(molecular weight) = 313.29 g/mol, CAS 31430-15-6; racemic mixture of reduced flubendazole {(±)-[5-(4-fluorophenyl)hydroxymethyl-1*H*-benzimidazole-2-yl]carbamic acid methyl ester, $C_{16}H_{14}FN_3O_3$, MW = 315.3 g/mol} and hydrolysed flubendazole {[(2-amino-1*H*-benzimidazole-5-yl)-4-fluorophenyl]methanone, decarbamoylated flubendazole, $C_{14}H_{10}FN_3O$, MW = 255.25 g/mol} were purchased from Janssen Pharmaceutica, Belgium.

Albendazole, 5-(propylthio)-2-benzimidazolecarbamic acid methyl ester ($C_{12}H_{15}N_3O_2S$, MW = 265.34 g/mol, CAS No.54965-21-8), was obtained from Sigma.

Acetonitrile (HPLC grade), *tert*-butyl methyl ether (analytical grade; both from Merck, Darmstadt, Germany), ammonium hydroxide (26% aqueous solution of NH_3), methanol (both of analytical grade, Lachema, Brno, Czech Republic), sodium perchlorate monohydrate (Lach-Ner s.r.o., Neratovice, Czech Republic), *N*,*N*-dimethyl formamide (DMF, 99.9+%, HPLC grade, Aldrich), ultra-high quality (UHQ) water (prepared using Elgastat UHQ PS apparatus, Elga Ltd., Bucks, England) were used in the sample preparation and chromatography of benzimidazole derivatives (see Sections 2.3 and 2.4).

The stock solutions (10^{-3} M) of flubendazole (6.26 mg/20 ml), hydrolysed flubendazole (5.11 mg/20 ml) and reduced flubendazole (racemic mixture of both enantiomers, 6.31 mg/20 ml) were dissolved in dimethyl formamide, albendazole (26.53 mg/100 ml) was soluble in acetonitrile. Lower concentrations (10^{-4} M, etc.) were prepared by diluting the stock solutions with the mobile phases used in the chiral separation.

FLUBENOL (50% flubendazole praemix ad us. vet., Janssen Pharmaceutica, Belgium) and Avicel RC-591, FMC, Belgium (microcrystalic cellulose) were used for the preparation of aqueous suspension administered to animals. Li-Heparin plastic tubes (Monovette, Sarstedt, Nümbrecht, Germany) were used for the blood sample collection during a pilot pharmacokinetic study.

2.2. A pilot pharmacokinetic study in sheep

To investigate the basic pharmacokinetic information concerning the plasma concentration of flubendazole and its metabolites in sheep and to specify the optimal sampling times for future pharmacokinetic studies, a pilot study on a single animal was undertaken.

A sexually non-matured ram (*Ovis aries*, Merinolandshaf breed) of 96 days of age and body weight 24 kg was experimentally treated with Flubenol. Flubenol 50% prm. ad us. vet. (Janssen, Belgium) was suspended in 1.5% aqueous microcrystalline cellulose, the concentration of the drug in the suspension was 2 g/100 ml. Suspended flubendazole was administered in a single dose of 30 mg/kg of body weight in 36 ml volume, a loader for the enteral administration of liquid pharmaceuticals was used. 10-ml blood samples were withdrawn (blank plasma, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 h) after the administration of the flubendazole suspension from *vena jugularis* into Li-Heparin plastic tubes (Monovette, Sarstedt). The blood samples were centrifuged (2500 × *g* for 10 min), the plasma was separated and stored (−70 °C) until the analysis. All experimental procedures were performed under the supervision of the Ethical Committee of the Charles University in Prague, Faculty of Pharmacy in Hradec Králové and the rules described in "The Guide for Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1996, Bethesda, USA) were applied to sheep used in this study.

The basic pharmacokinetic parameters for flubendazole, reduced flubendazole and hydrolyzed flubendazole were determined: maximum plasma concentration (c_{max}), time point at maximum plasma concentration (t_{max}), area under plasma concentration curve in the time interval 0–72 h ($AUC_{(0-72\,h)}$). The elimination rate constant (k_e) was expressed as the negative value of the slope k in the relationship $\ln(\text{concentration}) = k(\text{time}) + q$, which was constructed from the last six concentration values (withdrawal after 12, 24, 36, 48, 60 and 72 h). Plasma concentration half-life ($t_{(1/2)}$) was calculated using the equation $t_{(1/2)} = \ln(2)/k_e$.

2.3. Sample preparation: liquid–liquid extraction of ovine blood plasma

30 μl of 10^{-4} M albendazole (I.S.) in acetonitrile was transferred into a glass tube equipped with a ground-glass stopper, the solvent was evaporated to dryness. Plasma (1 ml, see Section 2.2) was added into the glass tube with albendazole, the sample was shortly sonicated, and 30 μl of ammonium hydroxide were added. The mixture was extracted with 3 ml of *tert*-butylmethyl ether. After centrifugation (2500 × *g* for 10 min), the glass tube was placed into a deep freezer (−80 °C for 30 min) until the bottom water layer froze to ice. Then the upper layer (ether) was decanted into another clean tube. The solvent was evaporated to dryness. The dry extract was reconstituted in 600 μl of the mobile phase and 100 μl were injected into the chromatograph.

2.4. Chiral HPLC with ultraviolet photodiode-array and fluorescence detector

The development and validation of the chiral HPLC method and routine chromatographic analyses were performed on a Thermo Electron (formerly Thermo Finnigan) chromatograph (San Jose, CA., USA). The chromatographic system was composed of an SCM1000 solvent degasser, P4000 quaternary gradient pump, AS3000 autosampler with a 100-μl sample loop, UV6000 LP photodiode array detector (UV-PDA) with Light Pipe Technology, FL3000 fluorescence detector, SN4000 system controller and a data station (Intel-Pentium 4CPU 1.6 GHz, RAM 256 MB, HDD 40GB) with the ChromQuest 4 analytical software (Thermo Electron, Inc., San Jose, CA, USA) working under the Windows 2000 operating system (Microsoft Corporation).

A 250-4.6 mm chromatographic column packed with a Chiralcel OD-R (Daicel, Japan) and mobile phase consisting of methanol-1 M aqueous $NaClO_4$, pH 6.85 (75:25, v/v) were employed for chiral chromatographic separations. The flow rate was 0.5 ml/min. Under these conditions, the mixture of hydrolysed flubendazole, both enantiomers of reduced flubendazole, albendazole and flubendazole was separated within 25 min.

UV detection was performed using a photodiode-array detector in the scan mode (in the range 195–385 nm with a 1 nm distance); single wavelength chromatograms at absorption maxima of 290 nm (for reduced flubendazole), 295 nm (for albendazole), 310 nm (for flubendazole) and 330 nm (for hydrolyzed flubendazole) were used for quantitative analysis. In addition, a more selective and sensitive fluorescence detection at λ(exc.)/λ(emis.) = 228 nm/310 nm (for reduced flubendazole) and λ(exc.)/λ(emis.) = 236 nm/346 nm (for albendazole as internal standard) was employed for the quantitative analysis of both enantiomers of reduced flubendazole.

2.5. Calibration

Nine-level calibration series of hydrolyzed flubendazole + reduced flubendazole (racemic mixture) + flubendazole/albendazole (I.S.) mixtures were prepared from 10^{-3} M solutions of each analyte (see Section 2.1) and from the mobile phase used in the HPLC analyses. The concentrations of flubendazole and its *phase I* metabolites at individual calibration levels were 5, 10, 50, 100, 500, 1000, 2000, 3000 and 5000 pmol/ml. Albendazole (internal standard) was present in each calibration sample in the concentration 3000 pmol/ml. Six individual samples were prepared at each calibration level. The same concentrations were used to prepare a plasma calibration sequence; drug-free ovine plasma samples were spiked with flubendazole, its two metabolites and albendazole (using appropriate concentrations in order to keep the volumes at minimum). The calibration series were measured using a tandem UV photodiode-array and fluorescence detector (see Section 2.4). The sample preparation procedure used for the plasma calibration samples was analogous as described in Section 2.3.

2.6. Validation of the analytical procedure

Statistical evaluation of the calibration analyses (see Section 2.5.) by the least-squares method was performed by the ChromQuest 4.0 software. The linearity of the calibration curves was tested and evaluated. Regression coefficients were calculated [$y = kx + q$, where x was the concentration ratio of flubendazole or its metabolite (hydrolyzed flubendazole or enantiomers of reduced flubendazole) to albendazole (I.S.) and y was the corresponding peak-area ratio of flubendazole or its metabolite to albendazole (I.S.). Coefficient of the determination (r^2) was expressed. The accuracy was determined as a relative bias [accuracy (%) = $100(C_{found} - C_{added})/C_{added}$] from the corresponding calibration curve equation. The precision was calculated as the relative standard deviation [R.S.D. (%) = 100 S.D./mean] from six identically prepared plasma calibration samples measured over 1 day and evaluated at various concentration levels. The range of the applicability of HPLC method was within the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The lower limit of quantification (LLOQ) was determined as the lowest concentration in the standard calibration curve which was measured with a precision of 20% and accuracy of ±20% [5]. Upper limit of quantification (ULOQ) was equal to the highest used concentration in plasma calibration. Limit of detection (LOD) and recovery for flubendazole and its individual metabolites were also calculated [5].

3. Results and discussion

The combination of achiral and chiral HPLC method described in our previous paper [1] afforded interesting qualitative and quantitative data from the *in vitro* flubendazole biotransformation experiments. However, for the HPLC determination in more complicated biomatrices (blood plasma), the methods were not enough sensitive and the chiral method could be used only in an external standard mode due to flubendazole–albendazole coelution. Hence, significant modifications of the former chiral HPLC method were needed. Better conditions for sufficient chromatographic separation of all individual analytes shown in Fig. 1 were explored, and the possibility of sensitivity and selectivity enhancement using a tandem UV-photodiode array (see Fig. 2) and fluorescence detection (see Fig. 3) was tested.

3.1. Chromatography

Chiralcel OD-R column used in our analyses contained a reversed-phase chiral selector, cellulose tris(3,5-dimethylphenyl carbamate) coated on 10 μm silica gel spheric particles. Various mixtures of acetonitrile and 0.5–1 M aqueous sodium perchlorate as the mobile phase were tested in our previous work for this type of chiral column.

The first issue to be addressed was the coelution of albendazole (internal standard) and flubendazole under the chiral chromatographic conditions mentioned in our previous paper [1]. The corresponding spectrochromatogram in Fig. 4A demonstrates a satisfactory separation of hydrolysed flubendazole (t_R = 10 min) and both reduced flubendazole enantiomers (t_R = 14 min for (−)-enantiomer and t_R = 17.2 min for (+)-enantiomer). On the other hand, the internal standard albendazole and the parent flubendazole were eluted in one concentration zone (t_R = 26 min). When bipolar aprotic acetonitrile in the mobile phase from the above-mentioned chiral method (see Fig. 4A) was replaced by the polar protic methanol, the coelution of flubendazole and albendazole was obviated, and all benzimidazole analytes under study were eluted in well-separated concentration zones (see Fig. 4B). The chromatographic conditions mentioned in Section 2.4 enabled the determination of flubendazole and its metabolites using an internal standard method (albendazole = I.S.) in a chiral chromatographic run lasting 27 min.

3.2. Fluorescence and multiwavelength UV detection

UV photodiode-array detector was found to be the most versatile tool in benzimidazole HPLC analyses with a view to the fact that individual analytes had very characteristic UV spectra (see Fig. 2). The identification of flubendazole, its metabolites and albendazole (IS) could be based not only on the retention

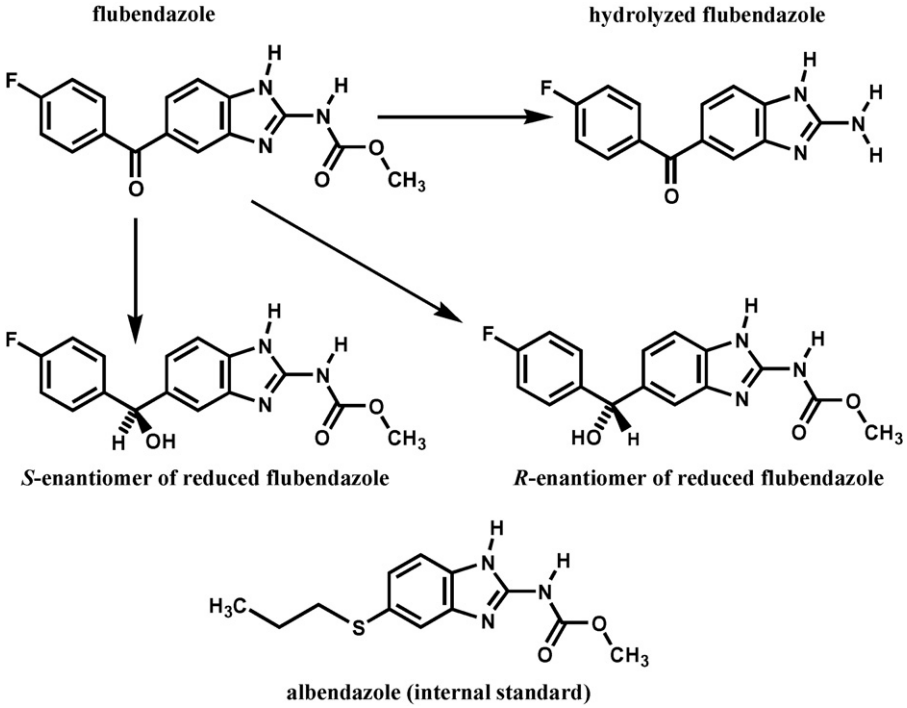

Fig. 1. Chemical structures of the benzimidazole carbamates under study.

times, but also on the UV spectra obtained from the spectrochromatograms (see Fig. 4). In order to suppress the possible spectral interferences of UV-absorbing eobiotics from the biomatrix with the benzimidazole analytes, more selective local UV-maxima near the visible part of the spectra were chosen for the quantification of the benzimidazole carbamates. In accordance with the UV-spectra demonstrated in Fig. 2, the wavelengths of 290 nm for reduced flubendazole, 295 nm for albendazole, 310 nm for flubendazole and 330 nm for hydrolyzed flubendazole were used during the UV photodiode-array determination of individual analytes. The shape of the UV-spectra of individual benzimidazole carbamates is pH-dependent, because the protonizable imidazole and carbamate nitrogens are located in the chromophore moiety of the molecule. Thus, the UV spectra of the benzimidazoles acquired during the achiral HPLC analysis in an acidic mobile phase (see Fig. 3 in paper [1]) are partially different from the UV spectra of the same benzimidazoles shown in Fig. 2 of this paper (neutral mobile phase was used in this case).

All benzimidazole carbamates (see Fig. 1) were also tested for possible fluorescence properties. 5-(4-Fluorobenzoyl)-1*H*-benzimidazole derivatives (i.e. flubendazole and hydrolyzed flubendazole) have no fluorescence spectra. On the other hand, both enantiomers of reduced flubendazole and albendazole were found to have analytically utilizable fluorescence spectra (see Fig. 3). Optimal excitation and emission wavelengths λ(exc.)/λ(emis.) = 228 nm/310 nm for reduced flubendazole and λ(exc.)/λ(emis.) = 236 nm/346 nm for albendazole were set on the fluorescence detector, which was connected in tandem behind the UV photodiode-array detector. The advantages of the fluorescence detection in comparison with the UV photodiode-array detection are evident from Fig. 5. The analysis of the standard mixture (hydrolyzed flubendazole, racemic reduced flubendazole, albendazole and flubendazole) using ultraviolet (280 nm, bottom grey chromatogram) and fluorescence (upper black chromatogram) detector is shown in Fig. 5A. Although the fluorescence detection did not enable the determination of flubendazole and hydrolyzed flubendazole, the fluorescence peak areas of reduced flubendazole and albendazole were approximately tenfold larger than the corresponding UV peak areas of these compounds at the wavelength of 280 nm (compare black *vs.* grey chromatogram in Fig. 5A). In addition, the fluorescence detection was much more selective in comparison with the UV detection. A suitable choice of the excitation and emission wavelength values was an important optical filter for the selective detection and determination of the mentioned

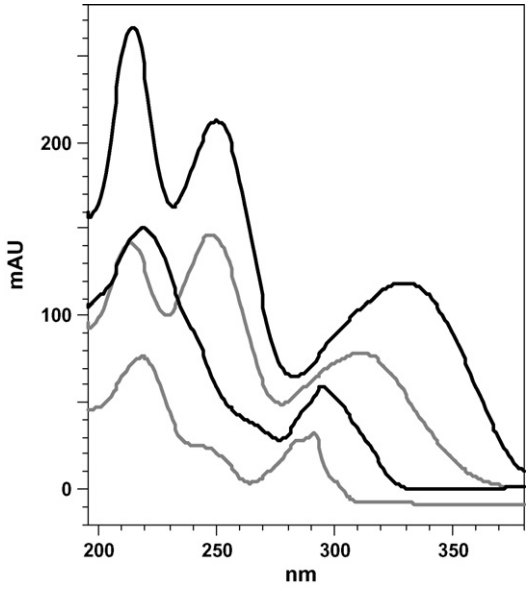

Fig. 2. Ultraviolet spectra of the hydrolyzed flubendazole, reduced flubendazole, albendazole and flubendazole in the mobile phase methanol-1 M $NaClO_4$ (75:25, v/v; pH 6.8). UV-absorption maxima: 214, 250, 330 nm for hydrolyzed flubendazole (upper black line); 218, 290 nm for reduced flubendazole (lower grey line); 219, 295 nm for albendazole (lower black line); 215, 250, 310 nm for flubendazole (upper grey line).

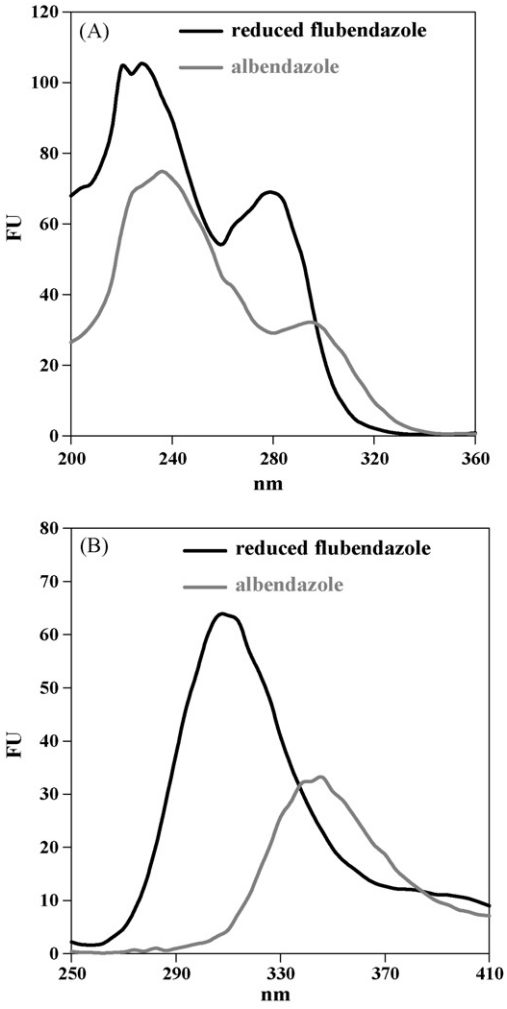

Fig. 3. Excitation (A) and emission spectra (B) of reduced flubendazole and albendazole. Maxima in fluorescence spectra: λ(excitation)/λ(emission) = 220, 228, 280 nm/310 nm for reduced flubendazole; λ(excitation)/λ(emission) = 236, 296 nm/346 nm for albendazole.

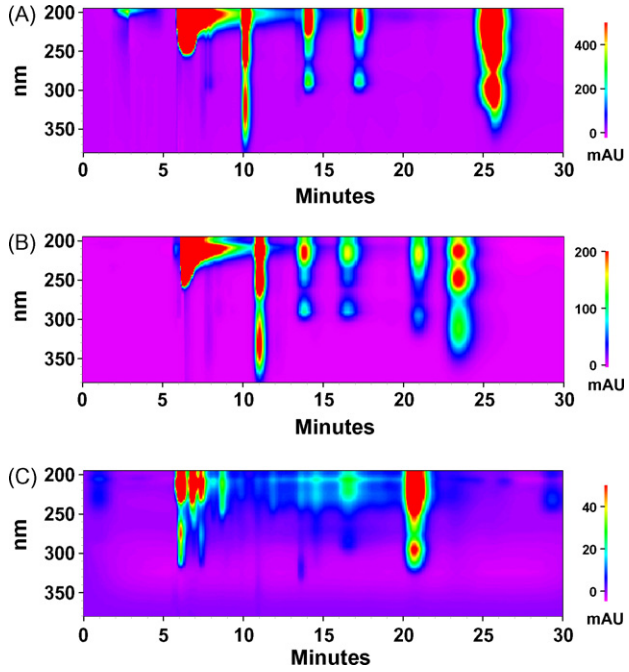

Fig. 4. (A) Spectrochromatogram of the 10^{-5} M mixture of hydrolyzed FLU (t_R = 10 min), (−)-reduced FLU (t_R = 14 min), (+)-reduced FLU (t_R = 17.2 min) and unresolved concentration zone of albendazole + flubendazole (ALB + FLU, t_R = 26 min) using the mobile phase acetonitrile-1 M aqueous $NaClO_4$, pH 6.8 (40:60, v/v). (B) Spectrochromatogram of the 10^{-5} M mixture of hydrolyzed FLU (t_R = 11 min), (−)-reduced FLU (t_R = 14 min), (+)-reduced FLU (t_R = 16.5 min), albendazole (t_R = 21 min, internal standard) and flubendazole (FLU; t_R = 23.5 min) using the mobile phase methanol-1 M aqueous $NaClO_4$, pH 6.8 (75:25, v/v). (C) Spectrochromatogram of the extract from the blood plasma of a ram treated with flubendazole (single oral dose 30 mg/kg). The blood sample was withdrawn 10 h after the administration (the same chromatographic conditions as in (B).

benzimidazole carbamates (reduced flubendazole and albendazole).

As shown in Fig. 4C (compare it with Fig. 4B), the photodiode-array UV detection of the extract from the blood plasma of a ram treated with flubendazole identified the presence of endogenous compounds originating from the blood plasma at t_R = 13–15 min. These compounds were coeluted or eluted very close to both enantiomers of the reduced flubendazole, and could be incorrectly interpreted and evaluated as the (−)- or (+)-reduced flubendazole in a single wavelength chromatogram. In the more selective and sensitive fluorescence detection (see Fig. 5B), the possibility of an incorrect identification and determination of both enantiomers of the reduced flubendazole was suppressed.

3.3. Validation results

The developed chiral LLE-HPLC-PDA-FL method was validated according to the recommendation of CDER and CVM Guidance (see Section 2.6 and reference [5]). The validation experiments were based on a 9-level plasma calibration series measured using both UV photodiode-array and fluorescence detection. The validation results are summarized in Tables 1A and 1B.

The intra-day variability (mean ± S.D., precision and accuracy) for hydrolyzed flubendazole, both enantiomers of reduced flubendazole and flubendazole calculated using an internal standard method (albendazole was used as I.S.) is presented in Table 1.

When the calibration data from the UV photodiode-array detector were processed, acceptable precision and accuracy results were found in the range of 10–2000 pmol ml^{-1} for the hydrolyzed flubendazole (λ = 330 nm) and flubendazole (λ = 310 nm), as shown in Table 1A. Plasma calibration results measured by the UV photodiode-array detector were well described by linear regression equations (see Table 2, UV detection).

On the other hand, the quantification of the reduced flubendazole enantiomers at lower concentration levels (under 25 pmol ml^{-1}) was subjected to a systematic error, which was probably caused by the presence of the interfering compounds coextracted from the plasma (see t_R = 13–15 min in Fig. 4C).

The calibration data from the fluorescence detector provided valid values of precision (<15%) and accuracy (<±15%) within the range 5–1000 pmol ml^{-1} for both enantiomers of the reduced flubendazole (λ(exc.)/λ(emis.) = 228 nm/310 nm). The fluorescence detection of the reduced flubendazole is more selective and sensitive then the UV photodiode-array detection. The fluorescence detection of the reduced flubendazole has non-linear response and the best fit of the calibration data was achieved using quadratic regression equations with the coefficients of determination 0.998 (see FL-detection in Table 2).

The tandem UV photodiode-array and fluorescence detection was found to be a suitable detection tool for flubendazole pharmacokinetic studies.

Table 1A
Intra-day precision and accuracy of hydrolyzed flubendazole, (−)-reduced flubendazole, (+)-reduced flubendazole and flubendazole determination in plasma calibration samples (six various concentration levels with six individually prepared samples at each calibration level; UV detection; linear fit).

UV detection linear fit ($y = Bx + C$)	Added (pmol ml^{-1})	Found (mean ± S.D.) (pmol ml^{-1})	Precision (R.S.D.) (%)	Accuracy (%)
Hydrolyzed flubendazole	10	12.04 ± 1.70	14.10	20.36
	50	53.65 ± 6.01	11.21	7.29
	100	102.13 ± 7.97	7.80	2.13
	500	547.25 ± 20.42	3.73	9.45
	1000	965.54 ± 24.39	2.53	−3.45
	2000	1957.51 ± 126.39	6.46	−2.12
(−)-Reduced flubendazole	5	9.37 ± 4.26	45.45	87.35
	25	20.27 ± 5.14	25.37	−18.93
	50	42.54 ± 4.72	11.10	−14.93
	250	251.57 ± 11.28	4.48	0.63
	500	498.11 ± 19.06	3.83	−0.38
	1000	1001.09 ± 54.17	5.41	0.11
(+)-Reduced flubendazole	5	6.48 ± 4.82	74.49	29.55
	25	24.03 ± 12.11	50.42	−3.89
	50	44.61 ± 3.78	8.46	−10.79
	250	260.75 ± 8.80	3.38	4.30
	500	489.49 ± 17.12	3.50	−2.10
	1000	1002.77 ± 58.24	5.81	0.28
Flubendazole	10	11.69 ± 1.82	15.55	16.93
	50	50.77 ± 7.59	14.95	1.55
	100	92.93 ± 7.08	7.62	−7.07
	500	525.27 ± 24.47	4.66	5.05
	1000	974.33 ± 33.22	3.41	−2.57
	2000	2006.80 ± 61.36	3.06	0.34

Table 1B
Intra-day precision and accuracy of (−)-reduced flubendazole and (+)-reduced flubendazole determination in plasma calibration samples (six various concentration levels with six individually prepared samples at each calibration level; fluorescence detection; quadratic fit).

Fluorescence detection quadratic fit ($y = Ax^2 + Bx + C$)	Added (pmol ml^{-1})	Found (mean ± S.D.) (pmol ml^{-1})	Precision (R.S.D.) (%)	Accuracy (%)
(−)-Reduced flubendazole	5	4.82 ± 0.34	7.06	−3.52
	25	25.18 ± 1.31	5.20	0.74
	50	48.59 ± 3.35	6.89	−2.81
	250	253.26 ± 10.38	4.10	1.30
	500	497.66 ± 21.72	4.36	−0.47
	1000	1000.77 ± 45.54	4.55	0.08
(+)-Reduced flubendazole	5	4.26 ± 0.27	6.23	−14.77
	25	24.59 ± 1.71	6.96	−1.63
	50	48.73 ± 3.53	7.25	−2.54
	250	256.52 ± 11.86	4.63	2.61
	500	495.23 ± 24.75	5.00	−0.95
	1000	1001.42 ± 54.29	5.42	0.14

3.4. Pharmacokinetic studies of flubendazole in sheep

In our previous study [1], the *in vitro* biotransformation studies in the subcellular fractions of hepatic and intestinal homogenates provided an important information about the location and coenzyme requirement of flubendazole-metabolizing enzymes. NADPH-dependent carbonyl reducing enzymes located in the liver cytosolic fractions were found to be mainly involved in flubendazole biotransformation. The flubendazole carbonyl reduction was stereospecific: (+)-reduced flubendazole was preferentially formed (with 93–97% enantiomeric excess of this enantiomer). The hydrolyzed flubendazole, second *phase I* metabolite of flubendazole, was formed in small amounts only in the hepatic cytosolic fractions [1].

In this study, flubendazole biotransformation was tested *in vivo* using chiral HPLC with a tandem UV photodiode-array and fluores-

Table 2
Internal standard calibration curve parameters for hydrolyzed flubendazole, (−)-reduced flubendazole, (+)-reduced flubendazole and flubendazole using UV detection and fluorescence detection. Based on 6-level ovine plasma calibration with six individually spiked drug-free plasma samples at each calibration level (see Tables 1A and 1B).

	Regression equation	r^2	Range (pmol ml^{-1})	Recovery (%)
UV-detection				
Hydrolyzed flubendazole	$y = 0.883x - 0.001$	0.998	10.5–2000	83.4
(−)-Reduced flubendazole	$y = 2.4674x + 0.0237$	0.9963	23–1000	87.5
(+)-Reduced flubendazole	$y = 2.4272x + 0.0327$	0.9957	43.4–1000	86.7
Flubendazole	$y = 1.2387x + 0.0033$	0.9982	9.95–2000	82.9
FL-detection				
(−)-Reduced flubendazole	$y = -2.0932x^2 + 4.7086x + 0.0028$	0.998	3.85–1000	87.5
(+)-Reduced flubendazole	$y = -1.8844x^2 + 4.6308x + 0.0075$	0.9979	3.42–1000	86.7

Table 3
Pharmacokinetic parameters for flubendazole and its two *phase I* metabolites (to the pharmacokinetics in Fig. 6).

Compound →	Hydrolyzed flubendazole	(+)-Reduced flubendazole	Flubendazole
$C_{(max)}$ (pmol ml^{-1})	56	327	40
$t_{(max)}$ (h)	10	10	10
$AUC_{(0-72\,h)}$ (pmol h ml^{-1})	1267	6287	830
k_e (h^{-1})	0.0302	0.0407	0.0127
$t_{(1/2)}$ (h)	23	17	55

cence detection. The pilot pharmacokinetic study of flubendazole was performed in a ram (see Section 2.2.) after the enteral administration of 30 mg dose of flubendazole (in the form of a suspense) per 1 kg of body weight. As flubendazole is poorly absorbed from the gastro-intestinal tract, low concentrations of this parent compound and its *phase I* metabolites in the blood stream were expected [1].

Ovine plasma samples, collected during the experiment, were processed and the concentrations of flubendazole and its principal metabolites were determined. The time course of the plasma concentrations of flubendazole and its individual *phase I* metabolites is displayed in Fig. 6 and the corresponding pharmacokinetic parameters were summarized in Table 3. The results showed that the parent flubendazole was initially absorbed from the forestomach and consecutively absorbed from the rest of the digestive tract. In ruminants, physiological long-term passage of the digestive content through the stomach and small intestine allows the absorption of a drug over a long period of time after administration. The absorbed flubendazole was consequently transported *via* blood to the liver where the main biotransformation occurred.

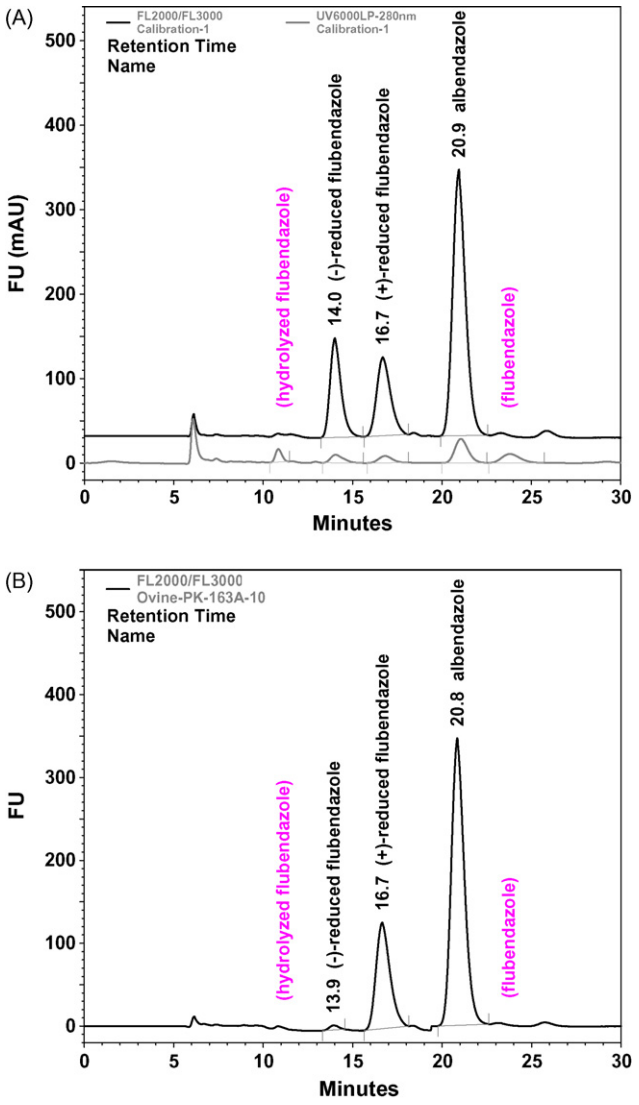

Fig. 5. (A) Chromatograms of the extract from a drug-free plasma spiked with flubendazole and its *phase I* metabolites (1 nmol/ml of each) and albendazole (3 nmol/ml, IS). Upper black chromatogram was acquired using fluorescence detection, the lower grey chromatogram using UV detection (at 280 nm). (B) Chromatogram (fluorescence detection) of the ovine plasma extract after 10 h following the administration of flubendazole (30 mg/kg).

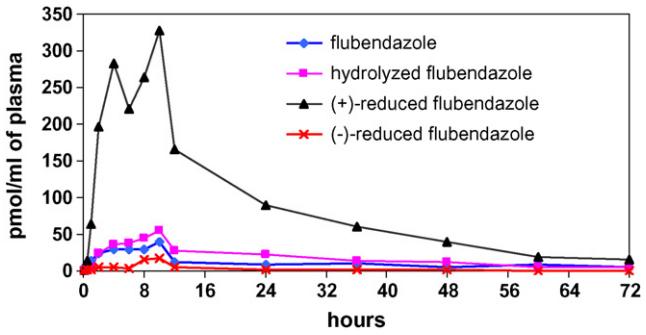

Fig. 6. Pharmacokinetics of flubendazole and its *phase I* metabolites in a young ram after a single oral administration of flubendazole (30 mg/kg, see Section 2.2).

As shown in Fig. 6, the (+)-enantiomer of the reduced flubendazole was found to be the principal *phase I* metabolite circulating in the ovine blood after the enteral administration of flubendazole. This enantiomer represents almost 75% of the total molar amount of benzimidazole carbamates (i.e. flubendazole + its *phase I* metabolites) found in the ovine blood plasma in the time interval 0–72 h after flubendazole administration. The enantiomeric excess (calculated as $AUC_{(0-72\,h)}$ for (−)-reduced flubendazole/$AUC_{(0-72\,h)}$ for (+)-reduced flubendazole = 2.5%/97.5%) shows a high stereospecificity of ovine carbonyl reductases. The pharmacokinetic curve for the (+)-reduced flubendazole is in accord with a very fast and active flubendazole reduction. On the other hand, very low concentrations of the parent drug (flubendazole) and the hydrolyzed flubendazole were found, and only traces of the (−)-reduced flubendazole were detected in the ovine blood plasma. These findings indicate high activities of the carbonyl reducing enzymes and minor importance of hydrolases in flubendazole biotransformation in sheep. The principal *phase I* metabolite, the (+)-reduced flubendazole probably undergoes conjugation, but no information about the structure of flubendazole conjugates in the sheep is known.

The question of flubendazole application in ruminants (especially in sheep) was recently dealt with by Moreno et al. [6]. However, in their study, the pharmacokinetics of flubendazole and its metabolites in sheep after an intravenous and intraruminal administrations of flubendazole (the same dosage 5 mg/kg) were evaluated using an achiral chromatographic method, and the stereospecificity in the formation of (+)-reduced flubendazole could not be evaluated.

4. Conclusions

Disposition studies of the anthelmintic flubendazole in animals required the development and validation of a selective and sensitive chiral HPLC method. The previously described analytical approach used for *in vitro* studies [1] was not suitable for a chiral HPLC determination of low plasmatic concentrations of flubendazole and its metabolites. In the newly developed chiral HPLC method, all analytes under study (i.e. hydrolyzed flubendazole, (−)-reduced flubendazole, (+)-reduced flubendazole, albendazole and flubendazole) were separated and determined using an internal standard method (with albendazole as an IS) in a chromatographic run lasting 27 min. All benzimidazole carbamates reported in this work were determinable by the UV photodiode array detector at individually selected wavelengths for each compound. In addition, the principal flubendazole metabolite, reduced flubendazole, and albendazole (internal standard) exhibited excitation and emission maxima and possessed fluorescence properties of analytical importance. The fluorescence response of the reduced flubendazole ($\lambda_{exc.}/\lambda_{emis.}$ = 228/310 nm) and albendazole ($\lambda_{exc.}/\lambda_{emis.}$ = 236/346 nm) was found to be ten times more intense than the ultraviolet response of these compounds at 280 nm, and also the selectivity of the fluorescence detection was better than that of the universal UV detection.

The applicability of the described chiral HPLC method armed by a tandem UV photodiode-array and fluorescence detection was tested on a pilot pharmacokinetics of flubendazole in sheep after the enteral administration of a dose of 30 mg/kg. The main pharmacokinetic parameters (c_{max}, t_{max}, $AUC_{0-72\,h}$, k_e and $t_{(1/2)}$) were measured and evaluated. The (+)-reduced flubendazole was found to be the prevailing flubendazole metabolite circulating in the ovine blood (73.6% of the total amount of flubendazole and its *phase I* metabolites determined in the plasma samples), the residual amounts are distributed among the hydrolyzed flubendazole (14.8%), parent flubendazole (9.7%) and (−)-reduced flubendazole (1.8%). The value of the enantiomeric excess (97.5% for the (+)-enantiomer of reduced flubendazole) shows a high stereospecificity of the ovine carbonyl reductases.

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