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Achiral and chiral high-performance liquid chromatographic determination of flubendazole and its metabolites in biomatrices using UV photodiode-array and mass spectrometric detection

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Abstract

Flubendazole, methyl ester of [5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl]carbamic acid, belongs to the group of benzimidazole anthelmintics, which are widely used in veterinary and human medicine. The *phase I* flubendazole biotransformation includes a hydrolysis of the carbamoyl methyl moiety accompanied by a decarboxylation (hydrolysed flubendazole) and a carbonyl reduction of flubendazole (reduced flubendazole). Flubendazole is a prochiral drug, hence a racemic mixture is formed during non-stereoselective reductions at the carbonyl group. Two bioanalytical HPLC methods were developed and validated for the determination of flubendazole and its metabolites in pig and pheasant hepatic microsomal and cytosolic fractions. Analytes were extracted from biomatrices into *tert*-butylmethyl ether. The first, achiral method employed a 250 mm × 4 mm column with octylsilyl silica gel (5 μm) and an isocratic mobile phase acetonitrile–0.025 M KH_2PO_4 buffer pH 3 (28:72, v/v). Albendazole was used as an internal standard. The whole analysis lasted 27 min at a flow rate of 1 ml/min. The second, chiral HPLC method, was performed on a Chiralcel OD-R 250 mm × 4.6 mm column with a mobile phase acetonitrile–1 M $NaClO_4$ (4:6, v/v). This method enabled the separation of both reduced flubendazole enantiomers. The enantiomer excess was evaluated. The column effluent was monitored using a photodiode-array detector (scan or single wavelength at λ = 246 nm). Each of the analytes under study had characteristic UV spectrum, in addition, their chemical structures were confirmed by high-performance liquid chromatography–mass spectrometry (HPLC–MS) experiments. Stereospecificity in the enzymatic carbonyl reduction of flubendazole was observed. While synthetic racemic mixture of reduced flubendazole was separated to equimolar amounts of both enantiomers, practically only one enantiomer was detected in the extracts from all incubates.

Keywords: Benzimidazole anthelmintics; *Phase I* flubendazole biotransformation; Pig; Pheasant; Achiral and chiral HPLC-DAD–MS

1. Introduction

Benzimidazole carbamates have been widely used since the 1960s as anthelmintic agents in veterinary and human medicine. Flubendazole, methyl ester of [5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl]carbamic acid (CAS 31430-15-6), has similar pharmacological properties as mebendazole [methyl ester of (5-benzoyl-1*H*-benzimidazol-2-yl)carbamic acid (CAS 31431-39-7)]. Both these compounds were synthesized in 1971 (van Gelder et al. in Janssen Pharmaceutica laboratories). Flubendazole and mebendazole are employed in similar therapeutic indications, for the treatment of various human and animal helminthoses (enterobiasis, ascariasis, hook-worm infections), the activity against some larval stages and ova has also been described [1].

The mode of pharmacodynamic action of these benzimidazole derivatives consists in the inhibition or destruction of cytoplasmic microtubules in the parasitic worm's intestinal or

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absorptive cells. Inhibition of glucose uptake and depletion of glycogen stores follow as do other inhibitory effects leading to death of the worm within several days [1,2].

For the understanding of flubendazole pharmacokinetics and disposition in organism, the evaluation of some physico-chemical properties of this compound was important. Flubendazole is almost insoluble in water (<10 mg/l), solubility in many organic solvents (ethanol, diethyl ether, dichloromethane, hexane, toluene) is also low. Flubendazole is better soluble in formic acid (340.5 g/l), in dimethyl sulfoxide (15 g/l) and dimethyl formamide (5.6 g/l). The octanol–water partition coefficient, expressed as log P, was found to be 3.0. Flubendazole has ionisation constants pK_{a1} = 3.6 (imidazole nitrogen) and pK_{a2} = 9.6 (carbamate nitrogen) [3]. Because of very low solubility in aqueous systems, flubendazole cannot be administered parenterally in practice, it is used in the form of oral formulations only.

Being administered orally, flubendazole is poorly absorbed from the gastro-intestinal tract. In liver as well as in gastrointestinal tract, it undergoes the biotransformation – hydrolysis to decarbamoylated (hydrolyzed) flubendazole and carbonyl reduction leading to reduced flubendazole [4–6]. Decarbamoylated metabolic product from reduced flubendazole has also been identified during *in vitro* metabolic studies with rat liver microsomes [5]. The metabolites together with unchanged parent compound are eliminated in the bile and excreted in the faeces [7,8]. Approximately, 2% of a dose is excreted unchanged or as metabolites in the urine. Flubendazole is highly protein bound.

Various analytical approaches to the sample preparation of biomatrices and to HPLC determination of flubendazole and its metabolites in biological samples have been published [4,5,9–17].

A radioimmunoassay of flubendazole in biological fluids has been described [7]. However, this method is not selective and sensitive, it cannot discriminate among benzimidazoles of similar structure for cross-reaction. Thus, it is not suitable for the simultaneous determination of low concetrations of flubendazole and its metabolites. This is why, the methods involving a separation of individual compounds are preferred.

Various bioanalytical HPLC methods have been developed and validated for flubendazole and its metabolites in whole blood [4], blood plasma [5,16], eggs [10,13], muscle tissue [11–13], foodstuffs of animal origin for human consumption [14], sheep liver microsomes [16], bovine liver [17] and parasite material [15,16].

After the homogenization of biomatrices, a liquid phase extraction procedure was performed using diethyl ether [4,5], acetonitrile [11,14], methanol [11,15] or ethyl acetate [12,13,17]. In some cases, the extracts were defatted with hexane [14,17] and finally cleaned up by solid phase extraction (SPE) [11,12,14,15,17] prior to HPLC analysis. Mebendazole [4,5], 2-*n*-butylmercaptobenzimidazole [12], benzthiazuron [14] or albendazole sulfone [15] were chosen as internal standards for HPLC determination.

Various detection principles were used during high-performance liquid chromatography. Fluorescence [11], ultraviolet [4,5,10,11,15,17], UV photodiode-array [14] or LC–MS/MS [12,13] detectors have been employed in the HPLC analysis of flubendazole and its metabolites.

This communication describes two bioanalytical HPLC methods involving a liquid–liquid extraction (LLE) of flubendazole and its metabolites from animal hepatic and intestinal microsomal and cytosolic fractions into *tert*-butylmethyl ether. Achiral HPLC method enabled the analysis of hydrolysed (decarbamoylated) flubendazole, reduced flubendazole and flubendazole in the extracts within 27 min with the employment of albendazole as an internal standard. Due to the fact that reduced flubendazole is an optical active compound and it could be present in the extracts in various ratios of individual enantiomers, the chiral HPLC method was developed. This chiral method allowed the resolution of decarbamoylated flubendazole, (−)-enantiomer of reduced flubendazole, (+)-enantiomer of reduced flubendazole and parent flubendazole in the extracts from biomatrices in 30 min. Use of this chiral HPLC method was necessary with a view to expected enzymatic stereoselectivity in the carbonyl reduction of flubendazole.

2. Experimental

2.1. Chemicals, preparations, solutions, materials

Flubendazole, [5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-y1]carbamic acid methyl ester, $C_{16}H_{12}FN_3O_3$, molecular weight (MW) = 313.29 g/mol, CAS 31430-15-6; racemic mixture of reduced flubendazole {(±)-[5-(4-fluorophenyl)hydroxymethyl-1*H*-benzimidazole-2-y1]-carbamic acid methyl ester, $C_{16}H_{14}FN_3O_3$, MW = 315.3 g/mol} and hydrolysed flubendazol {[(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), ammonium acetate (Fractopur), *tert*-butylmethyl ether (p.a.; all from Merck, Darmstadt, Germany), ammonium hydroxide (26% water solution of NH_3), formic acid (p.a., 85%), phosphoric acid (85%), methanol, sodium hydrogenphosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), potassium dihydrogenphosphate (KH_2PO_4, all chemicals of analytical grade, Lachema, Brno, Czech Republic), *N*,*N*-dimethyl formamide (DMF, >99.9%, HPLC grade, Aldrich), ultrahigh 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 2.5).

β-Nicotinamide adenine dinucleotide phosphate, reduced tetrasodium salt (NADPH), β-nicotinamide adenine dinucleotide, reduced disodium salt (NADH), bicinchoninic acid, dimethyl sulfoxide (DMSO) and glycerol (all from Sigma–Aldrich s.r.o., Prague, Czech Republic) were used in xenobiochemical reactions (the incubations *in vitro* described in Section 2.2).

An ammonium acetate buffer (0.005 M, pH 3) for HPLC–MS analyses was prepared by the dissolution of ammonium acetate (0.385 g) in UHQ water (990 ml), adjusting to pH 3 with 85% formic acid (approximately 2 ml) and filling to a total volume of 1000 ml with UHQ water.

A phosphate buffer (0.025 M, pH 3) for HPLC-DAD analyses was made by the dissolution of potassium dihydrogenphosphate (3.4 g) in UHQ water (990 ml), adjusting to pH 3 with a solution of phosphoric acid (2 mol/l) and filling to a total volume of 1000 ml with UHQ water.

A phosphate buffer (0.1 M, pH 7.4, made from sodium hydrogenphosphate, sodium dihydrogenphosphate and phosphoric acid) was used for xenobiochemical experiments.

The stock solutions (10^{-3} M) of flubendazole (6.26 mg/20 ml), hydrolysed flubendazole (5.11 mg/20 ml) and reduced flubendazole (the 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 mobile phases used in achiral or chiral separation.

2.2. Preparation of liver and intestinal microsomal and cytosolic fractions and their incubation with flubendazole

Frozen (liquid nitrogen) pig or pheasant liver or mucosa from intestine was homogenised (1:3, w/v) in 0.1 M sodium phosphate buffer, pH 7.4. The microsomal and cytosolic fractions were obtained by fractional ultracentrifugation of the homogenate. Obtained cytosol (105,000 × *g* supernatant) was stored at −80 °C. A re-washing step (follow by second ultracentrifugation) was added at the end of microsomal preparation. Microsomes were finally re-suspensed in homogenization buffer containing 20% (v/v) glycerol and stored at −80 °C. Total protein content in subcellular fractions was determined by the bicinchoninic acid assay [18].

The hepatic (intestinal) microsomal or cytosolic fraction was incubated with flubendazole (10 μM—presolved in dimethyl sulfoxide). The reaction mixture (total volume of 0.3 ml) contained 100 μl of microsomal suspension or 100 μl of cytosole, NADPH or NADH (1 mM) and 0.1 M sodium phosphate buffer, pH 7.4. Concentration of dimethyl sulfoxide in reaction mixture was 0.1%. The blank samples contained 0.1 M sodium phosphate buffer, pH 7.4, instead of microsomes or cytosol. The incubations were carried out at 37 °C for 30 min under aerobic conditions. At the end of incubation, 30 μl of concentrated (28%) NH_3 was added and the mixture was shaken and cooled to 0 °C.

2.3. Sample preparation of the microsomal and cytosolic incubates before HPLC

30 μl 10^{-3} M albendazole (I.S.) in acetonitrile tranferred into a glass tube equipped with a ground-glass stopper, acetonitrile was evaporated to dryness. 0.3 ml of alkalized incubates (see Section 2.2) was added and the mixture was sonificated. A liquid–liquid extraction into 3 ml of *tert*-butylmethyl ether was performed. 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. Decanted ether was evaporated to dryness and the residuum was reconstituted in 600 μl of the mobile phase.

2.4. HPLC with polarimetric detector and HPLC/MS analyses

A Shimadzu high-performance liquid chromatograph consisting of a CBM-10A Communication Bus Module, DGU-3A Degasser, high-pressure pump LC-10AD, SIL-10A Auto-Injector with a sample cooling, CTO-10A column oven, an SPD-M10A photodiode-array detector and a polarimetric detector Chiralyser (IBZ Messtechnik, Hannover) in tandem was employed in the identification of (+)-enantiomer and (−)-enantiomer of reduced flubendazole in the chiral chromatograms. An analytical software Shimadzu Class-LC10 was used in the evaluation of chromatograms. The conditions of chiral chromatography are described in Section 2.5.2.

High-performance liquid chromatography–mass spectrometry (HPLC–MS) analyses were performed on an Esquire 3000 ion trap analyzer (Bruker Daltonics, Bremen, Germany) and a Waters liquid chromatograph consisting of a Model 616 pump, a Model 717+ autosampler, a thermostated column compartment and a Model 996 photodiode-array detector (all from Waters, Milford, MA, USA). Electrospray ionization (ESI) mass spectra were recorded in the mass range *m/z* 50–1200 using both positive-ion and negative-ion modes and the following setting of tuning parameters: target mass *m/z* = 300, compound stability = 100%, pressure of the nebulizing gas = 70 psi, the drying gas flow rate = 12 l/min and temperature of ion source = 365 °C. UV spectra of all chromatographic peaks were recorded in the range 200–400 nm using a diode-array UV detector with the highest resolution 1.2 nm. The standards and samples were dissolved in the mobile phase and 100 μl of the solution was injected into HPLC/MS system.

Mobile phase for HPLC/MS experiments consisted of acetonitrile–0.005 M ammonium acetate buffer (28:72, v/v). The chromatographic column, length of analysis and flow rate were identical as described in Section 2.5.

2.4.1. ESI mass spectra

2.4.1.1. Desmethylcarboxy flubendazole, MW = 255.

Positive-ion ESI-MS: *m/z* 278 $[M+Na]^+$, 256 $[M+H]^+$ (100%).
Positive-ion ESI-MS/MS of *m/z* 256: *m/z* 239 $[M+H\text{-}NH_3]^+$ (100%).
Negative-ion ESI-MS: *m/z* 254 $[M-H]^-$ (100%).

2.4.1.2. Reduced flubendazole, MW = 315.

Positive-ion ESI-MS: *m/z* 338 $[M+Na]^+$, 316 $[M+H]^+$ (100%), 284 $[M+H\text{-}CH_3OH]^+$.

Positive-ion ESI-MS/MS of *m/z* 316: *m/z* 284 [M + H-CH_3OH]$^+$ (100%).
Negative-ion ESI-MS: *m/z* 651 [2M-2H + Na]$^-$, 619 [2M-2H-CH_3OH + Na]$^-$, 314 [M-H]$^-$, 282 [M-H-CH_3OH]$^-$ (100%), 264 [M-H-CH_3OH-H_2O]$^-$.
Negative-ion ESI-MS/MS of *m/z* 314: 282 [M-H-CH_3OH]$^-$ (100%).
Negative-ion ESI-MS/MS of *m/z* 282: 264 [M-H-CH_3OH-H_2O]$^-$ (100%).

2.4.1.3. Albendazole (internal standard), MW = 265.

Positive-ion ESI-MS: *m/z* 288 [M + Na]$^+$, 266 [M + H]$^+$ (100%), 234 [M + H-CH_3OH]$^+$.
Positive-ion ESI-MS/MS of *m/z* 266: *m/z* 234 [M + H-CH_3OH]$^+$ (100%).
Negative-ion ESI-MS: *m/z* 551 [2M-2H + Na]$^-$, 519 [2M-2H-CH_3OH + Na]$^-$, 487 [2M-2H-2CH_3OH + Na]$^-$, 264 [M − H]$^-$, 232 [M-H-CH_3OH]$^-$ (100%), 189 [M-H-CH_3OH-NHCO]$^-$.
Negative-ion ESI-MS/MS of *m/z* 264: 232 [M-H-CH_3OH]$^-$ (100%), 189 [M-H-CH_3OH-NHCO]$^-$.
Negative-ion ESI-MS/MS of *m/z* 232: 189 [M-H-CH_3OH-NHCO]$^-$ (100%).

2.4.1.4. Flubendazole, MW = 313.

Positive-ion ESI-MS: *m/z* 336 [M + Na]$^+$, 314 [M + H]$^+$ (100%), 282 [M + H-CH_3OH]$^+$.
Positive-ion ESI-MS/MS of *m/z* 314: *m/z* 282 [M + H-CH_3OH]$^+$ (100%).
Negative-ion ESI-MS: *m/z* 647 [2M-2H + Na]$^-$, 615 [2M-2H-CH_3OH + Na]$^-$, 312 [M − H]$^-$, 280 [M-H-CH_3OH]$^-$ (100%).
Negative-ion ESI-MS/MS of *m/z* 312: 280 [M-H-CH_3OH]$^-$ (100%).

2.5. HPLC with ultraviolet photodiode-array detector

The development of achiral and chiral HPLC methods and routine chromatographic analyses were performed using 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, SN4000 system controller and a data station (Intel-Pentium 4CPU 1.6 GHz, RAM 256 MB, HDD 40 GB) with the ChromQuest 4 analytical software (Thermo Electron, Inc., San Jose, CA, USA) working under the Windows 2000 operating system (Microsoft Corporation).

The chromatographic conditions for achiral and chiral separations are described in Sections 2.5.1 and 2.5.2.

UV detection was performed in dual wavelength mode (246 and 300 nm). For the collection of the UV spectra, a photodiode-array scan mode (in the range 195–385 nm with a 1 nm distance) was employed.

2.5.1. Achiral chromatographic separation

A LiChroCART 250 mm × 4 mm chromatographic column packed with LiChrospher 60 RP-selectB, 5 μm and precolumn LiChroCART 4 mm × 4 mm with the same stationary phase were used for achiral chromatographic separations. The composition of isocratic mobile phase was acetonitrile–0.025 M KH_2PO_4 buffer pH 3 (28:72, v/v). Ammonium acetate buffer instead of phosphate buffer was used in the case of HPLC–MS experiments. Flow rate was 1 ml/min. The whole analysis lasted 27 min.

2.5.2. Chiral chromatographic separation

A Daicel 250 mm × 4.6 mm chromatographic column packed with Chiralcel OD-R (Daicel, Japan) and mobile phase consisting of acetonitrile–1 M aqueous $NaClO_4$, pH 6.85 (4:6, v/v) were employed for chiral chromatographic separations. Flow rate was 0.5 ml/min. In this case, the mixture of decarbamoylated flubendazole, both enantiomers of reduced flubendazole and flubendazole was separated within 30 min.

2.6. Calibration

9-Level calibration series of hydrolyzed flubendazole + reduced flubendazole + 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 HPLC analyses. The concentrations of flubendazole and its metabolites in individual calibration levels were 0.5, 1, 2, 5, 8, 10, 20, 50 and 80 nmol/ml. Albendazole (internal standard) was present in each calibration sample in the concentration 50 nmol/ml. Six individual samples were prepared at each calibration level. The same concentrations were used to make a calibration curve with a drug-free cytosolic fraction spiked with flubendazole, its two metabolites and albendazole (using appropriate concentrations in order to keep the volumes at minimum). The calibration series was measured using UV photodiode-array detector (see Section 2.5). The sample preparation procedure used for calibration samples was analogous as described in Section 2.3.

2.7. Validation of the analytical procedure

Statistical evaluation of the calibration analyses (see Section 2.6.) by the least-squares method was performed using the ChromQuest 4.0 Software. The linearity of the calibration curves was tested and evaluated for aqueous solutions of hydrolyzed flubendazole + reduced flubendazole + flubendazole/albendazole series and for the cytosolic calibration series based on the hydrolysed flubendazole + reduced flubendazole + flubendazole/albendazole samples, which were extracted into *tert*-butylmethyl ether. Regression coefficients were calculated [$y = kx + q$, where x was the concentration ratio of flubendazole or its metabolite (hydrolyzed or reduced flubendazole) to albendazole (I.S.) and y was the corresponding peak-area ratio of flubendazole or its metabolite to albendazole (I.S.)]. The coefficient of the determination (r^2) was also expressed. The accuracy was determined as a relative error bias [accuracy (%) = $100 \times ((C_{real} - C_{determined})/C_{real})$] calcu-

lated from the corresponding calibration curve equation. The precision was calculated as the relative standard deviation [RSD (%) = 100 × (SD/mean)] from six identically prepared plasma calibration samples measured during 1 day and evaluated at three various (low, medium, high) concentration levels. The range of the applicability of HPLC method was enclosed 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% [19,20]. Upper limit of quantification (ULOQ) was equal to the highest concentration in 9-level cytosolic calibration. Limit of detection (LOD) was calculated as the concentration at a signal-to-noise ratio of 3. The recovery for flubendazole and its two metabolites was calculated [19,20].

3. Results and discussion

In order to understand some important aspects of *phase I* flubendazole metabolism, the *in vitro* biotransformation studies of this compound with microsomal and cytosolic fractions of cells from various sources, various species and performed under various incubation conditions were designed. The bioanalytical support of these xenobiochemical experiments consisted in the identification and determination of flubendazole and its principal *phase I* metabolites (decarbamoylated flubendazole, reduced flubendazole) in the incubation mixtures. For these purposes, an achiral LLE–HPLC-DAD–MS method was developed and validated for routine analyses of flubendazole and its two metabolites in biomatrices. With respect to the fact that reduced flubendazole is a chiral compound, the chiral HPLC method was employed for the enantiomeric excess evaluation of reduced flubendazole enantiomers in the incubation mixtures.

3.1. Chromatography

3.1.1. Sample preparation procedure

Microsomal and cytosolic fractions are *in vitro* subcellular systems usually used for the biotransformation studies. These fractions contain enzymes involved in the *phase I* flubendazole biotransformation leading to the metabolites displayed in Fig. 1. After the incubation of flubendazole with microsomes or cytosole, the sample preparation procedure consisted in the termination of enzymatic catalysis with aqueous ammonia followed by a liquid–liquid extraction of the analytes into suitable solvent immiscible with water.

Three extraction media (*tert*-butylmethyl ether, diethyl ether, ethyl acetate) for the liquid–liquid extraction of flubendazole and its metabolites were tested in our prevalidation experiments. The best results according to the recovery values were achieved with *tert*-butylmethyl ether (see Section 2.3). Separation of the aqueous and etheric phases by freezing of the aqueous phase was elegant and effective, because the upper organic layer was easily removable by decantation.

3.1.2. Routine achiral HPLC with UV photodiode-array

Three chromatographic columns containing various reversed phases were tested in the search of optimal separation conditions for the standards of hydrolysed (decarbamoylated) flubendazole, reduced flubendazole and flubendazole. In addition, a suitable internal standard had to be chosen. The analytes were separated on LiChrospher 100 RP-18, Purospher RP-18e and LiChrospher 60 RP-selectB columns (all from Merck, Germany) using isocratic mobile phase based on acetonitrile–phosphate buffer mixtures. Octadecylsilyl silica gel columns separated the benzimidazole analytes in asymmetric concentration zones (LiChrospher 100 RP-18) or the resolution of albendazole and flubendazole was not satisfactory (Purospher RP-18e). The best results (narrow symmetric concentration zones) were achieved

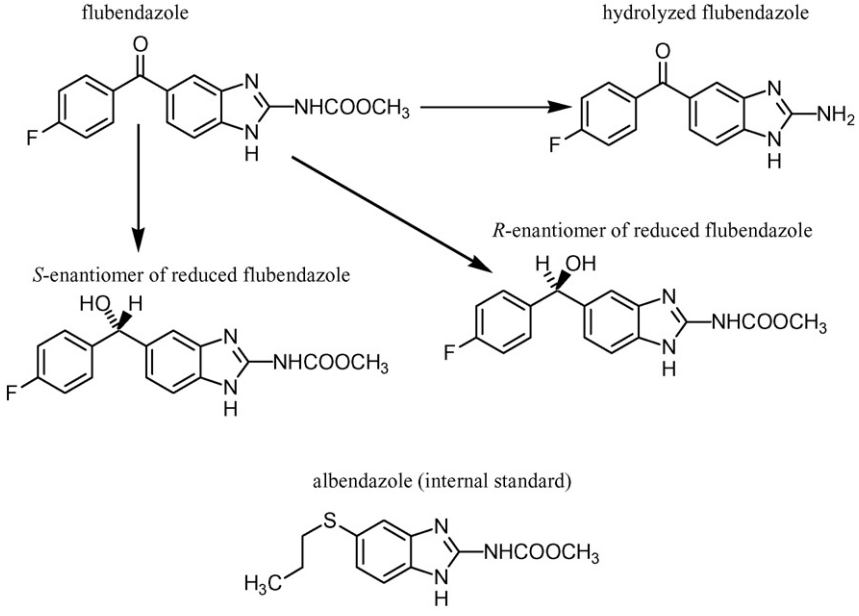

Fig. 1. Chemical structures of benzimidazole derivatives under study.

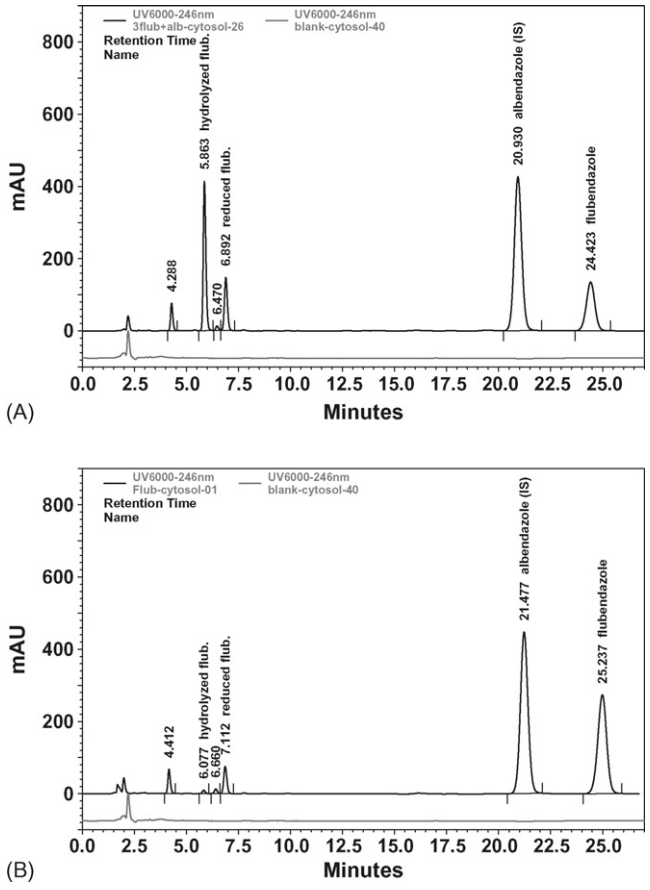

Fig. 2. Achiral chromatograms of the extracts of benzimidazole anthelmintics from cytosolic fractions: an extract of the cytosolic calibration sample spiked by hydrolysed flubendazole, reduced flubendazole and flubendazole (1 nmol of each) and 5 nmol of albendazole as internal standard (A); a real extract of cytosolic sample from pheasant liver (B). The gray bottom chromatogram in each of the picture is an extract from the blank cytosolic sample. See Section 2.5.1 for achiral chromatographic condition.

on octylsilyl silica gel LiChrospher 60 RP-selectB column for all analytes under study including albendazole, which was chosen as an internal standard (see Fig. 2). Chromatographic conditions for optimal achiral analyses were described in Section 2.5.1. The whole analysis lasted 27 min (see Fig. 2).

The analytes were detected using a photodiode-array detector in a single wavelength mode (the determination was performed at $\lambda = 246$ nm) or in a scan mode, which was employed for the acquisition of UV spectra. The individual analytes had very characteristic UV spectra (see Fig. 3), thus the identification of flubendazole, its metabolites and albendazole (I.S.) could be based not only on the retention times, but also on the UV spectra obtained from spectrochromatograms (see Fig. 4). The confirmation of the structures of individual flubendazole metabolites in biomatrix by HPLC-MS experiments was described and discussed in Sections 2.4 and 3.1.5.

The developed achiral LLE–HPLC-DAD method was validated as it was described in Section 2.7 and according to the recommendation CDER and CVM Guidance [20].

The selectivity of the method was tested by the analysis of blank cytosolic fraction extracts (see gray chromatograms in

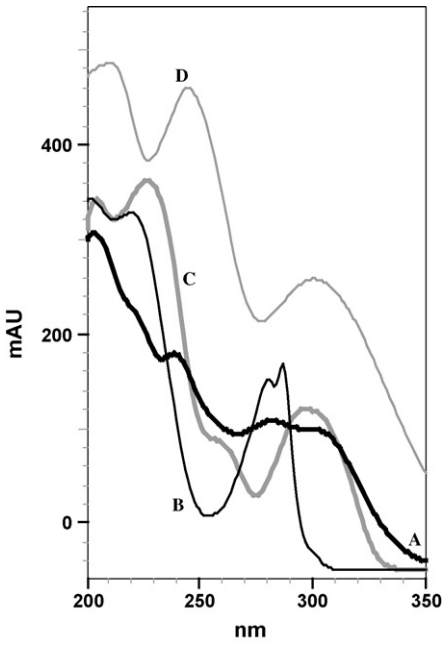

Fig. 3. Characteristic UV spectra of hydrolysed flubendazole (line A, $\lambda_{max} = 203, 239, 283, 300$ nm), reduced flubendazole (line B, $\lambda_{max} = 202, 220, 280, 287$ nm), albendazole (line C, $\lambda_{max} = 204, 227, 296$ nm) and flubendazole (line D, $\lambda_{max} = 210, 245, 300$ nm). The spectra were collected during a chromatographic analysis by photodiode array detector (see Section 2.5.1 for the achiral chromatographic conditions).

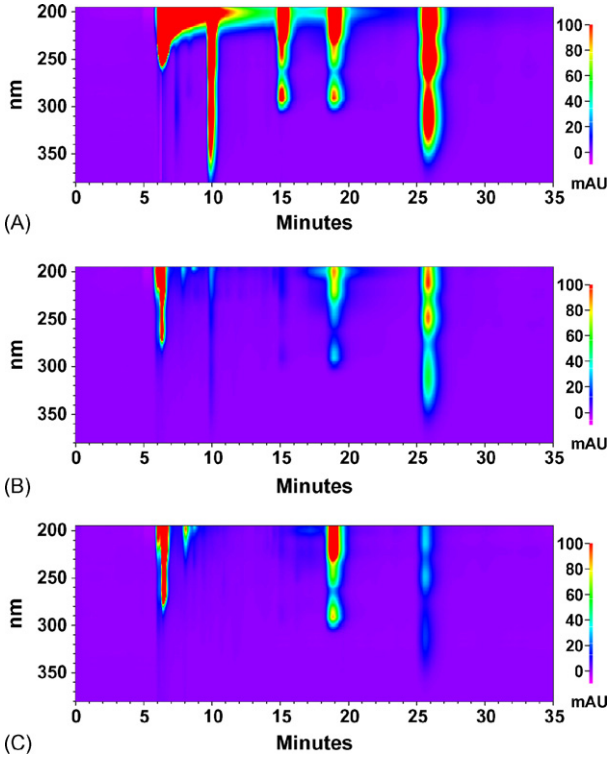

Fig. 4. Spectrochromatograms of the 10^{-5} M standard mixture (A), of the extract from pig liver cytosole fraction (B) and of the extract from pheasant liver cytosole fraction (C). See Section 2.5.2 for the chiral chromatographic conditions. Retention times of the individual compounds are following: $t_R = 10$ min for hydrolysed flubendazole; $t_R = 15$ min for (−)-reduced flubendazole; $t_R = 19$ min for (+)-reduced flubendazole; $t_R = 26$ min for flubendazole. The preparation of liver cytosole fractions is described in Section 2.2.

Table 1
Intra-day precision and accuracy of hydrolyzed flubendazole, reduced flubendazole and flubendazole determination in cytosolic calibration samples (three various concentration levels with six individually prepared samples at each calibration level were measured in 1 day)

	Added (nmol/ml)	Found (mean ± SD) (nmol/ml)	Precision (RSD, %)	Accuracy (%)
Hydrolyzed flubendazole	1	0.999 ± 0.043	4.3	−0.1
	8	8.23 ± 0.869	10.6	2.8
	80	76.81 ± 2.48	3.2	−4.0
Reduced flubendazole	1	0.852 ± 0.021	2.5	−7.0
	8	8.294 ± 0.397	4.8	3.1
	80	78.565 ± 0.989	1.3	−1.8
Flubendazole	1	1.166 ± 0.057	4.9	15.0
	8	8.054 ± 0.154	1.9	0.7
	80	82.489 ± 2.86	3.5	3.1

Fig. 2). No interferences were observed in the extracts from these samples. Also the resolution among individual benzimidazole carbamate peaks (hydrolysed flubendazole, reduced flubendazole, albendazole and flubendazole) was satisfactory (see Fig. 2). The UV photodiode-array detector had an important role in the identification of the individual derivatives according to their characteristic UV spectra and the retention times.

Validation results using UV detection at the single wavelength of 246 nm are summarized in Tables 1 and 2. The intra-day variability (mean ± SD, precision and accuracy) was calculated from cytosolic calibration for all hydrolyzed flubendazole, reduced flubendazole and flubendazole using an internal standard method with albendazole as I.S. (see Table 1). Acceptable intra-day results for the precision and accuracy were found in the range of 0.25–80 nmol/ml for hydrolyzed flubendazole and reduced flubendazole and 0.5–80 nmol/ml for flubendazole (see Tables 1 and 2). The parameters of calibration curve and the extraction recoveries for all three compounds are presented in Table 2. Limits of detection (LODs) were also determined (0.05 nmol/ml for flubendazole metabolites and 0.09 nmol/ml for flubendazole).

3.1.3. Chiral HPLC with UV photodiode-array detection

The use of Chiralcel OD-R column for the separation of reduced flubendazole enantiomers was based on our previous positive experiences with the chiral chromatography of secondary alcohols possessing stereogenic carbon atom [21]. This chiral selector is a reversed phase, 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 with the flow rate 0.5–1 ml/min are recommended by the producer of this type of chiral column as suitable mobile phases.

In the separation of hydrolysed (decarbamoylated) flubendazole, both reduced flubendazole enantiomers (used in the form of a racemic mixture) and flubendazole, an isocratic mobile phase acetonitrile–1 M aqueous $NaClO_4$ (4:6, v/v) with the flow rate of 0.5 ml/min was found to be optimal (see Fig. 4(A)). Under above-mentioned conditions, the retention times of individual analytes were $t_R = 10$ min for hydrolysed flubendazole, $t_R = 15$ min for first enantiomer of reduced flubendazole, $t_R = 19$ min for second enantiomer of reduced flubendazole and $t_R = 26$ min for flubendazole. For another (chiral) identification of both reduced flubendazole enantiomers, the experiments with polarimetric detector had to be performed.

3.1.4. Identification of (+)- and (−)-enantiomer using chiral HPLC with polarimetric detector

The separated enantiomers of reduced flubendazole (see chromatograms in Figs. 4(A) and 5(A)) were characterized only by various retention times ($t_R = 15$ and 19 min). The determination of the absolute configuration (according to Cahn–Ingold–Prelog *R*/*S* nomenclature) was impossible because the individual standards of *S*- and *R*-enantiomers of reduced flubendazole were not commercially available. Thus, the direction of the rotation of plane-polarized light when passing through the individual enantiomers was measured using a polarimetric detector.

Fig. 5 demonstrates chiral analysis of racemic mixture of reduced flubendazole measured by a polarimetric (Chiralyser) detector (Fig. 5(B)) and UV diode-array detector (Fig. 5(A)) for comparison. According to Fig. 5(B) chromatogram, the enantiomer having $t_R = 15$ min was identified as (−)-reduced flubendazole and the second eluted enantiomer ($t_R = 19$ min) was identified as (+)-reduced flubendazole.

A detector of circular-dichroism was also tested, but the intensity of the CD-signals of reduced flubendazole enantiomers was very low and is not included in this paper.

Table 2
Internal standard calibration curve parameters for hydrolyzed flubendazole, reduced flubendazole and flubendazole (based on 9-level cytosolic calibration with six individually spiked cytosolic samples at each calibration level)

	Slope	Intercept	Correlation coefficient	Range of applicability (nmol/ml)	Recovery (%)
Hydrolyzed flubendazole	1.1658	0.0017	0.996	0.25–80	83.4
Reduced flubendazole	0.5456	0.0127	0.999	0.25–80	88.5
Flubendazole	2.0499	0.0021	0.995	0.50–80	82.9

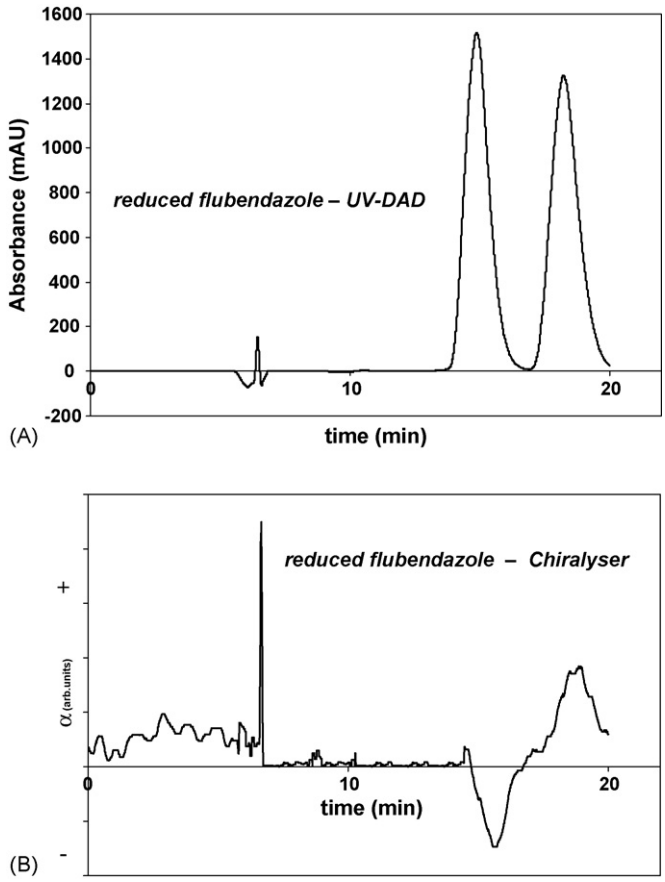

Fig. 5. Chiral analysis of the racemic mixture of (−)-reduced flubendazole (t_R = 15 min) and (+)-reduced flubendazole (t_R = 19 min) by a tandem of UV photodiode-array detector (λ = 250 nm, upper graph (A)) and polarimetric Chiralyser detector (λ = 430 nm, lower graph (B)).

3.1.5. HPLC with mass spectrometric detection

Although the standards of all analytes used were purchased from the companies providing the certificate of identity and purity, the chromatographic identification based on the retention times and UV spectra of individual metabolites in biomatrices were sometimes problematic as all metabolites were present in low concentrations. In these cases, the HPLC–MS analyses confirming the structure of such minor analytes were needed.

First, the electrospray ionisation mass spectra of parent drug (flubendazole), metabolites (desmethylcarboxy flubendazole and reduced flubendazole) and internal standard (albendazole) were measured using the direct infusion experiments in both polarity modes. All studied compounds provided characteristic ions in the positive-ion mode ($[M+H]^+$ and $[M+Na]^+$ ions) and in the negative-ion mode ($[M-H]^-$ ion and also dimeric ion $[2M\text{-}2H+Na]^-$), which enabled an easy molecular weight determination for individual compounds. Tandem mass spectra and mostly also the full scan mass spectra yielded some characteristic neutral losses, which can be correlated with the structures, such as methanol (Δ*m*/*z* 32), ammonia (Δ*m*/*z* 17) or water (Δ*m*/*z* 18). The presence of metabolites in studied samples were confirmed by HPLC with photodiode-array UV detection and ESI-MS detection techniques based on the agreement between UV spectra, ESI mass spectra and retention times of standards and sample peaks.

3.2. In vitro biotransformation studies of flubendazole

Flubendazole was incubated with microsomal or cytosolic fractions of pheasant or pig liver and small intestine. Using bioanalytical achiral HPLC-DAD–MS method, two *phase I* flubendazole metabolites (reduced flubendazole and hydrolysed flubendazole) in subcellular fractions incubates were detected (Fig. 2(B)). In both species, the activities of carbonyl reducing enzymes were significantly higher in cytosole comparing to microsomes. Flubendazole reductases preferred coenzyme NADPH. This result agrees with the literature data [22]. Hydrolysed flubendazole arose only in hepatic cytosolic fractions. More extensive formation of this metabolite was found in pig than in pheasant. In both species, the biotransformation of flubendazole was more extensive in hepatic than in small intestinal samples. None metabolites were detected in blank samples (without coenzyme NADPH or without subcellular fractions). Using the chiral HPLC-DAD method, a preferential formation of (+)-reduced flubendazole [approximately 93–97% of (+)-enantiomer to 3–7% of (−)-enantiomer] was registered (see Fig. 4(B and C)). In previous studies, concerning flubendazole bioanalyses, this stereospecificity in the carbonyl reduction of flubendazole has not yet been described [4–17].

4. Conclusions

Two new bioanalytical methods involving a simple liquid–liquid extraction of various biomatrices and subsequent isocratic high-performance liquid chromatographic analysis of flubendazole and its *phase I* metabolites using both UV photodiode-array and mass spectrometric detection were developed. The first, achiral HPLC method was validated and employed in routine analyses of the extracts from xenobiochemical *in vitro* experiments. The second, chiral HPLC method enabled the separation of hydrolyzed flubendazole, two enantiomers of reduced flubendazole and parent flubendazole and thus also the evaluation of the enantiomeric excess of reduced flubendazole in various biomatrices (liver and intestinal cytosolic and microsomal fractions from pigs and pheasants). *tert*-Butylmethyl ether was used for liquid–liquid extraction of biomatrices, albendazole was employed as an internal standard. UV photodiode-array, mass spectrometric and polarimetric detection were employed in the identification and determination of flubendazole and its metabolites.

Enzymatic reduction of prochiral flubendazole led to the prevalent stereospecific formation of the (+)-enantiomer of reduced flubendazole, which had higher retention time in chiral chromatogram (found in both species, pigs and pheasants). Higher amounts of this enantiomer were determined in cytosole fraction (in comparison with microsomal fraction). The reduction of flubendazole carbonyl was found to be NADPH-dependent. Higher activity of carbonyl reductases was found in pheasant cytosole (comparing with pig cytosole). Decarbamoy-

lated flubendazole was identified in higher amounts only in pig, not in pheasant.

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