Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 805 (2004) 267–274
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Reversed-phase liquid chromatographic method with fluorescence detection for the simultaneous determination of albendazole sulphoxide, albendazole sulphone and albendazole 2-aminosulphone in sheep plasma

Georgios C. Batzias*, Georgios A. Delis

Laboratory of Veterinary Pharmacology, School of Veterinary Medicine, Aristotle University of Thessaloniki, GR-54 124 Thessaloniki, Greece

Received 18 November 2003; received in revised form 5 March 2004; accepted 5 March 2004

Available online 12 April 2004

Abstract

A rapid and sensitive HPLC method for the simultaneous quantification of albendazole sulphoxide (ABZ-SO), albendazole sulphone (ABZ-SO_2) and albendazole 2-aminosulphone (ABZ-SO_2NH_2) in sheep blood plasma has been developed. Plasma samples were extracted with ethyl acetate under alkaline conditions. Separation was achieved on a C_{18} reversed-phase analytical column, in the presence of positively-(tetra-*n*-butylammonium hydrogen sulphate) and negatively-charged (octanesulphonate sodium) pairing ions, while detection was performed fluorometrically. Excitation and emission wavelengths were 290 and 320 nm, respectively. Limits of quantification were defined at 39 ng/ml for ABZ-SO, 4.95 ng/ml for ABZ-SO_2 and 4 ng/ml for ABZ-SO_2NH_2. Accuracy data, in terms of recovery efficiency showed overall values (±S.E.M.) of 85.6 ± 1.0% for ABZ-SO, 100.0 ± 1.0% for ABZ-SO_2 and 89.1 ± 0.6% for ABZ-SO_2NH_2. The method was successfully applied to quantitatively determine the three albendazole metabolites in plasma samples collected from sheep that had been orally administered albendazole.

Keywords: Albendazole sulphoxide; Albendazole sulphone; Albendazole 2-aminosulphone

1. Introduction

Albendazole, methyl [5-(propylthio)-1*H*-benzimidazol-2-yl] carbamate, is an anthelmintic benzimidazole widely used in veterinary and human medicine for the treatment of infections caused by helminthes [1,2].

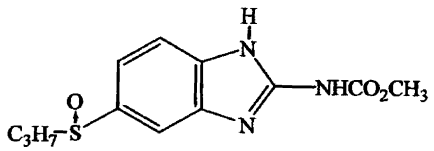
After oral administration to domestic animal species and humans, albendazole (ABZ) readily undergoes first-pass metabolism in enterocytes and liver cells. Sequential products of this metabolism are albendazole sulphoxide (ABZ-SO, present as a mixture of two enantiomers, *R* (+) and *S* (−)), formed after oxidation of the sulphide moiety of albendazole, albendazole sulphone (ABZ-SO_2), end-product of the two-step oxidation process and, finally, albendazole 2-aminosulphone (ABZ-SO_2NH_2), the *N*-deacetylation product of albendazole sulphone [3–7]. Chemical structures of albendazole metabolites are displayed in Fig. 1. The extent of albendazole first-pass metabolism is so great that the parent compound is undetectable in the vascular compartment after administration to sheep [8–10], cattle [11] and humans [12,13], although some studies report the detection of albendazole in sheep [9], mice [14] or humans [15] at concentrations much lower than the corresponding levels of metabolites.

Of all three metabolites, only ABZ-SO possesses anthelmintic along with teratogenic activity, whereas ABZ-SO_2 and ABZ-SO_2NH_2 are considered biologically inactive [16,17]. Thus, the anthelmintic effect observed after oral administration of albendazole can be attributed to the presence and activity of ABZ-SO, whereas detection and quantification of ABZ-SO_2 and ABZ-SO_2NH_2 provide information about the overall metabolism.

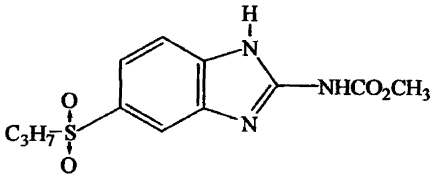
Literature offers published methods for the determination of the three metabolites in foodstuff or animal tissues, most of them using fluorometric detection [18–21]. However, most published methods concerning albendazole metabolites in animal or human plasma or serum are confined to the determination of ABZ-SO and ABZ-SO_2, with or without ABZ determination [7,14,22–26].

* Corresponding author. Tel.: +30-2310-999-855; fax: +30-2310-999-855.
E-mail address: batzias@vet.auth.gr (G.C. Batzias).

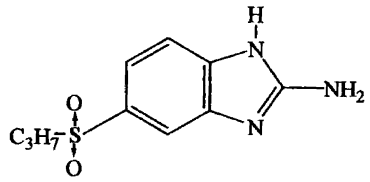
1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.03.012



albendazole sulphoxide



albendazole sulphone



albendazole 2-aminosulphone

Fig. 1. Chemical structures of albendazole sulphoxide, albendazole sulphone and albendazole 2-aminosulphone.

Mirfazaelian et al. [15] have described an HPLC assay for all three albendazole metabolites in human serum. However, the method involves the simultaneous use of both, a UV (ABZ-SO) and a fluorescence detector (ABZ-SO_2 and ABZ-SO_2NH_2), displays poor chromatographic characteristics, as can be deduced from chromatograms presented, and provides relatively high limits of quantification, along with laborious sample pretreatment.

The objective of this study was to develop a simple, inexpensive, sensitive and reliable high-performance liquid chromatographic (HPLC) method for the simultaneous determination of ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 in sheep plasma.

2. Experimental

2.1. Chemicals and reagents

Standard albendazole sulphoxide (purity 97%), albendazole sulphone (purity 98.8%) and albendazole 2-aminosulphone (purity 99.7%) were kindly donated by Glaxo-SmithKline (Worthing, UK). Standard of albendazole base was from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Analytical-grade ethyl acetate was from Lab-Scan Ltd. (Dublin, Ireland). Acetonitrile (gradient-grade), *ortho*-phosphoric acid (85% m/m), sodium hydroxide pellets and tetra-*n*-butylammonium hydrogen sulphate were from Merck (Darmstadt, Germany). Octanesulphonate sodium salt was from Fluka Chemie (Buchs, Germany). HPLC-grade water was produced in the laboratory by a Milli-Q (Millipore, Bedford, MA, USA) purification system.

2.2. Apparatus

A Shimadzu LC-10 series chromatographic system (Shimatzu Corporation, Kyoto, Japan) was used in this study. More precisely, the system consisted of a Model CBM-10A controller unit, a Model DGU-2A degasser, two Model LC-10AD piston pumps, a Model SIL-$10A_{XL}$ autosampler, a Model CTO-10A oven and a Model RF-551 spectrofluorometric detector. Chromatographic system operation and recording of data were performed with the use of Class-LC10 software (version 1.41, Shimadzu).

A Model Genie-2 Vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA), a Model Centra CL3R refrigerated centrifuge (Thermo IEC, Needman Heights, MA, USA) and a Model Reacti-Therm III evaporation unit (Pierce Chem., Rockford, IL, USA) were used during sample pretreatment.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–0.01 M *ortho*-phosphoric acid (25:75, v/v), containing 20 mM octanesulphonate sodium (OCT) and 2.5 mM tetra-*n*-butylammonium hydrogen sulphate (TBA), as ion-pair agents. The pH of the *ortho*-phosphoric acid solution, after the addition of the ion-pair agents and before the addition of acetonitrile, was approximately 3. Following its preparation, the mobile phase was passed through a 0.20 μm, Nylon 47 mm filter (Alltech Ass. Inc., Deerfield, IL, USA) and was degassed by passing helium for 10 min. The stationary phase was a Macherey-Nagel (Düren, Germany) Nucleosil 100-5, (250 mm × 4.6 mm i.d., 5 μm particle size), C_{18} reversed-phase analytical column, preceded by a Macherey–Nagel precolumn cartridge (8 mm × 4 mm i.d.) packed with the same packing material. The stationary phase was thoroughly equilibrated with mobile phase each time before use. Reproducible capacity factors (k') could be achieved after passage of at least 150 ml mobile phase through the column.

The LC system operated isocratically, at a flow rate of 1 ml/min, elution was performed at an oven temperature of 40 °C and the injection volume was 20 μl. Albendazole metabolites were detected with the use of a fluorescence detector, its excitation and emission wavelengths set at 290 and 320 nm, respectively. The sensitivity of the detector was set at "High" and the response time, at 1.5 s.

2.4. Stock and standard solutions

Stock solutions containing 1 mg/ml of ABZ-SO, ABZ-SO_2 or ABZ-SO_2NH_2 were prepared in 10 ml volumetric flasks by dissolving ca. 10 mg of each metabolite

and diluting to volume with acetonitrile. Corrections to the theoretical concentrations were performed, according to the degree of standard substances impurities.

Standard substances were weighed on a Model AX-105 analytical balance (Mettler Toledo Inc., Greifensee, Switzerland).

Mixed intermediate standard solutions were prepared by mixing aliquots of stock solutions and diluting with acetonitrile. Mixed calibration (working) standard solutions in the range of 39–7800 ng/ml for ABZ-SO, 4.95–990 ng/ml for ABZ-SO_2 and 4–800 ng/ml for ABZ-SO_2NH_2 were prepared by transferring appropriate aliquots (20–100 μl) of the mixed intermediate solutions in glass tubes, evaporating to dryness under a gentle stream of nitrogen at 40 °C, and reconstituting with 0.5 ml of mobile phase.

Mixed intermediate solutions were prepared weekly, while mixed calibration (working) standard solutions, used for the calibration curves, were prepared daily. Volumetric flasks were protected from light throughout analysis with the use of aluminium foil.

2.5. Validation control samples

Validation control (VC) samples were prepared by spiking blank samples with appropriate aliquots of the mixed intermediate solutions to achieve final concentrations of 39, 156, 195, 390, 1560 and 3900 ng/ml for ABZ-SO, 4.95, 19.80, 24.75, 49.50, 198 and 495 ng/ml for ABZ-SO_2 and 4, 16, 20, 40, 160 and 400 ng/ml for ABZ-SO_2NH_2. Aliquots (1 ml) of VC samples to be used for the evaluation of sample stability were transferred into 1.5-ml micro test tubes (Eppendorf AG, Hamburg, Germany) and were stored at −30 °C.

2.6. Sample extraction and cleanup

Into a 0.5-ml aliquot of sheep plasma, 50 μl of a 0.4N sodium hydroxide solution and 7 ml of ethyl acetate were added. After vortexing at high speed for 60 s and centrifugation at 3500 × *g* for 10 min, 6 ml of the clear supernatant were transferred into another tube and 1 ml of water was added. The sample was vortexed again for 15 s and centrifuged at 1000 × *g* for 2 min. Following this washing step, 5 ml of the top organic layer were evaporated to dryness at 40 °C, using a gentle nitrogen stream. The dried residue was reconstituted in 0.5 ml of mobile phase and 20 μl were injected into the LC system.

2.7. Determination

Calibration curves were generated for each of the three albendazole metabolites by running mixed calibration (working) standard solutions and plotting the recorded peak heights versus the corresponding mass of the analytes injected. Slope, intercept, correlation coefficients were determined by the least-square method and a lack-of-fit (LOF) test was performed. Quantification of albendazole metabolites in an unknown sample was achieved by back-referring the obtained peak heights to the appropriate corresponding calibration curve and multiplying the result of the equation $x = (y - b)/a$ (where x stands for ng of analyte/20 μl injected, y stands for peak height (μV), a stands for slope and b for intercept) by the overall mean recovery and the predetermined dilution factor.

3. Results

3.1. Chromatography and sample pretreatment

Preliminary experiments involving analysis of standard solutions of the parent compound (ABZ) and metabolites (ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2) were performed for the optimisation of mobile phase composition. As all compounds are weak bases, an effort was made to use TBA, an ion-pair agent, to eliminate their interaction and adsorption onto the free, ionised silanol groups of the stationary phase [27]. Suppression of silanol ionisation was attempted with the use of 0.01 mM *ortho*-phosphoric acid solution, instead of pure water in the mobile phase. By using mobile phases containing 10–30% acetonitrile, as organic solvent, a good separation of the investigational analytes could generally be achieved. However, in all cases, the peak attributable to the most polar ABZ-SO_2NH_2 metabolite appeared very early in the chromatograms, among the peaks of the solvent front.

In an attempt to increase the hydrophobicity of ABZ-SO_2NH_2 (cationic substance), that would lead to an increase in the retention time, OCT (a negatively-charged ion) was added into the mobile phase. Good, baseline separation was achieved for all three ABZ metabolites. Unfortunately, the parent compound could not be detected at any time point. A possible explanation for this phenomenon was that ABZ, as relatively more hydrophobic than the other compounds, was strongly retained by the stationary phase and eluted too slowly and gradually to provide a discrete, Gaussian peak.

Trials on the partitioning of albendazole and metabolites between an alkaline or acidic solution and an organic solvent showed that the substances, due to their basic structure, could be quantitatively extracted from blood plasma when alkalinised. A 0.4N sodium hydroxide solution was used for this purpose.

Since the investigated analytes have a relatively hydrophobic character, the choice of the appropriate organic solvent to perform extraction with, was directed to ethyl acetate and dichloromethane, two very potent, non-polar extraction solvents widely used for biological sample pretreatment. Further trials, performed in order to compare the extraction efficiency of ethyl acetate and dichloromethane proved the latter to yield a much lower recovery for ABZ-SO_2NH_2 (22%), while respective values for ABZ, ABZ-SO and ABZ-SO_2 were similar. No defatting was considered necessary, as samples provided clean chromatograms and reproducible reten-

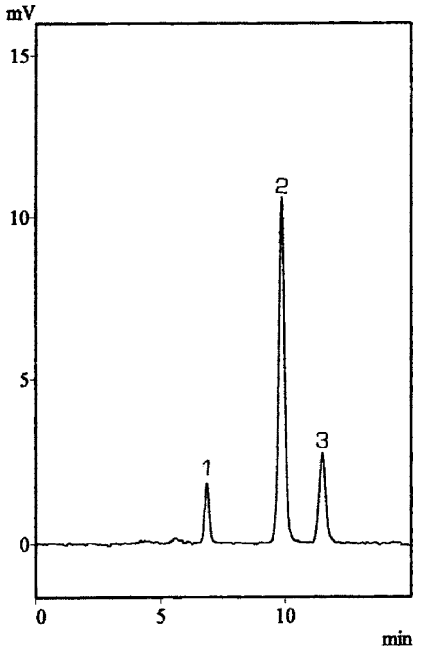


Fig. 2. Chromatogram of a calibration standard solution containing 7.8 ng/20 μl ABZ-SO (peak 1), 0.99 ng/20 μl ABZ-SO_2 (peak 2) and 0.8 ng/20 μl ABZ-SO_2NH_2 (peak 3). Stationary phase: Nucleosil 100-5; mobile phase: acetonitrile–0.01 M phosphoric acid solution (25:75, v/v) containing 20 mM octanesulphonate sodium and 2.5 mM tetra-*n*-butylammonium hydrogen sulphate; column temperature: 40 °C; flow rate: 1 ml/min; λ_{exc}: 290 nm, λ_{em}: 320 nm; injection volume: 20 μl.

tion times, in contrast to what is reported by Kitzman et al. [7] for some liquid–liquid extraction procedures. However, the washing step with water for the removal of polar endogenous compounds in plasma was deemed necessary, since they would otherwise interfere in analysis, as it was determined in trials by-passing this step.

Both, system pressure and column performance, remained stable after analysis of a great number of samples for the purpose of a pharmacokinetic study, during which it became evident that ABZ was not present in all animals and its inclusion in the method development would have no biological importance.

The final decision was to use the ion-pairing mixture (TBA, OCT) in the mobile phase, at the amounts mentioned in Section 2.3 and to validate the analytical method for albendazole metabolites only. Respective retention times were 7.0, 9.9 and 11.6 min for ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2. LC run time was 15 min (Fig. 2).

3.2. Calibration and linearity

The linearity of the detector response for the test compounds was evaluated by injecting a total of nine calibration (working) standard solutions of various concentrations covering the working range of the assay (39–7800 ng/ml for ABZ-SO, 4.95–990 ng/ml for ABZ-SO_2 and 4–800 ng/ml for ABZ-SO_2NH_2). Regression analysis of the data provided the following equations to describe the calibration curves: $y = 302.96(\pm 1.29)x - 12.80$, $r^2 = 0.999$, for ABZ-SO, $y = 15021(\pm 63.49)x + 313.16$, $r^2 = 0.999$, for ABZ-SO_2 and $y = 5293.5(\pm 24.86)x - 373.75$, $r^2 = 0.999$, for ABZ-SO_2NH_2, where y represents peak height (μV) and x is the quantity (in ng) of each compound, per 20 μl injected. All P values for intercepts were >0.05.

3.3. Selectivity

The method was checked to ensure that chromatograms obtained from blank plasma samples (collected from seven sheep) and spiked plasma samples (Fig. 3A and B) showed no interference, concerning the three metabolites, from matrix co-extractives and that the peaks attributable to the former substances were sufficiently resolved from all other peaks, if present, to ensure reliable quantification.

3.4. Limits of detection and limits of quantification

The efficiency of the ion-pairing chromatographic system, coupled with the cleanliness of the plasma extracts (Fig. 3B) after pretreatment and the high sensitivity of the fluorometric detector allowed for low limits of detection to be realised (19.5, 1 and 2 ng/ml for ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2, respectively, at a signal-to-noise ratio of 3:1).

The method was evaluated at the limit of quantification of each metabolite, generally defined as the lowest concentration of an investigational analyte in blood plasma with a precision, expressed as coefficient of variation (CV%) and a mean % bias below or equal to 20%. To determine the limits of quantification for ABZ-SO, ABZ-SO_2 or ABZ-SO_2NH_2, analysis was carried out on a series of six blank plasma samples spiked at the lowest end of the analytical range.

Analysis results permitted the establishment of limits of quantification as low as 39 ng/ml for ABZ-SO (CV ranging from 0.7 to 2.7%), 4.95 ng/ml for ABZ-SO_2 (CV ranging from 0.8 to 3.7%) and 4 ng/ml for ABZ-SO_2NH_2 (CV ranging from 1.5 to 3.9%). Values of % bias at the limits of quantification were 6.2, 1.4, and 1.4%, respectively.

3.5. Accuracy and precision

The accuracy (in terms of recovery efficiency) and the precision of the method were studied by spiking blank sheep plasma samples, at five fortification levels, with ABZ-SO (39, 195, 390, 1560 and 3900 ng/ml), ABZ-SO_2 (4.95, 24.75, 49.5, 198 and 495 ng/ml) and ABZ-SO_2NH_2 (4, 20, 40, 160 and 400 ng/ml). Seven replicates from each fortification level were analysed daily, for 3 successive days, to generate the relative data.

Least-squares regression analysis of the data presented in Table 1, showed that the relationship between ‘added’ (x) and ‘found’ (y) was adequately described by first order linear regression for each of the ana-

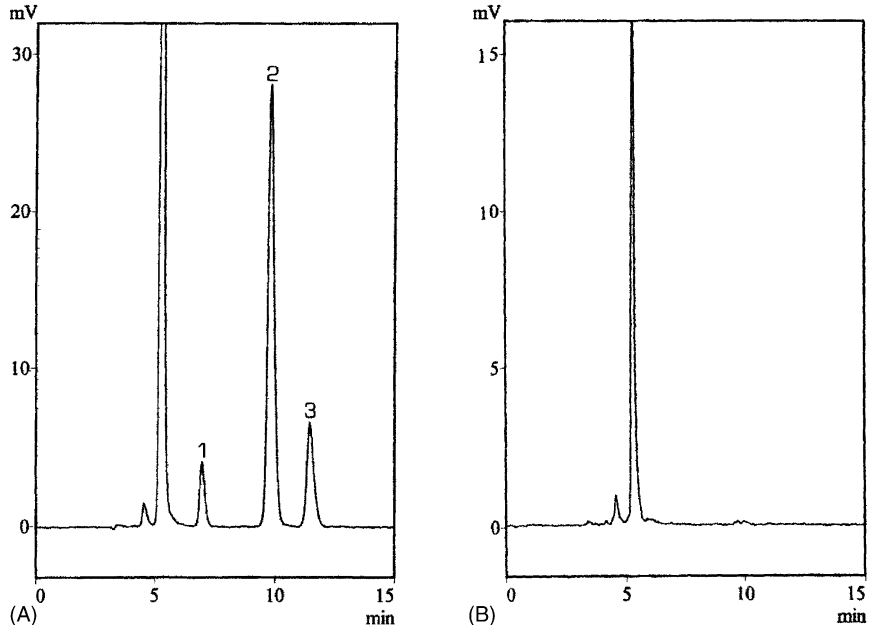


Fig. 3. Chromatograms obtained from (A) a blood plasma sample spiked with 1560 ng/ml ABZ-SO, 198 ng/ml ABZ-SO_2 and 160 ng/ml ABZ-SO_2NH_2 and (B) a drug-free sample. Chromatographic conditions and peak identification as in Fig. 2.

lytes: $y = 0.8564(\pm 0.0102)x + 43.36$ for ABZ-SO, $y = 1.0002(\pm 0.0104)x - 3.83$ for ABZ-SO_2, and $y = 0.8905(\pm 0.0059)x + 1.25$ for ABZ-SO_2NH_2. Therefore, the slope of each of these lines could be used as an estimate of the overall recovery—accuracy of the method, which was $85.6 \pm 1.0\%$ for ABZ-SO, $100.0 \pm 1.0\%$ for ABZ-SO_2, and $89.1 \pm 0.6\%$ for ABZ-SO_2NH_2.

The precision of the method was measured as the relative standard deviation—coefficient of variation (CV) of the concentrations determined in all replicates. Precision data are shown in Table 2. Results indicate totally acceptable recovery and precision (<15%) values.

3.6. Storage stability

The effect of storage, under different conditions, on the stability of albendazole metabolites in spiked plasma samples was assessed. After the development, and before the validation of the method, two stability experiments were performed.

One set of samples was extracted, transferred into autosampler inserts and analysed immediately and after a 24-h waiting period in the autosampler (room temperature, approximately 22 °C). The fortification level (of all three metabolites) was 156 ng/ml for ABZ-SO,

Table 1
Accuracy data of the analysis of sheep plasma spiked with ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2

	Analyte added (ng/ml)	Overall mean concentration found[a] (ng/ml ± S.D.)	Recovery (% ± S.D.)	Overall recovery (% ± S.E.M.)
ABZ-SO	39	39.8 ± 0.7	101.9 ± 1.7	85.6 ± 1.0
	195	193.3 ± 3.6	99.1 ± 1.8	
	390	367.6 ± 8.6	94.3 ± 2.2	
	1560	1482.2 ± 14.3	95.0 ± 0.9	
	3900	3344.4 ± 73.5	85.8 ± 1.9	
ABZ-SO_2	4.95	4.9 ± 0.1	100.3 ± 2.5	100.0 ± 1.0
	24.75	24.8 ± 0.5	100.1 ± 2.1	
	49.50	46.2 ± 1.3	93.3 ± 2.6	
	198	181.0 ± 3.8	91.5 ± 1.9	
	495	496.2 ± 11.1	100.3 ± 2.2	
ABZ-SO_2NH_2	4	4.1 ± 0.1	101.46 ± 2.1	89.1 ± 0.6
	20	18.9 ± 0.6	94.58 ± 2.7	
	40	37.4 ± 1.1	93.61 ± 2.8	
	160	144.3 ± 3.2	90.21 ± 2.0	
	400	357.2 ± 13.4	89.29 ± 3.4	

[a] Twenty-one replicates.

Table 2
Precision data of the analysis of sheep plasma spiked with ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 on three different days

	Analyte added (ng/ml)	Mean concentration found[a] (ng/ml ± S.D.)			CV (%)		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
ABZ-SO	39	39.6 ± 0.8	39.8 ± 0.6	39.63 ± 0.5	2.0	1.5	1.4
	195	195.2 ± 1.8	192.1 ± 4.6	192.45 ± 3.5	0.9	2.4	1.8
	390	364.4 ± 8.7	370.6 ± 10.1	367.92 ± 6.9	2.4	2.7	1.9
	1560	1487.2 ± 17.3	1482.8 ± 14.8	1476.5 ± 10.4	1.2	1.0	0.7
	3900	3348.1 ± 76.5	3365.8 ± 71.6	3319.4 ± 77.9	2.3	2.1	2.4
ABZ-SO_2	4.95	4.9 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	2.8	2.1	1.9
	24.75	24.8 ± 0.2	24.8 ± 0.5	24.7 ± 0.8	0.8	2.0	3.2
	49.50	46.0 ± 1.4	46.3 ± 1.7	46.2 ± 1.0	2.9	3.7	2.1
	198	182.5 ± 4.1	179.2 ± 3.4	181.5 ± 3.6	2.2	1.9	2.0
	495	492.9 ± 10.1	497.3 ± 13.3	498.6 ± 10.7	2.1	2.7	2.2
ABZ-SO_2NH_2	4	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	2.6	2.0	2.4
	20	18.6 ± 0.7	18.8 ± 0.5	19.1 ± 0.4	3.9	2.4	2.0
	40	37.9 ± 1.4	37.3 ± 0.7	37.2 ± 1.1	3.6	1.8	2.9
	160	145.3 ± 3.4	144.0 ± 3.2	143.9 ± 3.3	2.3	2.2	2.3
	400	356.0 ± 13.1	366.2 ± 15.5	349.2 ± 5.3	3.6	4.2	1.5

[a] Seven replicates.

19.8 ng/ml for ABZ-SO_2 and 16 ng/ml for ABZ-SO_2NH_2. Extracted samples analysed during the validation procedure or routine analysis should remain stable at room temperature, throughout daily analysis, to yield reliable quantification.

For the second experiment, samples containing 1560 ng/ml ABZ-SO, 198 ng/ml ABZ-SO_2 and 160 ng/ml ABZ-SO_2NH_2 were analysed to estimate the stability of albendazole metabolites after a three freeze-thaw cycle.

Finally, in order to test the applicability of the method for plasma samples frozen for a long period of time, as occasionally happens in the case of pharmacokinetic studies, samples fortified with ABZ-SO (156, 390 and 1560 ng/ml), ABZ-SO_2 (19.8, 49.5 and 198 ng/ml) and ABZ-SO_2NH_2

Table 3
Stability of albendazole metabolites in spiked plasma samples, after 24-h waiting time in the autosampler (room temperature, approximately 22 °C), after 4 months storage at −30 °C and after a three freeze-thaw cycle

	Analyte added (ng/ml)	Mean concentration found[a] (ng/ml ± S.D.)	
		0 h	24 h
Autosampler			
ABZ-SO	156	137.6 ± 4.8	136.5 ± 5.3
ABZ-SO_2	19.8	19.2 ± 0.3	19.2 ± 0.4
ABZ-SO_2NH_2	16	14.7 ± 0.2	14.8 ± 0.1
		0 months	4 months
Storage at −30 °C			
ABZ-SO	156	137.0 ± 5.1	137.5 ± 5.2
	390	348.7 ± 9.2	349.8 ± 10.5
	1560	1334.3 ± 22.6	1339.7 ± 25.5
ABZ-SO_2	19.8	19.1 ± 0.2	19.2 ± 0.4
	49.5	48.9 ± 1.5	48.4 ± 1.3
	198	191.6 ± 5.1	189.7 ± 4.3
ABZ-SO_2NH_2	16	14.7 ± 0.2	14.8 ± 0.1
	40	33.2 ± 1.1	32.7 ± 0.9
	160	134.1 ± 2.5	133.7 ± 2.0
		Before	After
Three freeze-thaw cycle			
ABZ-SO	1560	1333.3 ± 28.3	1335.9 ± 24.2
ABZ-SO_2	198	192.6 ± 3.9	194.0 ± 2.2
ABZ-SO_2NH_2	160	133.5 ± 2.4	132.4 ± 2.5

Significance level: $\alpha = 0.05$.
[a] Six replicates.

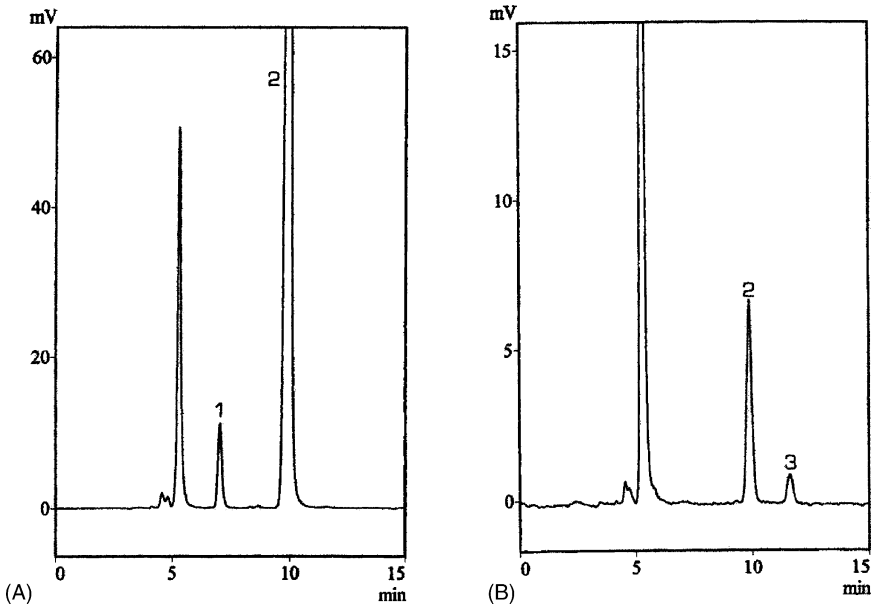


Fig. 4. Representative chromatograms of plasma samples collected from one animal at (A) 10 h and (B) 48 h after a single per os albendazole administration, at the dose of 11.5 mg/kg BW. Chromatographic conditions and peak identification as in Fig. 2.

(16, 40 and 160 ng/ml) were analysed before and after 4 months storage at −30 °C.

Results of stability experiments are presented in Table 3. Statistical analysis (paired *t*-test) of the mean values, in all cases, revealed no significant differences that would imply an effect of sample storage on metabolites' stability.

3.7. Applicability

To validate the method with real samples, a trial was undertaken to determine albendazole metabolites in the plasma of four sheep, that had been orally administered a single dose of an ABZ suspension, corresponding to 11.5 mg of ABZ per kg of body weight. Representative chromatograms obtained from one animal at 10 and 48 h after drug administration are shown in Fig. 4A and B, respectively.

The maximum concentration (C_{max}) of ABZ-SO (mean value of four animals) was 3297.7 ng/ml, and was observed 12 h after dosing, while the C_{max} of ABZ-SO_2 was 1394.8 ng/ml and occurred at 30 h. Finally, ABZ-SO_2NH_2 was detected between the 48th and the 72nd h, while C_{max} (82.2 ng/ml) was observed 66 h after dosing. All values are presented in Table 4.

Table 4
Albendazole metabolites plasma levels in four ($n = 4$) sheep, after a single per os administration of albendazole, at the dose of 11.5 mg/kg BW

Time after administration (h)	ABZ-SO plasma concentration (ng/ml ± S.D.)	ABZ-SO_2 plasma concentration (ng/ml ± S.D.)	ABZ-SO_2NH_2 plasma concentration (ng/ml ± S.D.)
1	416.5 ± 91.6	27.2 ± 2.9	ND
3	1267.5 ± 228.3	113.8 ± 20.1	ND
6	2554.4 ± 292.3	339.5 ± 83.8	ND
9	3241.8 ± 516.3	616.3 ± 155.4	ND
12	3297.7 ± 268.8	811.9 ± 183.5	ND
18	3030.2 ± 235.7	1040.3 ± 223.7	ND
24	2404.9 ± 439.7	1189.4 ± 294.1	ND
30	2368.4 ± 484.6	1394.8 ± 352.7	ND
36	1439.3 ± 545.2	1182.4 ± 182.4	ND
42	954.9 ± 520.9[a]	1063.9 ± 181.1	ND
48	562.5 ± 452.3[a]	834.8 ± 203.4[a]	62.6 ± 28.6[a]
54	ND	560.9 ± 578.8[a]	65.8 ± 27.6[a]
60	ND	227.8 ± 342.1[a]	71.1 ± 38.5[a]
66	ND	ND	82.2 ± 45.1[a]
72	ND	ND	55.0 ± 20.5[a]

ND: not detected.

[a] The very high S.D. values are due to a large inter-subject variability observed in the kinetics of albendazole metabolites, along with the small sample size ($n = 4$).

4. Discussion

In contrast to the sample pretreatment proposed by Mirfazaelian et al. [15], the current method involves only two steps of liquid–liquid extraction, namely extraction with ethyl acetate under alkaline conditions and washing with water for the removal of polar plasma endogenous compounds. In addition, recovery of the analytes is better (minimum value 80% for all metabolites, compared to 65% for ABZ-SO, 96% for ABZ-SO_2 and 70% for ABZ-SO_2NH_2 reported by Mirfazaelian et al. [15]) and this led to the establishment of substantially lower limits of quantification. Moreover, a single fluorometric detector was used for the determination of all three ABZ metabolites, without compromising assay sensitivity. Chromatograms obtained show that investigational analytes displayed almost excellent chromatographic behaviour, yielding sharp, symmetric peaks and a clear baseline resolution, two properties rather ambiguous in the method of Mirfazaelian et al. [15].

5. Conclusion

The RP-HPLC method described in this study has excellent analytical characteristics with respect to recovery, sensitivity and repeatability. It is also rapid since a daily throughput of 70 sheep plasma samples could be easily achieved by manual sample preparation and automated LC analysis. This method can prove to be very useful in pharmacokinetic and bioequivalence studies, in which large numbers of samples are usually analysed.

Acknowledgements

The authors would like to express their gratitude to GlaxoSmithKline (Worthing, UK) for kindly donating pure standards of ABZ metabolites.

References

[1] V.J. Theodorides, R.J. Gyurik, W.D. Kingsbury, R.C. Parish, Experientia 32 (1976) 702.
[2] Q. McKellar, E. Scott, J. Vet. Pharmacol. Ther. 13 (1990) 223.
[3] H. Souhaili-El-Amri, X. Fargetton, P. Delatour, A.M. Batt, Xenobiotica 17 (1987) 1159.
[4] H. Souhaili-El-Amri, O. Mothe, M. Totis, C. Masson, A.M. Batt, P. Delatour, G. Siest, J. Pharmacol. Exp. Ther. 246 (1988) 758.
[5] P. Moroni, T. Buronfosse, C. Longin-Sauvageon, P. Delatour, E. Benoit, Drug Metab. Dispos. 23 (1995) 160.
[6] P. Delatïur, M.C. Cure, E. Benoit, F. Garnier, J. Vet. Pharmacol. Ther. 9 (1986) 230.
[7] D. Kitzman, K.-J. Cheng, L. Fleckenstein, J. Pharm. Biomed. Anal. 30 (2002) 801.
[8] C.P. Swarnkar, P.K. Sanyal, D. Singh, F.A. Khan, P.S.K. Bhagwan, Vet. Res. Commun. 22 (1998) 545.
[9] S.E. Marriner, J.A. Bogan, Am. J. Vet. Res. 41 (1980) 1126.
[10] L.I. Alvares, S.F. Sánchez, C.E. Lanusse, Vet. Parasitol. 69 (1997) 241.
[11] R.K. Prichard, D.R. Hennessy, J.W. Steel, E. Lacet, Res. Vet. Sci. 39 (1985) 173.
[12] S.E. Mariner, D.L. Morris, B. Dickson, J.A. Bogan, Eur. J. Pharmacol. 30 (1986) 705.
[13] A. Mirfazaelian, S. Dadashzadeh, M.R. Rouini, Eur. J. Clin. Pharmacol. 58 (2002) 403.
[14] J.J. Garcia, F. Bolás-Fernández, J.J. Torrado, J. Chromatogr. B 723 (1999) 265.
[15] A. Mirfazaelian, S. Dadashzadeh, M.R. Rouini, J. Pharm. Biomed. Anal. 30 (2002) 1249.
[16] P. Delatour, R. Parish, in: A.G. Rico (Ed.), Drug Residues in Animals, Academic Press, Orlando, FL, 1986, p. 175.
[17] M. Navvaro, L. Canut, A. Carretero, C. Cristòfol, F.J. Pérez-Aparicio, M. Arboix, J. Ruberte, Reprod. Toxicol. 13 (1999) 295.
[18] D. Fletouris, N.A. Botsoglou, I.E. Psomas, A.I. Mantis, J. Dairy Sci. 80 (1997) 2695.
[19] D. Fletouris, N.A. Botsoglou, I.E. Psomas, A.I. Mantis, Anal. Chim. Acta 345 (1997) 111.
[20] B. Shaikh, N. Rummel, R. Reimschuessel, J. Agric. Food Chem. 51 (2003) 3254.
[21] G. Balizs, J. Chromatogr. B: Biomed. Sci. Appl. 727 (1999) 167.
[22] M. Alvinerie, P. Galtier, J. Pharm. Biomed. Anal. 2 (1984) 73.
[23] P.E. Hoaksey, K. Awadzi, S.A. Ward, P.A. Coventry, M.L.E. Orme, G. Edwards, J. Chromatogr. B: Biomed. Sci. Appl. 566 (1991) 244.
[24] M.E. Valois, O.M. Takayanagui, P.S. Bonato, V.L. Lanchote, D. Carvalho, J. Anal. Toxicol. 18 (1994) 86.
[25] V.L. Lanchote, M.P. Marques, O.M. Takayanagui, R. de Carvalho, F.O. Paias, P.S. Bonato, J. Chromatogr. B 709 (1998) 273.
[26] P. Chiap, B. Evrand, M.A. Bimazubute, P. de Tullio, P. Hubert, L. Delattre, J. Crommen, J. Chromatogr. A 870 (2000) 121.
[27] D.J. Fletouris, N.A. Botsoglou, I.E. Psomas, A.I. Mantis, J. Chromatogr. B 687 (1996) 427.