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Development and optimization of a rapid HPLC method for analysis of ricobendazole and albendazole sulfone in sheep plasma

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Abstract

A simple, rapid and reliable high performance liquid chromatographic (HPLC) method has been developed and validated for simultaneous determination of ricobendazole (RBZ) and its main metabolite albendazole sulfone ($ABZSO_2$) in sheep plasma using an isocratic system with UV detection. The method involved solid phase extraction followed by separation on a reversed phase C-18 column. Internal standard was selected by quantitative structure retention relationships (QSRRs) analysis. A method to optimize the composition of ternary components mobile phase with the assistance of multiple linear regression is described. Retention times were within 10 min. The calibration curves were linear over a concentration range of 10–1000 ng/ml for both RBZ and $ABZSO_2$ ($r > 0.999$). Intra-day relative standard deviation at low, medium and high concentration levels were <5.5% for RBZ and <4.6% for $ABZSO_2$; average accuracies were 98.3, 101.0 and 100.5% for RBZ and 101.0, 102.4 and 100.8% for $ABZSO_2$. The inter-day variations at the same concentrations were <5.9% for RBZ and <6.4% for $ABZSO_2$. The extraction recoveries at these concentrations for RBZ, $ABZSO_2$ and the internal standard were all over 96%. The limit of detection and limit of quantitation were 2.4 and 7.1 ng/ml, respectively for RBZ, and 10 ng/ml for both analytes.
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Keywords: Ricobendazole; Metabolites; Reversed-phase HPLC; log *P*; Solid phase extraction; QSRRs; Multiple linear regression

1. Introduction

Albendazole (ABZ), a benzimidazole methylcarbamate, is widely used in both human and veterinary medicine for the prevention and treatment of parasitic diseases [1]. In vivo studies suggested that albendazole is normally detected in plasma only when injected intravenously. For other routes, only the two metabolites, albendazole sulphoxide and albendazole sulfone ($ABZSO_2$) are detectable as a result of its rapid metabolism [2,3].

Albendazole sulphoxide, which is also known as ricobendazole (RBZ), has been found to be the only active metabolite of albendazole. The presence of the oxidised sulphur enhances the water solubility of RBZ (62 μg/ml) compared with the parent drug ABZ (0.61 μg/ml) and enables the development of injectable aqueous solutions for cattle [4]. It is currently marketed as injectable products such as Bayverm PI® (Bayer Argentina) [4,5], and Sintyotal-R® (Biogenesis Argentina) [6].

Pharmacokinetic studies have shown that following treatment with RBZ in rodent, chicken, cattle and sheep, RBZ is slowly oxidised to $ABZSO_2$, and then hydrolyzed to albendazole sulfone amine ($ABZSO_2NH_2$) due to the cleavage of the amide linkage [7]. These two metabolites have also been detected in human serum after an oral administration of ABZ [8]. Although there are no reports that RBZ undergoes hydrolysis to form ricobendazole amine ($RBZNH_2$) in vivo, it is possible due to the presence of amide linkage (Fig. 1), which can potentially hydrolyze enzymatically or non-enzymatically [9]. A preliminary study by mass spectrometry (MS) showed that this reaction occurred in aqueous solution, particularly in basic media. Fig. 1 shows the potential metabolites and degradation products of RBZ.

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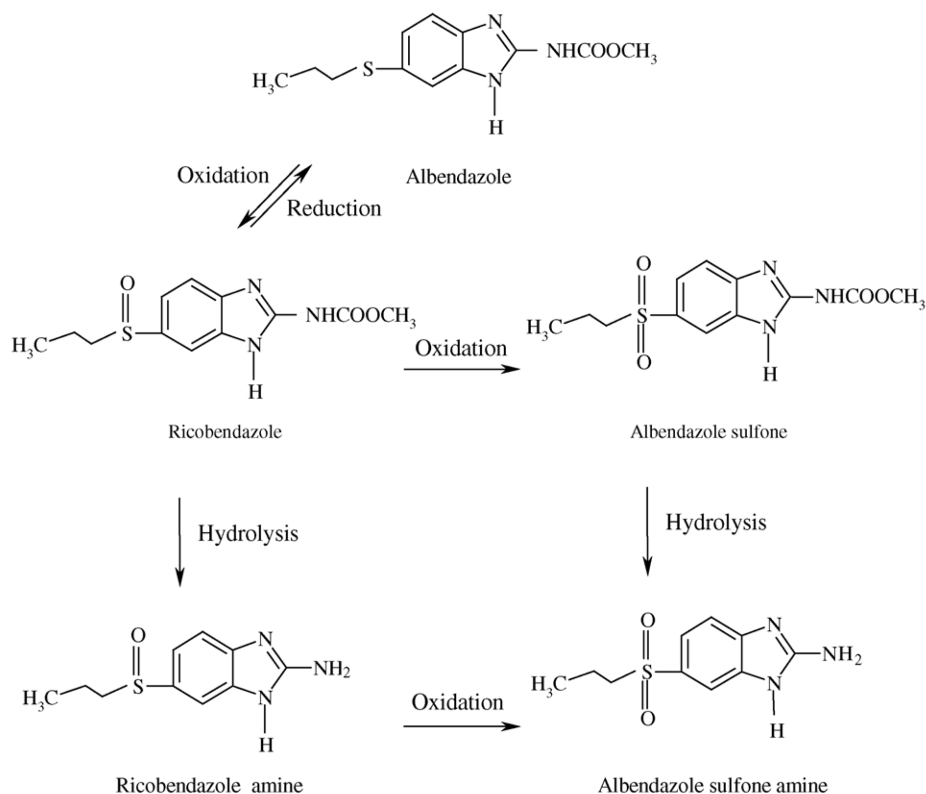

Fig. 1. The structures of ricobendazole and its potential metabolites and degradation products.

Various analytical methods have been reported for RBZ pharmacokinetic studies, mainly using reverse phase HPLC with UV detection [3–6,8,10–13]. Spectrofluorometric detection [12], or a combination of UV and fluorescence [8] or LC–MS–MS methods [13], have also been reported. Most of these methods used mebendazole [8,14] or oxibendazole [4–6,10,11] (Fig. 2) as the internal standard (IS). These compounds differ in polarity compared to RBZ and $ABZSO_2$, necessitating the use of gradient mobile phase systems. One method [3], which used an isocratic binary solvent composed of buffer and methanol, was unable to resolve RBZ and $ABZSO_2$. In addition, except for the study by Mirfazaeliana et al. [8] which used UV detection for RBZ and mebendazole (IS), and fluorescence detection for $ABZSO_2$ and $ABZSO_2NH_2$, most literature has focused on the assay of RBZ and $ABZSO_2$, and less attention has been given to the interference from $RBZNH_2$ and $ABZSO_2NH_2$, which are potential metabolites or degradation products.

This paper reports a simple, rapid and reliable isocratic HPLC method using UV detection for the simultaneous determination of RBZ and its metabolite $RBZSO_2$ in sheep plasma, without interference from the potential metabolites or degradation products associated with RBZ. The internal standard was selected by quantitative structure retention relationships (QSRRs) analysis. A method to optimize the composition of the ternary mobile phase with the assistance of multiple linear regression is described.

2. Experimental

2.1. Chemicals

Ricobendazole (99.3%) was a gift from Transchem Limited, Ambernath, India. Albendazole sulfone (99.4%) was

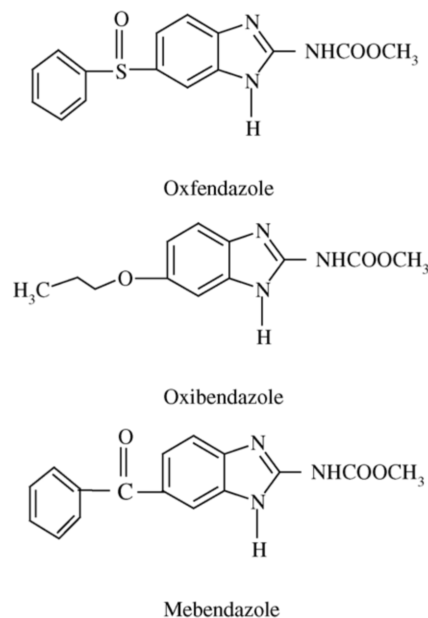

Fig. 2. The chemical structures of the internal standards used in literature and this study.

kindly donated by Uquifa Mexico, SA DE CV. Oxfendazole (99.6%) was purchased from Haimen Pharmaceutical Factory, China. Ricobendazole amine and albendazole sulfone amine were prepared by the hydrolysis of 10 μg/ml solutions of ricobendazole and albendazole sulfone, respectively, in NaOH solutions (pH 9), stored at 50 °C for 3–5 h. Reaction products were separated by LC and then identified by MS. HPLC grade acetonitrile and methanol were from J.T. Baker and Mallinckrodt (USA). All other chemicals and solvents were reagent grade (BDH Chemicals Ltd, England).

2.2. Instrumentation

The method development and validation was performed on a Shimadzu (Kyoto, Japan) HPLC system comprising an LC 10-AT quaternary pump equipped with a SIL-10AD auto-sampler injector (injection volume was 20 μl), a SPD-10A variable wavelength detector (set at 292 nm) and a CTO-10ASVP column oven all controlled by a computer using Class-VP 6.1 software. Separation was carried out on a C18 Prodigy 5 μm, 4.6 mm × 250 mm column connected with a C18 Prodigy 5 μm, 4.6 mm × 30 mm pre-column (Phenomenex®, New Zealand) maintained at 30 °C. Unless otherwise stated, the mobile phase used for the final method validation consisted of methanol–acetonitrile–phosphate buffer (pH 6.8; 20 mM) (22:22:56, v/v/v) and the flow rate was 1 ml/min.

2.3. Choice for internal standard

Quantitative structure retention relationships analysis was applied to predict reversed-phase HPLC retention times of the compounds using logarithm of octanol–water partition coefficient (log *P*) as a primary descriptor of the structures. The calculated log *P* (clog *P*) values were obtained using the on-line interactive programme (http://www.syrres.com/esc/est_kowdemo.htm), which is based on the atom/fragment contribution method. The methodology has been described by Meylan and Howard [15]. Several benzimidazole derivatives were compared for the choice of internal standard and the compound with a clog *P* close to those of the analytes was selected (Fig. 2).

2.4. Mobile phase optimization

The mobile phase was optimised by simultaneously varying 'solvent selectivity' (solvent type) and 'solvent strength' (volume fraction of organic solvent(s) in the mobile phase) for the separation of all the analytes and other interfering peaks on the C-18 column with a flow rate of 1 ml/min.

The following steps were needed to obtain the data for the optimization of mobile phase:

(i) Determination of the approximate solvent strength of mobile phase needed so that the retention time of the first-eluting component did not elute before 4 min, in order to avoid endogenous plasma peaks, and the last-eluting component did not elute after 20 min.

(ii) Determination of the retention times of all analytes with 6–7 different mobile phases with various volume ratios of methanol to acetonitrile (from 1:0.8 to 1:3) at the appropriate solvent strength as determined by step (i).

(iii) Regression of the retention time of each analyte as the function of the percentage of each component in the mobile phase with the multiple linear regression model (Eq. (1)) using Minitab version 12.1 (Minitab Inc., PA, USA):

$$R_t = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + e \quad (1)$$

where R_t is the retention time, X_1 and X_2 are percentages (by volume) of methanol and acetonitrile in a given mobile phase, β_0, β_1 and β_2 are model coefficients determined by regression analysis, and e is the experimental residual error. The variable X_3, denoting the third component of the mobile phase, phosphate buffer (pH 6.8; 20 mM), was removed from the equation, since $X_1 + X_2 + X_3 = 100$.

For each analyte, coefficient β_0 denotes the R_t corresponding to the phosphate buffer as a hypothetical mobile phase, β_1 and β_2 are negative values and depend on the eluting power of each organic solvent used in the mobile phase.

(iv) The regression equations were used to predict the modification of the mobile phase components required to achieve appropriate retention times for the analytes.

Separation of overlapping peaks, a and b (if any), could be obtained by varying the solvents ratio (Eq. (2)), to achieve an appropriate difference in retention times ($R_{ta} - R_{tb}$) between the two peaks:

$$R_{ta} - R_{tb} = \beta_{0a} - \beta_{0b} + (\beta_{1a} - \beta_{1b})\, X_1 + (\beta_{2a} - \beta_{2b}) X_2 \quad (2)$$

The criterion for resolution between the two peaks was $R_s > 2$, where R_s is defined as [16]:

$$R_s = 2\left(\frac{R_{ta} - R_{tb}}{W_a + W_b}\right) \quad (3)$$

where W_a and W_b are the width of peaks at the baseline, obtained from the chromatograms at step (i).

2.5. Stock solutions and standards

A stock solution of RBZ was prepared in methanol at 1 mg/ml and kept at −20 °C. Working standards were prepared by diluting the stock solution to obtain the concentrations of 1, 2, 5, 10, 20, 50 and 100 μg/ml. A set of quality control (QC) samples at low (20 μg/ml), medium (40 μg/ml) and high (80 μg/ml) levels were prepared from different stock solution.

$ABZSO_2$ standard solutions and QC solutions were prepared at the same concentrations as for RBZ.

An oxfendazole (internal standard) working solution was prepared by diluting a 1 mg/ml stock solution to 50 μg/ml with methanol.

2.6. Solid phase extraction (SPE) procedure

SPE cartridges (Strata® C 18-E, 200 mg, 3 ml reservoir, Phenomenex) were conditioned by washing with 3 ml of methanol followed by 3 ml of water. Control sheep plasma (1 ml) was spiked with aliquots (10 μl) of working standards of RBZ and $ABZSO_2$ along with 10 μl internal standard solution (oxfendazole, 50 μg/ml) to obtain plasma calibration standards. Samples were diluted 1:1 with water to reduce the viscosity, votexed for 1 min and then loaded onto the preconditioned SPE cartridges. Cartridges were washed with 0.5 ml of 40% methanol, then 9 ml of water and dried by vacuum (20 kPa for 5 min) using a vacuum manifold (Phenomenex®, New Zealand). Analytes were eluted with 1.5 ml methanol into Eppendorf (2 ml) safety-lock tubes. Solvent was evaporated with a stream of nitrogen gas and the residues dissolved in 200 μl mobile phase. The tubes were centrifuged at 7000 × *g* using a Mini-centrifuge (Denver Instruments, USA). The supernatant was transferred into the glass insert vials for HPLC assay.

2.7. Extraction efficiency

The extraction efficiencies of RBZ, $ABZSO_2$ and IS oxfendazole were determined at low, medium and high concentrations by comparing the HPLC peak areas of the standard solutions without extraction and the samples with extraction. The extraction recovery was calculated using Eq. (4):

$$\text{Recovery (\%)} = \frac{\text{Peak area found in the extracted plasma sample}}{\text{Peak area of the standard solution without extraction}} \times 100 \quad (4)$$

2.8. Validation method

The specificity of the method was determined by comparing the chromatograms of drug free plasma samples with those plasma samples spiked with standard solutions.

The linearity of the response was determined by chromatographing five replicates of seven standards at concentrations of 10, 20, 50, 100, 200, 500, 1000 ng/ml to produce a measure of pure error for testing of linearity [17]. Weighted ($1/\sigma^2$) least squares linear regression was performed on the ratios of peak areas of RBZ or $ABZSO_2$ to that of the IS versus concentrations. Analysis of variance (ANOVA) with a lack-of-fit test, which is designed to evaluate if the selected model (linear) is adequate to describe the observed data, was done using Minitab (version 12.1). Intra-day variability was determined by five repeated analyses of the QC samples on the same day, and inter-day variability on five consecutive days. A new calibration curve was prepared on each day. Limit of detection (LOD) and quantification (LOQ) were estimated using Eqs. (5) and (6), respectively [18].

$$\text{LOD} = \frac{3.3\sigma}{S} \quad (5)$$

$$\text{LOQ} = \frac{10\sigma}{S} \quad (6)$$

where σ is the residual standard deviation of the regression line, S is the slope of the standard curve.

Assay precision was assessed by the percent relative standard deviation (%R.S.D.) and accuracy was calculated as the estimated concentration as the percentage of the nominal concentration. Accuracy was assessed by comparing the predicted concentrations of the QC samples, using the calibration curve, with the respective theoretical concentration.

2.9. Stability

QC samples containing 800 ng/ml RBZ and $ABZSO_2$ in sheep plasma, were stored at −20 °C for 3 weeks to assess stability. Also another three sets of these samples were freeze-thawed (4 cycles, −20 °C to melt) for stability assessment.

2.10. Pharmacokinetic studies in sheep

Pharmacokinetic studies of two RBZ formulations, an aqueous low pH solution and a microemulsion, were performed in sheep at a dose of 5 mg/kg (RBZ base). Blood samples were taken at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 18, 24, 30, 36, 48, 60 and 72 h post subcutaneous administration. Samples were centrifuged at 3000 rpm for 10 min, and the plasma were transferred into Eppendorf tubes and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Choice of internal standard using QSRRs [19]

The clog *P* values of analytes and IS clearly correlate with the reported retention times (Table 1). The higher the log *P* of the compound, the stronger the affinity for the stationary phase (column), which is in agreement with many reported studies [19,20]. Since clog *P* values of the analytes in this study were in the range of 0.3–1.1, oxfendazole (clog *P* 1.63) was selected as the most appropriate IS. Internal standards used in the literature have higher clog *P* values (2.6–2.7), and required the use of a stronger eluent in a gradient system to reduce the analysis time, especially when analysing ABZ, with a clog *P* of 3.14.

The second consideration for the choice of oxfendazole as IS was that compounds with similar log *P* values elute simultaneously from the solid phase extraction column.

Table 1
Comparison of clog P values and retention times for RBZ, its metabolites and potential internal standards in reversed-phase HPLC systems

Analytes	clog P	R_t (min)		
		Gradient method [5]	Gradient method [11]	Isocratic method of this study
RBZ NH_2	0.30	–	–	3.68
$ABZSO_2NH_2$	0.43	–	–	4.65
RBZ	0.97	5.9	4.2	5.48
$ABZSO_2$	1.1	7.4	5.8	7.50
Oxfendazole	1.63	–	–	8.47
Oxibendazole (IS)	2.62	10.3	–	–
Mebendazole (IS)	2.71	–	10.3	–
ABZ	3.14	12.9	12	19.8

In addition, with respect to its spectral properties, the same concentrations of RBZ and oxfendazole produced similar peak areas at 292 nm.

Quantitative structure retention relationships (QSRRs) describe the relationships between chromatographic parameters of analytes and the quantitative difference among their structures. They are determined statistically from a series of analytes in a given separation system [19,20]. log P (octanol/water) is the most fundamental quantitative structural descriptor that can be related to the retention times of the analytes in both gradient and isocratic reversed-phase HPLC systems [20–22]. The following relationship has been established [20].

$$\text{Retention parameter} = k_1 + k_2 \log P \quad (7)$$

where k_1 and k_2 are the regression constants determined from a series of compounds in a given separation system.

In our study the correlation between log P and retention time using a previously developed reversed-phase HPLC stability-indicating method [23] was poor. The mobile phase in this method consisted of acetonitrile and phosphate buffer (pH 3; 20 mM) (30:70, v/v). The IS peak was found to overlap with the peak of $ABZSO_2$ irrespective of the fraction of acetonitrile (varying from 25 to 40%) in the mobile phase. Consequently a third component, methanol, was included in the mobile phase to provide a better resolution (see Section 3.2). This was not surprising as Cimpan et al. [21] has reported that a satisfactory correlation between retention parameters and log P data was obtained when methanol–buffer binary systems were used as eluents, but not consistently when acetonitrile–water systems were used.

3.2. Optimization of mobile phase by multiple regression analysis

In this study, the total organic solvent(s) volume fraction in the mobile phase for appropriate retention times for all analytes was in the range 35–45%. Table 2 shows the retention times obtained with seven mobile phases with varying ‘solvent strengths’ and solvent ratios (methanol:acetonitrile 1:0.8–1:3, v/v).

QSRR linear regression equations were generated from the data (Table 2) according to Eq. (1) (Table 3). In all cases, regressions were highly significant ($P \le 0.001$) and the regression coefficients were good ($r < 0.99$).

The regression equations suggest that the organic solvents have different eluting strength for each analyte. To resolve the overlapping pair, IS and $ABZSO_2$, the difference in retention time, $R_{t_{\text{oxf}}\text{-ABZSO}_2}$ was expressed as a function of the fractions of the organic solvents: $R_{t_{\text{oxf}}\text{-ABZSO}_2} = 3.40 - 0.008\text{M} - 0.118\text{A}$. An optimized mobile phase containing methanol–acetonitrile–buffer (22:22:56, v/v/v) was found to separate all the analytes with satisfactory resolution particularly between the IS and $ABZSO_2$ (Table 2). It is well known that a multiple component mobile phase gives better separation efficiency than a binary component mobile phase, as it is convenient to vary ‘solvent strength’ and selectivity simultaneously to obtain desired retention times [24–26]. It is particularly useful in the resolution of overlapping peaks. Interestingly, the overlapping of oxfendazole and $ABZSO_2$ peaks with the acetonitrile–buffer binary eluting systems (25–40% acetonitrile) was predictable using the QSSR equations.

A number of studies [21,27,28] have demonstrated linear correlations between analyte retention parameters and solvent strength, as described by Eq. (8), when using a binary eluent in reversed-phase HPLC systems:

$$\log R_t = \log k_w - \beta X \quad (8)$$

where $\log k_w$ denotes the retention parameter corresponding to pure water as eluent, X is the organic solvent volume fraction in a given mobile phase, β is a parameter which depend on properties of the analyte in a given solvent system, such as total dipole moment, electrical charge and water accessible molecular surface area [28]. In our study we found that retention parameters (using retention times) with multi-component mobile phases could be as described by Eq. (9):

$$\log R_t = \log R_t - \beta_1 X_1 - \beta_2 X_2 \quad (9)$$

where $\log R_{tw}$ is the corresponding retention time with pure buffer as eluent, X_1 and X_2 are the two organic solvents volume fractions in a given ternary mobile phase, β_1 and β_2 are the respective regression parameters. The results suggested that regressions were highly significant ($P < 0.001$) and all the regression coefficients, r, were >0.998. However, in this study the retention times were within a narrow range (4–10 min)

Table 2
Retention times with various mobile phases at a flow rate 1 ml/min

Methanol/acetonitrile (%, v/v)	Observed R_t (min)				Predicted R_t (min)			
	$ABZSO_2NH_2$	RBZ	$ABZSO_2$	IS	$ABZSO_2NH_2$	RBZ	$ABZSO_2$	IS
10/25	4.86	5.53	9.30	9.80	4.85	5.55	9.19	9.72
10/30	3.97	4.37	6.51	6.50	3.94	4.35	6.56	6.50
12/25	4.73	5.46	8.59	9.13	4.77	5.41	8.64	9.18
15/25	4.60	5.20	7.68	8.22	4.64	5.22	7.81	8.38
20/25	4.37	4.88	6.56	7.08	4.42	4.89	6.43	7.04
25/20	5.14	5.79	7.64	8.91	5.11	5.76	7.68	8.92
22/22[a]	4.65	5.48	7.45	8.37	4.88	5.48	7.46	8.44

[a] The optimum mobile phase subjected for validation in this study.

and retention times (Eq. (1)) instead of logarithm retention times were by the linear model (Table 3).

The multiple linear regression method for ternary mobile phase composition optimization described in this study is simple and direct since equations are expressed in retention times. It is useful particularly for dealing with the separation of interfering peaks. The regression equations can be derived from only a few chromatographic experiments in which the two organic eluent concentrations are varied simultaneously. However, it should be noted that the model might be only adequate when the retention times are within a small range, and further application of the regression model (Eq. (1)) to a wider range of solvent strengths or other solvent types has not been investigated. The application of Eq. (9) to the other analytes in ternary mobile phases would also be of interest for further study.

3.3. Extraction efficiency

The extraction recoveries of RBZ at 20, 400 and 800 ng/ml were 96.6 ± 3.9, 97.2 ± 1.9 and $101.6 \pm 0.7\%$, respectively ($n = 3$). The corresponding values for $ABZSO_2$ at the same concentrations were 98.5 ± 1.5, 101.4 ± 0.2 and $102.7 \pm 0.7\%$, respectively ($n = 3$). The internal standard recovery at 500 ng/ml was $97.2 \pm 4.2\%$ ($n = 9$).

The optimal eluting volume for solvent phase extraction was investigated by analysing the eluate every 0.5 ml. It was found that >90% of both RBZ and oxfendazole were eluted in the first 0.5 ml, and <10% in the second 0.5 ml. Therefore, a volume of 1.5 ml methanol was chosen to elute the analytes from the SPE columns.

The results show that the recovery at both low and high concentrations was nearly complete and highly reproducible.

3.4. Validation of the assay

3.4.1. Specificity

Fig. 3 shows the chromatograms of blank sheep plasma spiked with IS (a), plasma spiked with RBZ NH_2, $ABZSO_2NH_2$, RBZ, $ABZSO_2$ and IS (b), and a sample from the pharmacokinetic studies (c). All eluted within 10 min (Table 1). There were no endogenous peaks in the blank plasma that co-eluted with RBZ, $ABZSO_2$ and IS indicating that the method is selective and specific.

3.4.2. Linearity

The assay for RBZ and $ABZSO_2$ were found to be linear in the range of 10–1000 ng/ml ($r > 0.999$ for both RBZ and $ABZSO_2$) with no significant curvature (ANOVA, lack of fit $P > 0.05$).

3.4.3. Accuracy and precision

Table 4 summarises the precision and accuracy of the simultaneous assay of RBZ and $ABZSO_2$ in sheep plasma.

The results suggest satisfactory intra-day and inter-day precision (%R.S.D. between 2.1 and 7.4) and accuracy (98.3–103.2%) for both RBZ and $ABZSO_2$.

3.4.4. Limit of detection and quantification

The LOD and LOQ were estimated as 2.4 and 7.1 ng/ml for RBZ, and 3 and 10 ng/ml for $ABZSO_2$, respectively using Eqs. (5) and (6). Since the lowest standard for RBZ was 10 ng/ml, the LOQ is 10 ng/ml for both analytes.

3.4.5. Stability

Samples stored in mobile phase at 4 °C were stable at least for 1 week. RBZ and $ABZSO_2$ in plasma were stable when stored at −20 °C over three weeks (Table 5). However, there

Table 3
QSRR equations generated from the six mobile phases in Table 2

Regression equations[a] (R_t) (min)	Correlation coefficient (r)	ANOVA (P-value)
$R_{t_{ABZSO_2NH_2}} = 9.84 - 0.0436M - 0.182A$	0.998	0.001
$R_{t_{RBZ}} = 12.2 - 0.0655M - 0.240A$	0.995	<0.001
$R_{t_{ABZSO_2}} = 25.1 - 0.276M - 0.526A$	0.996	0.001
$R_{t_{IS}} = 28.5 - 0.268M - 0.644A$	0.998	<0.001

[a] M and A are the vol.% fractions of methanol and acetonitrile in the mobile phases, respectively.

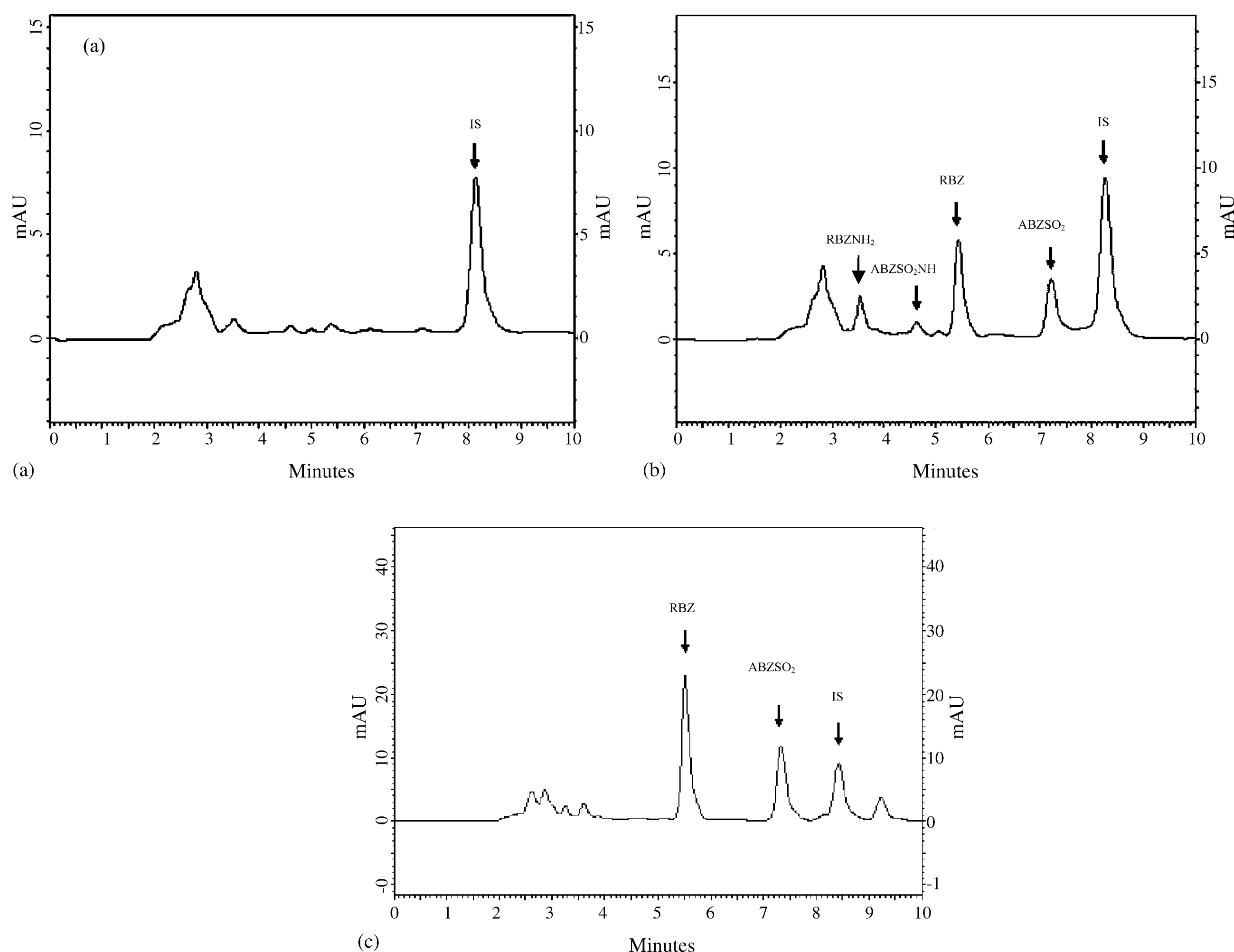

Fig. 3. Chromatograms of (a) blank plasma with IS (500 ng/ml); (b) plasma spiked with RBZ NH_2, $ABZSO_2NH_2$ (unknown concentration), RBZ (200 ng/ml), $ABZSO_2$ (200 ng/ml) and IS (500 ng/ml); and (c) a plasma sample taken 18 h after subcutaneous injection of a ricobendazole formulation in sheep.

Table 4
Intra-day and inter-day precision and accuracy of ricobendazole and ricobendazole sulfone

Nominal conc. (ng/ml)	Conc. found (mean ± S.D.) (ng/ml)	Precision (%R.S.D.)	Accuracy (percentage of nominal)
(RBZ) intra-day ($n = 5$)			
20	19.7 ± 1.1	5.5	98.3 ± 5.4
400	403.8 ± 9.5	2.4	101.0 ± 2.4
800	808.6 ± 35.7	4.4	100.5 ± 4.5
inter-day (n=5)			
20	20.2 ± 1.2	5.9	101.0 ± 6.0
400	409.4 ± 10.1	2.5	102.4 ± 2.5
800	806.3 ± 17.1	2.1	100.8 ± 2.1
$ABZSO_2$ intra-day ($n = 5$)			
20	20.4 ± 1.5	7.4	102.0 ± 4.1
400	398.7 ± 20.0	5.0	99.7 ± 4.6
800	798.8 ± 46.7	5.9	99.8 ± 4.1
inter-day (n=5)			
20	20.6 ± 1.3	6.4	103.2 ± 6.6
400	406.4 ± 9.4	2.3	101.6 ± 2.3
800	801.9 ± 22.0	2.7	100.2 ± 2.7

Accuracy = found/nominal × 100%.

Table 5
Stability of RBZ and albendazole sulphone in sheep plasma ($n = 3$)

Storage condition	Recovery (%)	RBZ	$ABZSO_2$
3 weeks at −20 °C	Mean	99.8	100.1
	S.D.	8.6	8.6
	R.S.D.	8.6	8.6
Four freeze-thaw cycles	Mean	94.0	94.6
	S.D.	1.7	2.4
	R.S.D.	1.8	2.5

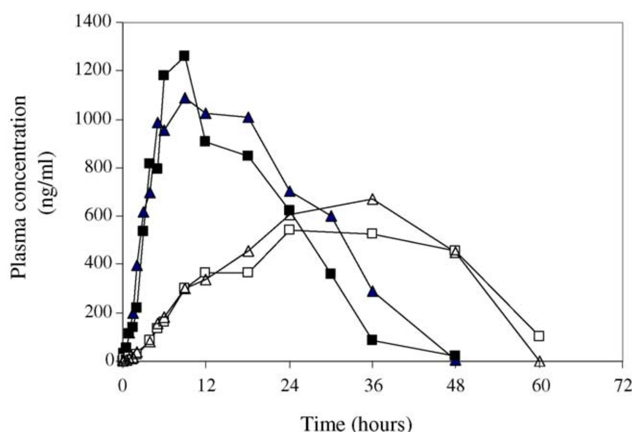

Fig. 4. Typical plasma concentration–time profiles of RBZ (solid symbols) and $ABZSO_2$ (open symbols). RBZ was administrated subcutaneously to sheep as an aqueous low pH solution (triangles) or a microemulsion (squares).

was some degradation in samples which were subjected to four freeze-thaw cycles although this was not statistically significant ($P > 0.05$).

3.4.6. Application of the assay to pharmacokinetic studies in sheep

The assay has been applied for analysis of plasma of sheep administrated RBZ subcutaneously in various formulations (Fig. 4). RBZ and $ABZSO_2$ were simultaneously quantified, whereas both $RBZNH_2$ and $ABZSO_2NH_2$ remained at low levels or were not visible in the chromatograms.

4. Conclusions

In this study a rapid and reliable reversed-phase HPLC method with UV detection, involving solid phased extraction, has been developed and optimized, for simultaneous determination of RBZ and its main metabolite $ABZSO_2$ in sheep plasma. This method is more specific and simpler than existing methods because it uses isocratic elution versus gradient, and is free of interference from the other potential metabolites of RBZ, namely $RBZNH_2$, $ABZSO_2NH_2$. The composition of the multiple component mobile phase was optimized to obtain the desired resolutions by multiple linear regression, and the internal standard was selected according to QSRRs enabling use of an isocratic elution.

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References

[1] Q.A. McKellar, E.W. Scott, J. Vet. Pharmacol. Ther. 13 (1990) 223–247.
[2] P. Galtier, M. Alvinerie, J.L. Steimer, P. Francheteau, Y. Plusquellec, G. Houin, J. Pharm. Sci. 80 (1991) 3–10.
[3] S.E. Marriner, J.A. Bogan, Am. J. Vet. Res. 41 (1980) 1126–1129.
[4] C.E. Lanusse, G.L. Virkel, S.F. Sanchez, L.I. Alvarez, A. Lifschitz, F. Imperiale, A. Monfrinotti, Vet. Sci. 65 (1998) 5–10.
[5] C. Cristofol, G. Virkel, L. Alvarez, M. Arboix, C.E. Lanusse, Biopharm. Drug Dispos. 21 (2000) 303–311.
[6] E.A. Formentini, O.N. Mestorino, E.L. Marino, J.O. Errecalde, J. Vet. Pharmacol. Ther. 24 (2001) 199–202.
[7] The European agency for the evaluation of medicinal products, www.emea.eu.int/pdfs/vet/mrls/055599en.pdf.EMEA/MRL/555/99-FINAL, 1999.
[8] A. Mirfazaeliana, S. Dadashzadehb, M.R. Rouini, J. Pharm. Biomed. Anal. 30 (2002) 1249–1254.
[9] T. Bernard, M.M. Joachim, Hydrolysis in drug and prodrug metabolism: chemistry, biochemistry and enzymology, Zurich, Verlag Helvetica Chimica Acta (2003) 81–162.
[10] D. Kitzman, K.J. Cheng, L. Fleckenstein, J. Pharm. Biomed. Anal. 30 (2002) 801–813.
[11] C. Cristofol, G. Virkel, L. Alvarez, S. Sanchez, M. Arboix, C. Lanusse, J. Vet. Pharmacol. Ther. 24 (2001) 117–124.
[12] V.L. Lanchote, M.P. Marques, O.M. Takayanagui, R. de Carvalho, F.O. Paias, P.S. Bonato, J. Chromatogr. B 709 (1998) 273–279.
[13] P.S. Bonato, V.L. Lanchote, O.M. Takayanagui, J. Chromatogr. B 783 (2003) 237–245.
[14] P.A. Redondo, A.I. Alvarez, J.L. Garcia, C. Villaverde, J.G. Prieto, Biopharm. Drug Dispos. 19 (1998) 65–70.
[15] W.M. Meylan, P.H. Howard, J. Pharm. Sci. 84 (1995) 83–92.
[16] IUPAC. www.iupac.org/goldbook/P04465.pdf. IUPAC Compendium of Chemical Terminology, second ed., 1997.
[17] N.R. Draper, H. Smith, Applied Regression Analysis, third ed., Wiley, New York, 1998, pp. 47–50.
[18] ICH guideline Q2B, Validation of Analytical Procedures: Methodology, November 1996.
[19] M. Ahmed Al-Haj, R. Kaliszan, B. Buszewski, J. Chromatogr. Sci. 39 (2001) 29–38.
[20] T. Baczek, R. Kaliszan, J. Chromatogr. A 987 (2003) 29–37.
[21] G. Cimpan, F. Irimie, S. Gocan, H.A. Claessens, J. Chromatogr. B 714 (1998) 247–261.
[22] N.C. Dias, M.I. Nawas, C.F. Poole, Analyst 128 (2003) 427–433.
[23] Z. Wu, M. Razzak, I.G. Tucker, N.J. Medlicott, J. Pharm. Sci., 94 (2005) 983–993.
[24] M. Gazdag, G. Szepesi, E. Szeleczki, J. Chromatogr. 454 (1988) 83–94.
[25] G. D'Agostino, F. Mitchell, L. Castagnetta, M.J. O'Hare, J. Chromatogr. A 305 (1984) 13–26.
[26] E.H. Jansen, R. Both-Miedema, R.H. van den Berg, J. Chromatogr. 489 (1989) 57–64.
[27] J.C. Ford, J. Ko, J. Chromatogr. A 727 (1996) 1–11.
[28] T. Baczek, R. Kaliszan, J. Chromatogr. A 962 (2002) 41–55.