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Quantitative determination of albendazole metabolites in sheep spermatozoa and seminal plasma by liquid chromatographic analysis with fluorescence detection

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Abstract

A new analytical method for the simultaneous quantitative determination of albendazole metabolites in sheep spermatozoa and seminal plasma at levels down to 46.5 ng/mL for albendazole sulphoxide (ABZ-SO), 7.5 ng/mL for albendazole sulphone (ABZ-SO_2) and 12 ng/mL for albendazole 2-aminosulphone (ABZ-SO_2NH_2) has been developed. Analytes were extracted from alkalinized samples with ethyl acetate. Separation was carried out on a C_{18} column in the presence of tetra-*n*-butylammonium (TBA) hydrogen sulphate and octanesulphonate sodium (OCT), as ion-pair agents. Fluorometric detection was performed with excitation and emission wavelengths set at 290 and 320 nm, respectively. Accuracy data showed overall recoveries (±S.E.M.) of $83.1 \pm 1.2\%$ for ABZ-SO, $98.8 \pm 0.6\%$ for ABZ-SO_2 and $85.3 \pm 0.7\%$ for ABZ-SO_2NH_2, in spermatozoa. Respective values in seminal plasma were $88.0 \pm 0.9\%$, $97.7 \pm 0.5\%$ and $93.2 \pm 1.7\%$. Precision data suggested coefficient of variation (CV%) values lower than 5.9% for spermatozoa and 3.8% for seminal plasma. The method was successfully applied for the determination of the three albendazole metabolites in semen samples collected from rams that had been orally administered albendazole.

Keywords: Spermatozoa; Seminal plasma; Albendazole sulphoxide; Albendazole sulphone; Albendazole 2-aminosulphone

1. Introduction

Albendazole, methyl[5-(propylthio)-1*H*-benzimidazol-2-yl] carbamate, is a widely used anthelmintic in both human and veterinary medicine, active against trematode, cestode and nematode parasites [1,2].

Albendazole, as the parent compound, is undetected [3–8] or present at extremely low concentrations [4,9] in blood plasma after oral administration in various animal species and man, due to an extensive first-pass metabolism occurring in the enterocytes and liver cells. Albendazole sulphoxide (ABZ-SO), present as mixture of two enantiomers, *R* (+) and *S* (−) [9–11], albendazole sulphone (ABZ-SO_2) and albendazole 2-aminosulphone (ABZ-SO_2NH_2) are the sequential products of this metabolism [12–15], the first one being considered biologically active [16].

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Several HPLC methods have been published for the determination of albendazole metabolites in foodstuff, animal tissues [17–20] and in human or animal blood plasma or serum [9–11,21–25]. However, to our knowledge, there are no data concerning the presence of albendazole metabolites in the ejaculate (semen—spermatozoa and seminal plasma).

Albendazole and ABZ-SO have been reported to possess teratogenic [26] and possibly mutagenic properties, although the latter has not been entirely elucidated [27]. Especially for ABZ-SO, it has been demonstrated that it is transported transplacentaly [28,29] and exerts its teratogenic effect on the fetus [26]. Respective effects of albendazole metabolites on the male reproductive system have also been studied in a few papers. Otubanjo and Mosuro [27] report that albendazole administered to mice failed to yield significant increase in the frequency of sperm head abnormalities. In accordance, Berndtson et al. [30] discovered no effect of albendazole administration on the reproductive function of bulls. A serious drawback of these observations is that they were not correlated with corresponding levels of albendazole metabolites in whole ejaculate or semen fractions.

The possible presence of albendazole metabolites, especially ABZ-SO, in spermatozoa and/or seminal plasma and the impact this could have on fertility, led to the consideration of developing an HPLC method for the determination of albendazole metabolites in the former media and this was the purpose of the present study.

2. Experimental

2.1. Chemicals

Standard albendazole sulphoxide (purity 97%), albendazole sulphone (purity 98.8%) and albendazole 2-aminosulphone (purity 99.7%) were kindly donated by GlaxoSmithKline (Worthing, UK).

Trizma hydrochloride buffer and Triton X-100 were from Sigma–Aldrich Chemie (Steinheim, Germany). Acetonitrile (gradient-grade), ortho-phosphoric acid (85%, m/m), sodium hydroxide pellets and tetra-*n*-butylammonium hydrogen sulphate, were from Merck (Darmstadt, Germany). Ethyl acetate of analytical grade was from LabScan Ltd. (Dublin, Ireland). Octanesulphonate sodium salt was from Fluka Chemie (Buchs, Germany). HPLC-grade water was produced, using a Milli-Q (Millipore, Bedford, MA, USA) purification system.

2.2. Biological materials

Control ram semen was collected from healthy, young adult animals by ejaculation into an artificial vagina. The semen was immediately centrifuged at $3000 \times g$ for 20 min at 4 °C. The supernatant seminal plasma was transferred into 1.5 mL micro-test tubes (Eppendorf AG, Hamburg, Germany) and was stored at −30 °C, pending analysis. The sediment, consisting of spermatozoa, was washed three times with normal saline and was extracted with 0.1 M Trizma hydrochloride buffer containing 1% Triton X-100, pH 7.4 [31]. Final concentration was 5×10^8 spermatozoa/mL. Aliquots of 1 mL were transferred into glass tubes and were also stored at −30 °C.

2.3. Instrumentation

The HPLC 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, all from Shimadzu Corp. (Kyoto, Japan). The controller unit was linked to a PC and chromatographic system operation was controlled by Class-LC10 software (version 1.41, Shimadzu).

A Model Genie-2 Vortex mixer (Scientific Industries Inc., Bohemia, NY, USA), a Model UCI-50 ultrasonic bath (R. Espinar, S.L., Barcelona, Spain), 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 for sample preparation. Standard substances were weighed on a Model AX-105 analytical balance (Mettler Toledo Inc., Greifensee, Switzerland).

2.4. Chromatographic conditions

A mobile phase, mixture of acetonitrile–0.01 M ortho-phosphoric acid solution (25:75 v/v), containing 20 mM octanesulphonate sodium (OCT) and

2.5 mM tetra-*n*-butylammonium (TBA) hydrogen sulphate was used to elute the samples through 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 column packing. The pH of the ortho-phosphoric acid solution, after the addition of the ion-pair agents, was adjusted to 3 prior to the addition of acetonitrile. The mobile phase was filtered through a 0.20 μm Nylon 47 mm filter (Alltech Ass. Inc., Deerfield, IL, USA) and was degassed by passing helium for 10 min before use. Reproducible capacity factors (k') could be achieved after passage of at least 150 mL mobile phase through the column before daily analysis.

The LC system flow rate was 1 mL/min and elution was performed at 40 °C. Injection volume was 20 μL. Fluorescence detection was used for the determination of albendazole metabolites with excitation and emission wavelengths set at 290 and 320 nm, respectively. The sensitivity of the detector was set at "high" and the response time was 1.5 s.

2.5. Spermatozoa pretreatment

All frozen spermatozoa samples were allowed to thaw for 1 h at room temperature. They were vigorously vortexed for 5 min and sonicated for 10 min. By the end of the sonication, at least 95% of spermatozoa were disrupted and their content was diluted (extracted) in the liquid medium, as assessed by microscopical observation.

2.6. Stock and standard solutions

Individual stock solutions, at the nominal concentration of 1 mg/mL of ABZ-SO, ABZ-SO_2 and 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. Impurity of the standard substances was measured and was found to be almost in accordance with the values referred by the manufacturer. Corrections to the theoretical amounts added in the stock solutions were performed and actual concentrations were obtained. Two mixed intermediate standard solutions containing, respectively, 1247 and 14 050 ng/mL ABZ-SO, 375 and 16 300 ng/mL ABZ-SO_2, and 485 and 6075 ng/mL ABZ-SO_2NH_2 were prepared in 10 mL volumetric flasks by mixing aliquots of stock standard solutions and diluting with acetonitrile. All above solutions were stored at −15 °C and flasks were protected from light throughout analysis with the use of aluminium foil.

Eight mixed calibration standard solutions (used for the construction of the calibration curves) in the range of 49.9–2248 ng/mL for ABZ-SO, 15–2608 ng/mL for ABZ-SO_2 and 19.4–972 ng/mL for ABZ-SO_2NH_2 were prepared for the determination of concentrations in the seminal plasma samples by transferring appropriate aliquots (10–100 μL) of the mixed intermediate solutions in glass tubes, evaporating to dryness under a gentle stream of N_2 at 40 °C, and reconstituting with 0.5 mL of mobile phase. This range of concentrations for albendazole metabolites was not absolutely suitable for their determination in spermatozoa extract samples, as was confirmed in a pilot experiment employing real samples. Therefore, another series of three mixed intermediate standard solutions containing, respectively, 580, 1163 and 12 975 ng/mL ABZ-SO, 50, 187.5 and 13 125 ng/mL ABZ-SO_2, and 120, 300 and 6075 ng/mL ABZ-SO_2NH_2 were prepared for this purpose. The range of concentrations for the mixed calibration standard solutions was 23.2–519 ng/mL for ABZ-SO, 2–525 ng/mL for ABZ-SO_2 and 4.8–243 ng/mL for ABZ-SO_2NH_2. Mixed intermediate solutions were prepared weekly, while mixed calibration (working) standard solutions, used for the calibration curves, were prepared daily.

2.7. Sample extraction and cleanup

The procedures for sample extraction and cleanup were similar for spermatozoa extracts and seminal plasma. Into a 1 mL (spermatozoa extract) or 0.25 mL (seminal plasma) aliquot of sample, 100 μL (spermatozoa extract) or 50 μL (seminal plasma) of a 0.4 N sodium hydroxide solution and 7 mL of ethyl acetate were added. After vortex-mixing at high speed for 60 s and centrifugation at 3500 × *g* for 10 min, 6 mL of the clear supernatant (containing the investigational analytes) were transferred into another tube and a water volume of 1 mL was added. The sample was vortexed

again for 15 s and centrifuged at 1000 × *g* for 2 min. A 5 mL aliquot of the top organic layer was transferred into another tube and was evaporated to dryness at 40 °C, under a nitrogen stream. The dried residue was reconstituted in 0.5 mL of mobile phase and was vortex-mixed for 15 s. After transferring the samples to autosampler inserts, 20 μL were injected in the LC system.

2.8. Validation control samples

Validation control (VC) samples were prepared in blank media (spermatozoa and seminal plasma) at various concentrations by adding aliquots of the mixed intermediate solutions. For spermatozoa extract samples, the fortification levels were 46.5, 69.7, 139.5 and 348.5 mg/mL for ABZ-SO, 7.5, 11.2, 22.5 and 56.2 ng/mL for ABZ-SO_2 and 12, 18, 36 and 93.7 ng/mL for ABZ-SO_2NH_2. Fortification levels concerning seminal plasma were 49.9, 562, 1686 and 2248 ng/mL for ABZ-SO, 15, 652, 1956 and 2608 ng/mL for ABZ-SO_2 and finally, 19.4, 243, 729 and 972 ng/mL for ABZ-SO_2NH_2. Depending on the phase of the validation study, the samples were either stored at −30 °C (evaluation of stability) or preceded for analysis immediately (selectivity, sensitivity, accuracy and precision determination).

2.9. Determination

Calibration curves covering the range mentioned in Section 2.6 were generated daily, by using the mixed calibration standard solutions. Determination of the equations best describing the curves was performed by the least-squares method, after plotting the recorded peak heights (μV) versus the corresponding concentration of the analytes injected (ng/20 μL injected). Quantification of albendazole metabolites in an unknown sample was achieved by back-referring the displayed peak heights to the appropriate corresponding calibration curve. The equation $y = ax + b$ (where x stands for ng of analyte per volume injected, y for peak height, a for slope and b for intercept) was used to obtain the value of the independent variable x which was further multiplied by the appropriate dilution and recovery factor.

3. Results and discussion

3.1. Chromatography and sample pretreatment

The chemical structures of ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 are shown in Fig. 1. The nature of the molecular structure and chemical characteristics of the investigational analytes (weak bases) led to the assumption that an adsorptive interaction between the compounds and the free silanol groups present on the surface of the stationary phase should be anticipated. An effort was made to suppress the ionization of the latter by using an acidic mobile phase (containing 0.01 M ortho-phosphoric acid) while simultaneously attempting to "mask" the negative charge of the residual silanols with the addition of a positively-charged ion-pair agent (TBA, concentration 2.5 mM). Unfortunately, the most polar metabolite, ABZ-SO_2NH_2, appeared very early in the chromatograms obtained from standard solutions, almost independently of the percentage of the organic solvent (acetonitrile) in the mobile phase [17]. The co-addition of a negatively-charged ion-pair agent (OCT, concen-

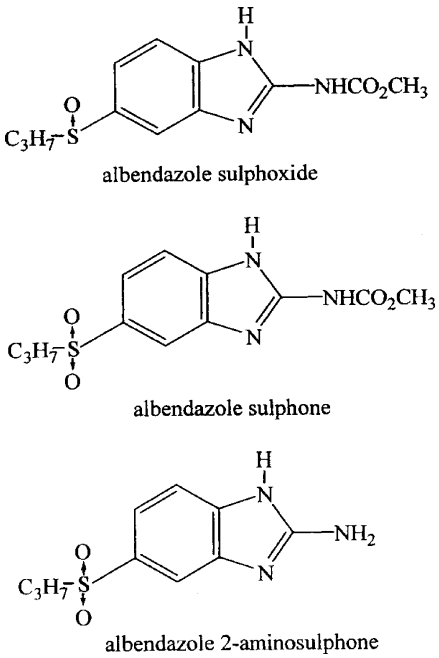

Fig. 1. Chemical structures of albendazole sulphoxide, albendazole sulphone and albendazole 2-aminosulphone.

tration 20 mM) showed that the hydrophobicity of ABZ-SO_2NH_2 was remarkably increased, possibly due to the formation of a larger complex after binding of OCT to the primary amine group, present in the metabolite molecule. As a result of the addition of both ion-pair agents (TBA and OCT) into the mobile phase, an excellent baseline resolution of all chromatographic peaks was achieved. Furthermore, by increasing column temperature up to 40 °C, the shape of the peaks was improved and this was most likely due to a better transfer of the analytes between the stationary and mobile phase. In addition, after testing both, a UV and a RF detector, it was concluded that the maximum response (and therefore, sensitivity) was clearly offered by fluorescence detection, especially for ABZ-SO_2 and ABZ-SO_2NH_2 metabolites. Under the established conditions, the peaks of ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 are adequately resolved with respective retention times 7.4, 10.5 and 12.4 min. A representative chromatogram of a standard solution containing the three metabolites is presented in Fig. 2.

Early trials concerning the partitioning of albendazole metabolites under alkaline or acidic conditions into organic solvents showed that they were best extracted when the aqueous phase was alkalinized. A sodium hydroxide solution was selected for this purpose, as mentioned in Section 2.7. However, by trial and error, it was concluded that the amount of sodium hydroxide added should differ between spermatozoa extract and seminal plasma. Ethyl acetate was selected for the extraction of albendazole metabolites from spermatozoa extract and seminal plasma, because of the high solvating power it displayed for these weak basic compounds. Data concerning the extraction efficiency, in terms of analyte recovery are mentioned in Section 3.2.4. Chromatograms of spiked spermatozoa extract and seminal plasma samples are presented in Figs. 3(A) and 4(A), respectively.

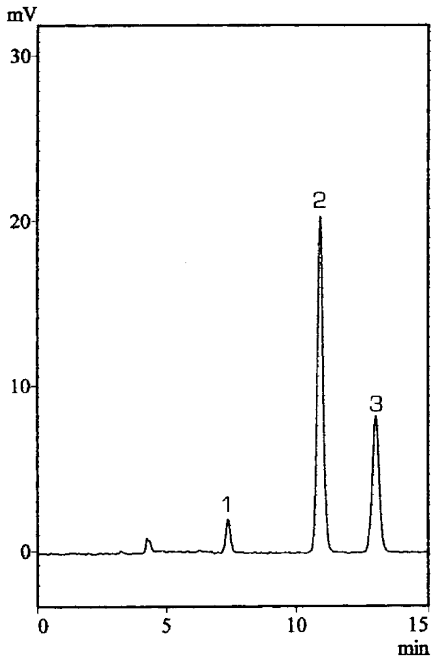

Fig. 2. Chromatogram of a calibration standard solution containing 4.65 ng/20 μL ABZ-SO (peak 1), 0.75 ng/20 μL ABZ-SO_2 (peak 2) and 1.2 ng/20 μL ABZ-SO_2NH_2 (peak 3). Stationary phase: Nucleosil 100-5; mobile phase: acetonitrile–0.01 M phosphoric acid solution (25:75 v/v) containing 20 mM octanesulphonate sodium and 2.5 mM tetra-*n*-butylammonium hydrogen sulphate; column temperature: 40 °C; flow rate: 1 mL/min; excitation wavelength: 290 nm; emission wavelength: 320 nm; injection volume: 20 μL.

3.2. Method validation

The method was validated for use, in a 3-day validation study, with respect to linearity, selectivity, sensitivity (limit of detection and limit of quantification), accuracy, precision, recovery, stability and applicability aspects. It is reminded that concentrations (ng/mL) of albendazole metabolites in spermatozoa extract samples can be back-referred as amount (ng) contained in 5×10^8 spermatozoa.

3.2.1. Calibration and linearity

The linearity of the detector response for the test compounds was evaluated by injecting mixed calibration standard solutions of various concentrations covering the working range of the assay. Data from the calibration curves obtained in the three days of the validation study were analysed. The following equations were found to best describe the calibration curves for all analytes (y represents peak height (μV) and x the quantity (ng) of each compound per 20 μL injected): (a) *Spermatozoa extract*: $y = 297.14(\pm 4.26)x - 16.55$, $r^2 = 0.995$ for ABZ-SO, $y = 19116.87(\pm 374.03)x - 641.31$, $r^2 = 0.998$ for ABZ-SO_2 and $y = 4765.22(\pm 60.78)x - 151.15$, $r^2 = 0.999$ for ABZ-SO_2NH_2. (b) *Seminal plasma*: $y =$

347.40(±0.97)x − 21.78, r^2 = 0.999 for ABZ-SO, y = 22817(±150.12)x + 999.65, r^2 = 0.999 for ABZ-SO_2 and y = 5865.62(±55.85)x + 130.7, r^2 = 0.999 for ABZ-SO_2NH_2. Numbers in parentheses represent S.E.M., as obtained from ANOVA tables. All P-values for intercepts were >0.05.

3.2.2. Selectivity

The selectivity of the method was demonstrated by analyzing control blank spermatozoa extract and seminal plasma samples, collected from eight animals. Chromatograms obtained showed no interferences between albendazole metabolites peaks and peaks attributable to sample endogenous compounds and/or chemicals used for sample cleanup and chromatography (Figs. 3(B) and 4(B)).

3.2.3. Limits of detection and limits of quantification

The limits of detection (LOD) of the method were defined using the signal-to-noise ratio 3:1 rule and were set at 20 ng/mL for ABZ-SO, 1 ng/mL for ABZ-SO_2 and 2 ng/mL for ABZ-SO_2NH_2, in both investigational media.

The method of analysis was evaluated at the limits of quantification (LOQ) of the three metabolites, generally defined as the lowest concentration of an analyte in a sample with precision (expressed as coefficient of variation, CV%) and accuracy (percentage of bias) values within the limits of 80–120%. The limits of quantification in the present method were defined as the lowest in concentration validated fortified samples.

During the 3 days of the validation study, 21 (n = 21) replicates of spiked spermatozoa and seminal plasma samples were analysed. In the case of spermatozoa samples LOQs were set at 46.5 ng/mL for ABZ-SO (CV 4.6%, bias 1.8%), 7.5 ng/mL for ABZ-SO_2 (CV 2.6%, bias 1.7%), and 12 ng/mL for ABZ-SO_2NH_2 (CV 5.9%, bias 1.8%). Respective values for seminal plasma were 49.9 ng/mL (CV 3.8%, bias 4.8%), 15 ng/mL (CV 3.4%, bias 0.6%), and 19.4 ng/mL (CV 2.8%, bias 7.2%).

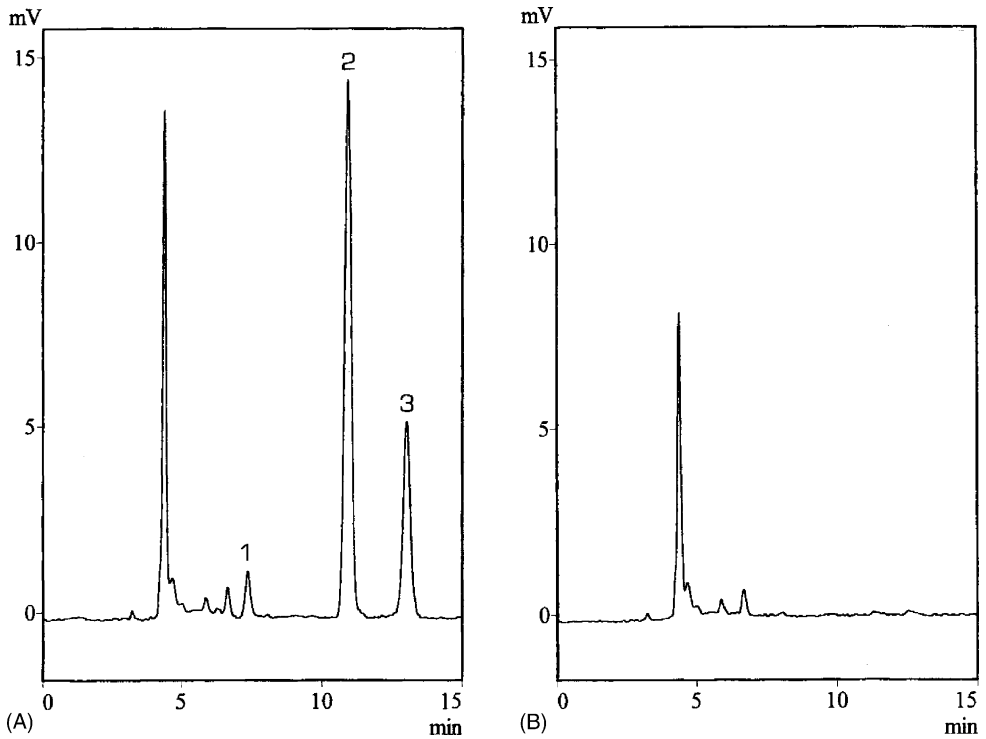

Fig. 3. Chromatograms obtained from (A) a spermatozoa extract sample spiked with 348.5 ng/mL ABZ-SO, 56.2 ng/mL ABZ-SO_2 and 93.7 ng/mL ABZ-SO_2NH_2 and (B) a drug-free spermatozoa extract sample. Chromatographic conditions and peak identification as in Fig. 2.

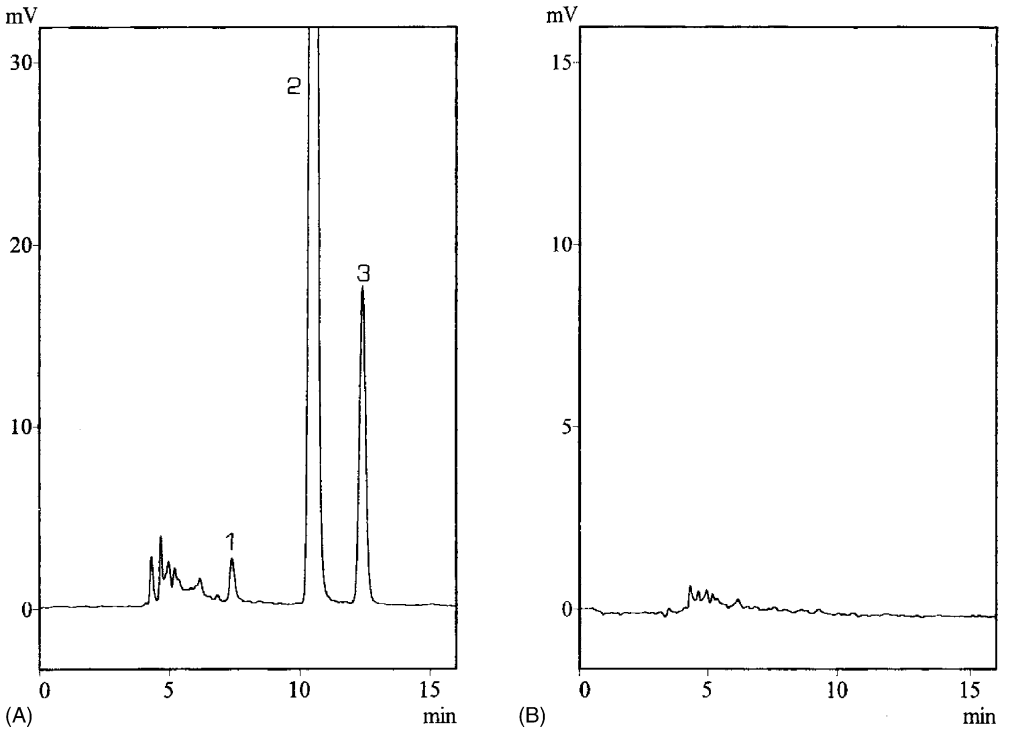

Fig. 4. Chromatograms obtained from (A) a seminal plasma sample spiked with 562 ng/mL ABZ-SO, 652 ng/mL ABZ-SO_2 and 243 ng/mL ABZ-SO_2NH_2 and (B) a drug-free seminal plasma sample. Chromatographic conditions and peak identification as in Fig. 2.

3.2.4. Accuracy and precision

Since the calibration curves were prepared in mobile phase and not in a biological matrix, the accuracy of the method was measured in terms of recovery efficiency. Control blank samples were fortified at three (spermatozoa: 46.5, 139.5 and 348.5 ng/mL for ABZ-SO, 7.5, 22.5 and 56.2 ng/mL for ABZ-SO_2, and 12, 36 and 93.7 ng/mL for ABZ-SO_2NH_2) or four (seminal plasma: 49.9, 562, 1686 and 2248 ng/mL for ABZ-SO, 15, 652, 1956 and 2608 ng/mL for ABZ-SO_2, and 19.4, 243, 729 and 972 ng/mL for ABZ-SO_2NH_2) concentration levels, for each metabolite, by adding appropriate aliquots of the mixed intermediate standard solutions. The precision of the method, measured as the coefficient of variation (CV%) was evaluated at the same fortification levels.

Data concerning the accuracy and precision of the method are presented in Tables 1–4. Regression of the data obtained from spermatozoa extract samples revealed overall mean recoveries (±S.E.M.) 83.1 ± 1.2% for ABZ-SO, 98.8 ± 0.6% for ABZ-SO_2, and 85.3 ± 0.7% for ABZ-SO_2NH_2. Respective values in seminal plasma samples were 88.0 ± 0.9, 97.7 ± 0.5, and 93.2 ± 1.7%. Precision values all fell within acceptable criteria (<15%). Maximum CV

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

Analyte added (ng/mL)	Concentration found (ng/mL ± S.D.)[a]	Mean recovery (% ± S.D.)
ABZ-SO		
46.5	44.4 ± 1.7	95.4 ± 4.0
139.5	130.7 ± 5.6	93.7 ± 4.3
348.5	297.3 ± 8.3	85.3 ± 2.2
ABZ-SO_2		
7.5	7.5 ± 0.2	100.3 ± 3.0
22.5	23.0 ± 0.7	102.2 ± 3.2
56.2	55.8 ± 0.3	99.3 ± 0.6
ABZ-SO_2NH_2		
12	11.6 ± 0.5	97.6 ± 3.7
36	33.0 ± 1.0	91.9 ± 2.7
93.7	81.6 ± 1.4	86.9 ± 1.4

[a] Seven replicates.

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

Analyte added (ng/mL)	Overall mean concentration found (ng/mL ± S.D.)[a]	CV (%)
ABZ-SO		
46.5	45.6 ± 2.1	4.6
139.5	130.9 ± 4.8	3.7
348.5	283.9 ± 9.6	3.4
ABZ-SO_2		
7.5	7.6 ± 0.2	2.6
22.5	23.2 ± 0.8	3.4
56.2	55.2 ± 1.5	2.7
ABZ-SO_2NH_2		
12	11.8 ± 0.7	5.9
36	33.4 ± 1.3	3.9
93.7	80.6 ± 2.3	2.8

[a] Seven replicates per day.

values were 5.9% in spermatozoa and 3.8% in seminal plasma.

3.2.5. Storage stability

The stability of albendazole metabolites in samples stored under different conditions was evaluated by performing three experiments. For the first experiment, fortified spermatozoa extract (139.5 ng/mL ABZ-SO, 22.5 ng/mL ABZ-SO_2 and 36 ng/mL ABZ-SO_2NH_2) and seminal plasma (562 ng/mL ABZ-SO, 652 ng/mL ABZ-SO_2 and 243 ng/mL ABZ-SO_2NH_2) samples were extracted, transferred into autosampler vials and analysed before and after a 24 h waiting time (room temperature) in the LC autosampler. Assessment of sample stability would guarantee reliable quantification during the main validation phase and routine analysis. For the purpose of the second experiment, the stability of the investigational analytes (spermatozoa samples: 69.7 and 139.5 ng/mL ABZ-SO, 11.2 and 22.5 ng/mL ABZ-SO_2, 18 and 36 ng/mL ABZ-SO_2NH_2—seminal plasma: 562 and 2248 ng/mL ABZ-SO, 652 and 2608 ng/mL ABZ-SO_2, 243 and 972 ng/mL ABZ-SO_2NH_2) was evaluated after 4 months storage at −30 °C, while for the third experiment, a three-freeze–thaw cycle was performed on the tested samples (139.5 ng/mL ABZ-SO, 22.5 ng/mL ABZ-SO_2 and 36 ng/mL ABZ-SO_2NH_2 in spermatozoa, 562 ng/mL ABZ-SO, 652 ng/mL ABZ-SO_2 and 243 ng/mL ABZ-SO_2NH_2 in seminal plasma). Data are presented in Tables 5 and 6. In all cases, no statistically significant differences (paired *t*-test) were observed and samples were considered stable under all tested conditions.

Table 3
Accuracy data of the analysis of ram seminal plasma spiked with ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2

Analyte added (ng/mL)	Concentration found (ng/mL ± S.D.)[a]	Mean recovery (% ± S.D.)
ABZ-SO		
49.9	46.8 ± 1.8	93.7 ± 3.7
562	530.1 ± 12.5	94.3 ± 2.2
1686	1493.4 ± 18.0	88.6 ± 1.1
2248	1994.5 ± 59.5	88.7 ± 2.7
ABZ-SO_2		
15	14.9 ± 0.5	99.8 ± 3.0
652	628.9 ± 17.6	96.5 ± 2.7
1956	1934.1 ± 29.5	98.9 ± 1.5
2608	2533.4 ± 34.3	97.1 ± 1.3
ABZ-SO_2NH_2		
19.4	17.9 ± 0.5	91.77 ± 2.4
243	227.3 ± 5.8	93.55 ± 2.4
729	646.2 ± 15.8	88.64 ± 2.2
972	883.0 ± 18.1	90.84 ± 1.9

[a] Seven replicates.

Table 4
Precision data of the analysis of sheep seminal plasma spiked with ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 on three successive days

Analyte added (ng/mL)	Overall mean concentration found (ng/mL ± S.D.)[a]	CV (%)
ABZ-SO		
49.9	46.8 ± 1.8	3.8
562	530.1 ± 12.5	2.4
1686	1493.3 ± 18.0	1.2
2248	1994.5 ± 59.5	3.0
ABZ-SO_2		
15	14.9 ± 0.5	3.4
652	628.9 ± 17.6	2.8
1956	1934.1 ± 29.5	1.5
2608	2533.4 ± 34.3	1.4
ABZ-SO_2NH_2		
19.4	17.8 ± 0.5	2.8
243	227.3 ± 5.7	2.5
729	646.2 ± 15.8	2.4
972	883.0 ± 18.1	2.0

[a] Seven replicates per day.

Table 5
Stability of albendazole metabolites in spiked spermatozoa 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 (ng/mL ± S.D.)[a]	
		0 h	24 h
Autosampler			
ABZ-SO	139.5	133.8 ± 2.3	133.3 ± 1.4
ABZ-SO_2	22.5	22.1 ± 0.3	22.2 ± 0.4
ABZ-SO_2NH_2	36	33.9 ± 1.4	33.9 ± 2.0
		Mean concentration found (ng/mL ± S.D.)[a]	
		0 months	4 months
Storage at −30 °C			
ABZ-SO	69.7	63.4 ± 2.2	62.3 ± 4.4
	139.5	123.4 ± 5.9	125.2 ± 3.9
ABZ-SO_2	11.2	11.2 ± 0.2	11.2 ± 0.2
	22.5	22.7 ± 0.5	22.5 ± 0.5
ABZ-SO_2NH_2	18	16.7 ± 0.9	16.7 ± 0.6
	36	32.8 ± 1.1	33.3 ± 0.6
		Mean concentration found (ng/mL ± S.D.)[a]	
		Before	After
Three-freeze–thaw cycle			
ABZ-SO	139.5	119.9 ± 5.2	120.3 ± 5.5
ABZ-SO_2	22.5	22.1 ± 0.6	21.9 ± 0.6
ABZ-SO_2NH_2	36	32.1 ± 1.9	32.8 ± 1.1

[a] Six replicates.

Table 6
Stability of albendazole metabolites in spiked seminal 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 (ng/mL ± S.D.)[a]	
		0 h	24 h
Autosampler			
ABZ-SO	562	534.9 ± 15.6	542.0 ± 20.4
ABZ-SO_2	652	638.4 ± 12.3	622.4 ± 11.4
ABZ-SO_2NH_2	243	225.6 ± 2.7	223.0 ± 3.3
		Mean concentration found (ng/mL ± S.D.)[a]	
		0 months	4 months
Storage at −30 °C			
ABZ-SO	562	537.4 ± 11.5	540.6 ± 22.8
	2248	2081.3 ± 52.3	2107.2 ± 46.5
ABZ-SO_2	652	617.8 ± 10.7	625.4 ± 24.6
	2608	2531.1 ± 40.7	2516.5 ± 21.7
ABZ-SO_2NH_2	243	227.7 ± 4.7	226.8 ± 5.7
	972	883.5 ± 25.4	877.6 ± 20.7
		Mean concentration found (ng/mL ± S.D.)[a]	
		Before	After
Three-freeze–thaw cycle			
ABZ-SO	562	536.6 ± 10.7	527.9 ± 13.5
ABZ-SO_2	652	635.2 ± 17.7	634.1 ± 10.4
ABZ-SO_2NH_2	243	228.8 ± 8.8	227.1 ± 5.6

[a] Six replicates.

3.3. Applicability

To evaluate the applicability of the method in real samples and for the purpose of a pharmacokinetic study, albendazole was administered per os to four rams, at the dose of 11.5 mg/kg body weight. Sperm was collected at fixed time intervals during a 72 h period after drug administration. Concentration data

Table 7
Mean levels of albendazole metabolites in spermatozoa (1 mL = 5×10^8 spermatozoa) and seminal plasma of four rams, after a single per os administration of albendazole, at the dose of 11.5 mg/kg b.w.

Time after administration (h)	ABZ-SO concentration (ng/mL ± S.D.)		ABZ-SO_2 concentration (ng/mL ± S.D.)		ABZ-SO_2NH_2 concentration (ng/mL ± S.D.)	
	Spermatozoa	Seminal plasma	Spermatozoa	Seminal plasma	Spermatozoa	Seminal plasma
12	122.8 ± 33.7	2857.4 ± 392.3	22.7 ± 6.6	396.0 ± 64.8	ND	ND
24	111.5 ± 11.8	2372.1 ± 745.7	39.5 ± 2.6	555.8 ± 86.6	ND	ND
36	65.9 ± 15.1	1128.5 ± 451.5	36.5 ± 10.3	519.2 ± 161.7	ND	ND
48	53.6 ± 17.0	398.7 ± 186.5	34.3 ± 3.8	401.3 ± 76.5	4.6 ± 2.2 >LOD	52.5 ± 11.9
60	ND	ND	ND	ND	5.4 ± 2.3 >LOD	61.5 ± 11.2

ND: not detected; >LOD: higher than the limit of detection.

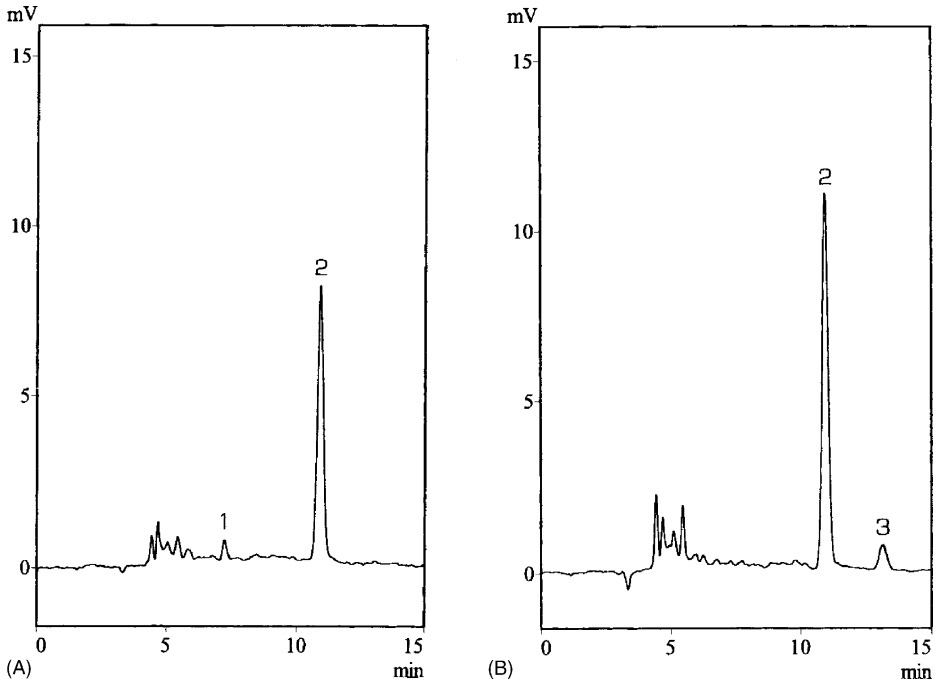

Fig. 5. Representative chromatograms of spermatozoa extract samples collected from a ram at (A) 12 h and (B) 48 h after a single per os albendazole administration, at the dose of 11.5 mg/kg b.w. Chromatographic conditions and peak identification as in Fig. 2.

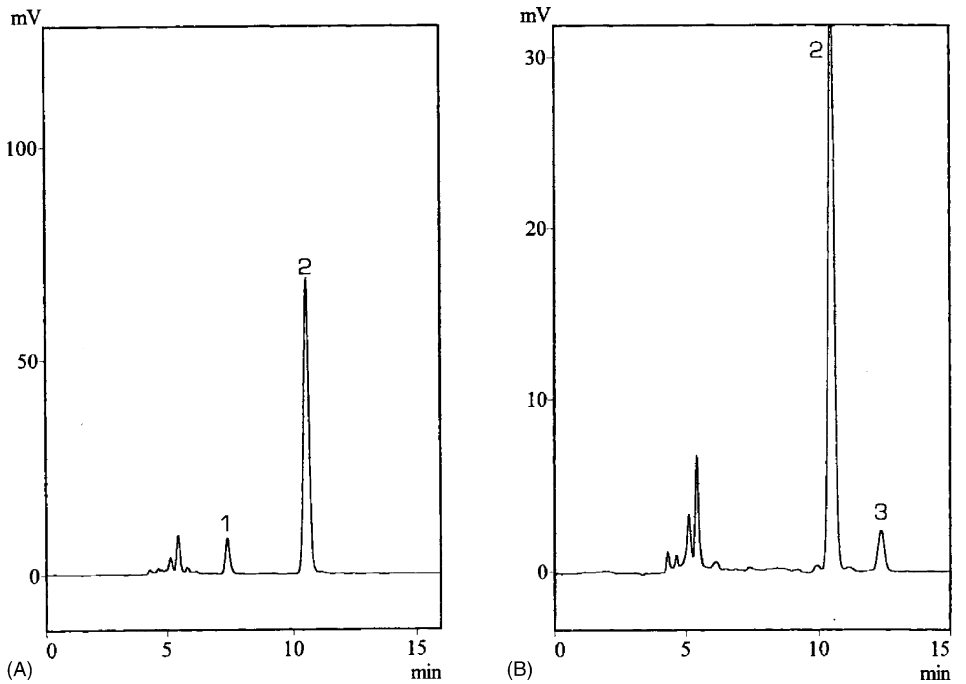

Fig. 6. Representative chromatograms of seminal plasma samples collected from a ram at (A) 12 h and (B) 48 h after a single per os albendazole administration, at the dose of 11.5 mg/kg b.w. Chromatographic conditions and peak identification as in Fig. 2.

of albendazole metabolites in spermatozoa and seminal plasma are presented in Table 7 and representative chromatograms are displayed in Figs. 5 and 6. Briefly, ABZ-SO maximum concentration (C_{max}) was (±S.D.) 122.8 ± 33.7 ng/mL in spermatozoa samples, and 2857.4 ± 392.3 ng/mL in seminal plasma, and was observed during the first sperm collection (12 h post-administration). Respective levels of ABZ-SO_2 were 39.5 ± 2.6 and 555.8 ± 86.6 ng/mL and occurred 24 h post-administration. Finally, ABZ-SO_2NH_2 concentrations were considered quantifiable only in seminal plasma samples. C_{max} was 61.5 ± 11.2 ng/mL and occurred at 60 h. Typical chromatograms obtained are displayed in Figs. 5 and 6. As shown in Fig. 5(B), ABZ-SO_2NH_2 yielded a discrete, Gaussian peak in the 48 h spermatozoa sample. The mean spermatozoa extract concentrations calculated from all animals were 4.6 ng/mL at 48 h and 5.4 ng/mL at 60 h. These values fall within the range of the calibration curve (they are therefore clearly higher than the limit of detection); however, they are lower than the limit of quantification of the method, based on definition (the lowest in concentration validated sample). Nevertheless, the presence of albendazole 2-aminosulphone in ram spermatozoa can be considered a fact.

4. Conclusion

The relative simplicity of the liquid–liquid extraction procedure, the low cost of materials required for sample cleanup and chromatography, combined with the sensitivity of fluorescence detection (as defined by the low limits of quantification for all albendazole metabolites), the cleanliness of the chromatograms (no rise in the LC system pressure or deterioration of the column performance were observed during analysis of samples from the validation and the pharmacokinetic study) offer the present method favorable characteristics. A throughput of 70–100 samples, by a single analyst, in 4–5 h (time for spermatozoa pretreatment not included) is considered feasible.

The method described in this paper is currently the only tool for the determination of albendazole metabolites in sperm. With its use in the aforementioned pharmacokinetic study, the presence of ABZ-SO, ABZ-SO_2 and ABZ-SO_2NH_2 in both fractions of ovine sperm (spermatozoa and seminal plasma) has been reported for the first time.

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