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Quantitative chromatographic determination of several benzimidazole anthelmintic molecules in parasite material

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Abstract

A specific, precise and accurate high-performance liquid chromatographic (HPLC) analytical method has been developed for the quantitative determination of different benzimidazole (BZD) anthelmintics in parasite material (*Moniezia benedeni*). Mebendazole (MBZ), oxibendazole (OBZ), flubendazole (FLBZ), albendazole (ABZ) ricobendazole (RBZ), albendazole sulphone ($ABZSO_2$), fenbendazole (FBZ), oxfendazole (OFZ) and fenbendazole sulphone ($FBZSO_2$) were measured simultaneously in *M. benedeni*, a sheep and cattle cestode parasite used as a model of the biological matrix. The recovery, linearity, precision, accuracy, limits of detection and quantification of the method were determined. Drug extraction from the parasite's tissue homogenate was performed using methanol (liquid phase extraction), and after solvent evaporation, the residual material was cleaned up by solid phase extraction prior to analysis by reversed-phase HPLC. The resolution of all the BZD molecules assayed was obtained on a C_{18} reversed-phase (5 μm, 250 mm × 4.6 mm) column using acetonitrile and ammonium acetate as the mobile phase and ultraviolet (UV) detection at 292 nm. Regression analyses for all the BZD compounds assayed were linear at concentrations ranging from 1.61 to 64.21 nmol/100 mg protein (triplicate determinations) showing correlation coefficients greater than 0.9922. The developed method is efficient for the simultaneous determination of several benzimidazole anthelmintic molecules in parasite material and useful for the ex vivo and in vivo characterisation of the kinetics of drug uptake/diffusion in target parasites, which seems to be relevant to optimise parasite control both in human and veterinary medicine.

Keywords: Benzimidazole anthelmintics

1. Introduction

Benzimidazole (BZD) anthelmintics are effective against nematode, cestode and trematode parasites, and they are widely used as antiparasitic drugs in veterinary and human medicine [1,2]. The BZD structure is a bicyclic ring system in which benzene has been fused to the 4 and 5 positions of the heterocycle (imidazole) [3]. Different modifications at positions 2 and 5 of the BZD ring system (Fig. 1) have provided the most anthelmintically active drugs [4]. These compounds can be chemically classified as BZD thiazolyls (thiabendazole and cambendazole); halogenated BZD thiols (triclabendazole); pro-BZD (febantel, netobimin and thiophanate) and BZD methylcarbamates (albendazole (ABZ), ricobendazole (RBZ), fenbendazole (FBZ), oxfendazole (OFZ), mebendazole (MBZ), flubendazole (FLBZ), oxibendazole (OBZ), etc.) [3]. RBZ and OFZ are the active sulphoxide metabolites of ABZ and FBZ, respectively.

Since BZD anthelmintics need to reach their specific receptor inside the parasite cell to exert their action, concentrations achieved inside the intracellular space are critical to assure clinical efficacy. There have been many publications describing the determination of BZD concentrations by high performance liquid chromatography (HPLC) in plasma [5–10], in biological fluids/tissues of different animal species [11–17] and in other biological matrices [18]. The concentration profiles of the drug/metabolites measured in plasma strongly correlate with those achieved in different tissues/fluids of treated animals. However, the quantification of drug/metabolites concentrations in target parasite

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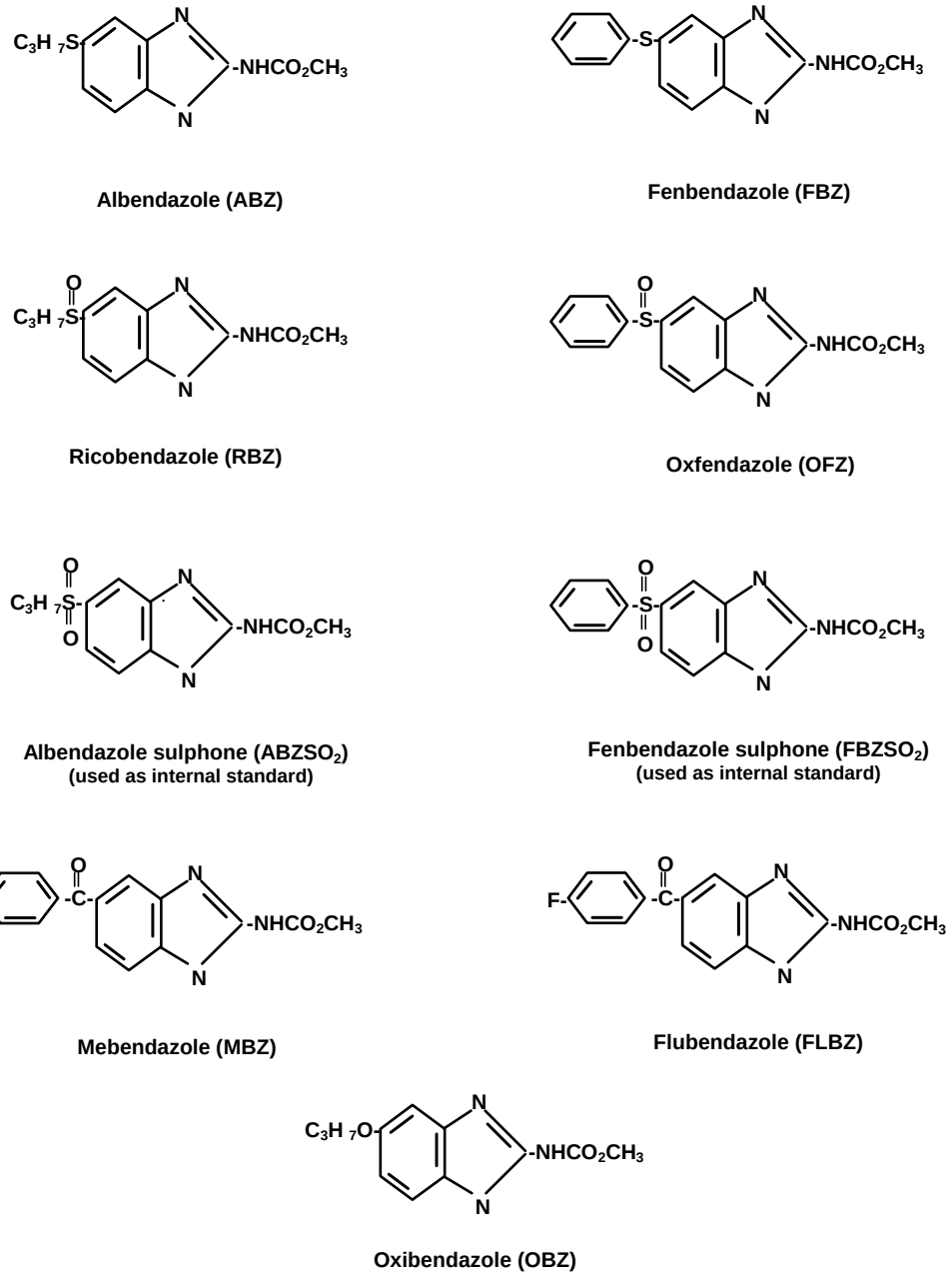

Fig. 1. Chemical structures of benzimidazole methylcarbamate molecules assayed in the current work.

material after a conventional anthelmintic treatment is required to correlate information on host pharmacokinetics with the pattern of drug uptake by the target parasite. Additionally, the evaluation of the pattern of ex vivo diffusion of the BZD molecules into helminth parasites leads to a greater comprehension of the processes and the factors influencing the in vivo drug uptake and resultant clinical efficacy. Although information about drug availability in target parasites is important and useful to optimise anthelmintic therapy and to delay the development of anthelmintic resistance, limited information is available on the extraction and HPLC analysis of BZD molecules from parasite material [19,20], where only the parent drug and one or two metabolites have been measured. The aim of the present work was to develop and validate a method to extract, analyse and simultaneously quantify the therapeutically most important BZD methylcarbamate molecules in *Moniezia benedeni*, a sheep and cattle cestode parasite used as a model of the biological matrix. The developed methodology includes the chromatographic determination of MBZ, OBZ, FLBZ, ABZ and FBZ, including their sulphoxide (RBZ and OFZ, respectively) and sulphone metabolites (albendazole sulphone ($ABZSO_2$) and fenbendazole sulphone ($FBZSO_2$), respectively).

2. Experimental

2.1. Chemicals

Pure reference standards of MBZ, OBZ, ABZ, RBZ, $ABZSO_2$ (Schering Plough, Kenilworth, USA), FBZ, OFZ, $FBZSO_2$ (Rhone Merieux, Lyon, France) and FLBZ (Janssen Animal Health, Beerse, Belgium) were used for the validation of the analytical methodology. All the solvents (acetonitrile and methanol) used during the extraction and drug analysis were HPLC grade and purchased from Sintorgan® (Buenos Aires, Argentina). Water was double distilled and deionized using a water purification system (Simplicity®, Millipore, Brazil).

2.2. Equipment and chromatographic conditions

Chromatography was performed on a Shimadzu HPLC equipment (Shimadzu Corporation, Kyoto, Japan), with two LC-10AS solvent pumps, an automatic sample injector (SIL-10A) with a 50 μl loop, an ultraviolet visible spectophotometric detector (UV) (SPD-10A), a column oven (Eppendorf TC-45, Eppendorf, Madison, WI, USA) set at 30 °C, and a CBM-10A integrator. Data and chromatograms were collected and analysed using the Class LC10 software (SPD-10A, Shimadzu Corporation, Kyoto, Japan). The C_{18} reversed-phase column was Phenosphere (Phenomenex®, Torrance, CA, USA). The length of the separation column was 250 mm × 4.6 mm with 5 μm particle size. Elution from the stationary phase was carried out at a flow rate of 1.2 ml/min using acetonitrile and ammonium acetate buffer (0.025 M, pH 6.6) as a mobile phase. The elution gradient linearly changed from 27:73 (acetonitrile:ammonium acetate buffer) to 50:50 in 5 min, then maintained for 5 min and modified to 27:73 in 3 min, which was then maintained over 3 min. The detection of the BZD compounds under evaluation was done at a wavelength of 292 nm.

2.3. Calibration standards

Stock solutions for the different molecules were prepared in methanol to reach final concentrations ranging from 50 to 1000 μM for all the molecules assayed. The solutions were stored at 4 °C.

2.4. Sample preparation and extraction procedures

M. benedeni specimens were collected from the small intestine of untreated cattle killed at the local abattoir. The parasite material was rinsed extensively with saline solution to remove intestinal debris and adherence materials. Drug concentrations are expressed as nmol/100 mg protein. The determination of parasite protein concentrations was carried out according to the methodology described by Smith et al. [21]. Protein concentration in *M. benedeni* ($n = 5$) was 31.15 ± 4.43 mg/g of cestode parasite. Drug-free *M. benedeni* material (0.5 g) was spiked with each molecule target (MBZ, OBZ, FLBZ, ABZ, FBZ, RBZ, and OFZ) to reach the following final concentrations: 1.61, 3.21, 6.42, 16.05, 32.10, and 64.21 nmol/100 mg protein. After 10 min, the parasite material was homogenised (15 s, at 4 °C) (Ultraturrax®, T 25, Ika Works Inc., Labortechnik, USA) and spiked with the chosen internal standard (IS) compound. $ABZSO_2$ (10 μl, stock solution of 500 μM) was used as IS to measure the concentration of RBZ, FLBZ and ABZ. $FBZSO_2$ (10 μl, stock solution of 500 μM) was the chosen IS to determine the concentration of OFZ, OBZ, MBZ and FBZ. The parasite material homogenate was mixed with 1.5 ml of methanol (twice) and shaken (multi-tube vortexer, VWR Scientific Products, USA) over 5 min to extract the drug analyte(s) present in the sample, and then centrifuged (Jouan®, BR 4i Centrifuge, France) to allow phase separation (2000 × *g*, 10 min, 10 °C). The collected methanol phase (3 ml total) was concentrated to dryness in a vacuum concentrator (Speed-Vac®, Savant, CE). The residue obtained was dissolved in 1 ml methanol/water (20/80 (v/v)). Then, the molecules were extracted using disposable C_{18} Supelclean cartridges (Supelco Inc., Bellefonte, PA, USA) previously conditioned with 0.5 ml of methanol, followed by 0.5 ml of water. All the dissolved samples were applied to the cartridge and then washed with 0.5 ml of water and eluted with 3 ml of methanol, concentrated to dryness in the vacuum concentrator, and then reconstituted with 300 μl of mobile phase. Fifty microliter of each solution were injected into the chromatographic system. Spiked parasite material samples were analysed by HPLC to measure the concentrations of each drug assayed. Blank unspiked parasite samples were prepared with the same extraction procedure.

2.5. Ex vivo incubation assays

The collected specimens of *M. benedeni* were maintained for 2 h before starting the incubation in a Kreb's Ringer Tris (KRT) buffer (pH 7.4) at 37 °C [22]. The tapeworm material (0.5 g) was incubated at 37 °C in 5 ml of the KRT buffer containing either FBZ, OFZ or OBZ at a final concentration of 5 nmol/ml. The following incubation times were used to evaluate the diffusion of the different BZD anthelmintics into *M. benedeni*: 5, 10, 15, 30, 60, 90 and 120 min. There were four replicate assays for each incubation time. Blank samples containing parasite material and medium without drug, and, drug-spiked medium without parasite material, were incubated over the same time intervals and served as controls. Once the appropriate incubation time had elapsed, the tapeworm material was rinsed thoroughly with saline solution, blotted on coarse filter paper and prepared for HPLC analysis to determine drug concentrations. The parasite material was processed shortly after the incubation assays.

2.6. Method validation

2.6.1. Absolute analyte recoveries

The absolute recovery of each compound was assessed at three concentration levels (3.21, 16.05, and 32.10 nmol/100 mg protein) by triplicate analysis. The extraction efficiency of the nine molecules under study was determined by comparison of the detector responses obtained for fortified blank samples peak areas with the peak areas resulting from direct injections of equivalent quantities of standard solutions. The coefficient of variation (%CV) for recovery of the method was calculated.

2.6.2. Linearity of standard curves

Linearity of a method is a measure of how a calibration plot of response versus concentration approximates a straight line. A linear calibration gives evidence that the system is performing properly through the concentration range of interest [23]. The linearity was tested by constructing calibration curves for each compound. The peak area ratio between the molecule under study and the chosen IS was determined for each drug to prepare the calibration curves ranging from 1.61 to 64.21 nmol/100 mg protein ($n = 3$). For the most hydrophilic molecules, RBZ and OFZ (lower lipid-to-water partition coefficients [27]), calibration curves were prepared with lowest concentration values (0.32 and 0.64 nmol/100 mg protein, respectively), which allowed the quantification of lower concentrations achieved inside the parasites after the ex vivo incubations. The data were analysed for linearity using the least-squares regression method, using the Run Test and ANOVA to determine if the data differed from a straight line.

2.6.3. Precision and accuracy

Precision (intra-day and inter-day) and accuracy of the method were determined by evaluation of replicates of drug-free parasite material fortified ($n = 5$) with each compound at three different concentrations (3.21, 16.05, and 32.10 nmol/100 mg protein).

The evaluation of the intra-day precision involved five ($n = 5$) measurements of the same sample at the three different concentrations within a single run. The inter-day precision was determined to estimate the run to run extraction and chromatographic variation in the method. Inter-day variation was measured during five ($n = 5$) consecutive working days for a parasite sample at the three concentrations mentioned above. Precision was expressed as %CV.

Accuracy is defined as the closeness between the experimentally measured and true value [23]. Accuracy of the method was measured by the differences between observed and calculated concentration results, and expressed as the relative error (%RE).

2.6.4. Limit of detection (LOD) and limit of quantification (LOQ)

The minimum detectable concentration, often referred as the LOD, is the smallest concentration that can be reliably detected [23]. The theoretical LOD was estimated integrating the baseline noise of the system in the area covering the mean retention time of each compound in five ($n = 5$) blank parasite samples spiked with IS. The theoretical LOD was defined as the mean baseline noise/IS peak area ratio plus three standard deviations (S.D.). The LOQ was calculated ($n = 5$) as the lowest drug concentration on the standard curve that could be quantitated with precision not exceeding 20% and accuracy within 20% of nominal [23].

3. Results and discussion

A simple, fast and reliable reversed-phase HPLC method was developed to quantify BZD anthelmintics in cestode parasite samples. The C_{18} reversed-phase stationary phases are among the most widely used in the resolution of BZD anthelmintics compounds by HPLC. The HPLC separation of the analytes was obtained with a gradient of 0.025 M ammonium acetate (pH 6.6) and acetonitrile. Under the described chromatographic conditions, the retention times were 4.50 (RBZ), 5.89 ($ABZSO_2$), 6.37 (OFZ), 7.72 ($FBZSO_2$), 9.10 (OBZ), 9.11 (FLBZ), 10.77 (MBZ), 10.77 (ABZ) and 12.41 (FBZ) min. The total run time for the method was 16 min. The procedure employed for the extraction of the nine molecules from parasite material was simple and highly efficient. Representative chromatograms are shown in Fig. 2. Those chromatograms were obtained after analysis of *M. benedeni* samples without drug (blank) (Fig. 2a), fortified with different BZD molecules (Fig. 2b and c) and ex vivo incubated with OFZ over 90 min (Fig. 2d). The *M. benedeni* blank was free of interferences in the time regions of analytical interest. Good peak shape was obtained for the compounds. Due to the short gradient run time it was necessary to analyse the compounds as two separate sets (Fig. 2b and c) due to co-elution of several of the compounds. Fig. 2b displays a chromatogram of *M. benedeni* spiked with 32.10 nmol/100 mg protein of RBZ, $ABZSO_2$, FLBZ and ABZ. Fig. 2c shows a chromatogram of *M. benedeni* spiked with 32.10 nmol/100 mg protein of OFZ, $FBZSO_2$, OBZ, MBZ and FBZ.

Mean absolute recoveries assessed at three concentrations levels (3.21, 16.05, 32.10 nmol/100 mg protein) (triplicate determinations) for all the molecules assayed are shown in Table 1. The mean recovery calculated for each compound was within 70 and 84%, with CV $\leq$17%. BZD molecules are lipophilic drugs, which could affect their extraction from the lipoid tissues of *M. benedeni*. However, high recoveries were obtained using a simple method based on liquid phase extraction with low methanol volumes (2 ml × 1.5 ml). Comparatively, other methods previously described require much more complex processes for sample preparation [24],

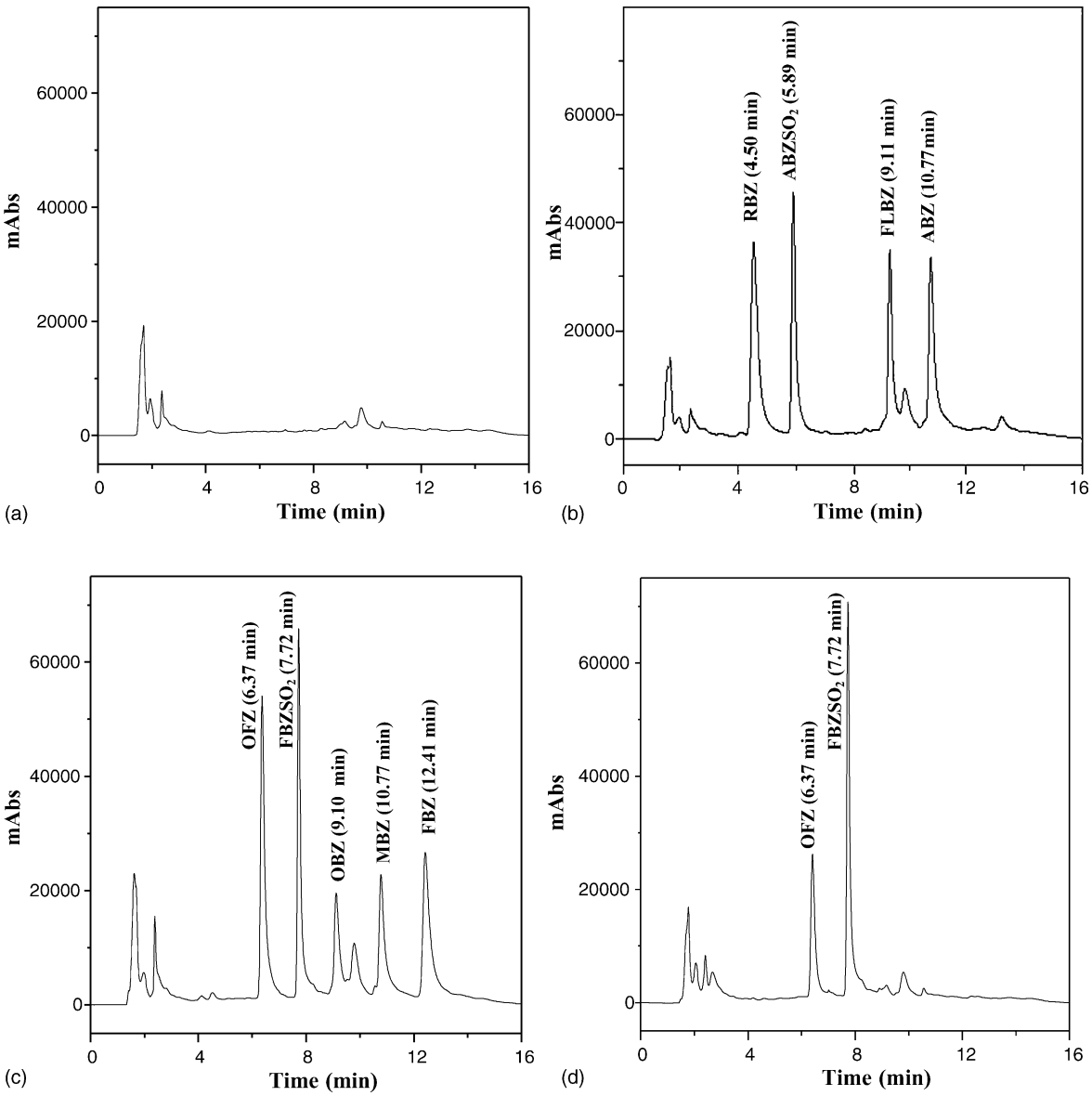

Fig. 2. Chromatograms obtained from (a) drug-free *Moniezia benedeni* parasite sample; (b) *M. benedeni* spiked with 32.10 nmol/100 mg protein of ricobendazole (RBZ) (4.50 min), albendazole sulphone ($ABZSO_2$) (5.89 min) (as internal standard), flubendazole (FLBZ) (9.11 min) and albendazole (ABZ) (10.77 min); (c) *M. benedeni* spiked with 32.10 nmol/100 mg protein of oxfendazole (OFZ) (6.37 min), fenbendazole sulphone ($FBZSO_2$) (7.72 min) (as internal standard), oxibendazole (OBZ) (9.10 min), mebendazole (MBZ) (10.77 min) and fenbendazole (FBZ) (12.41 min); (d) *M. benedeni* incubated with OFZ during 90 min in a Kreb's Ringer Tris buffer incubation medium. ($FBZSO_2$ used as internal standard).

high solvent volumes (10–15 ml of ethyl acetate) [20,25] or further repetition of the liquid phase extraction process (3 ml three times [19,26]). Changing the extraction solvent (from ethylacetate to methanol) presented advantages and disadvantages. The main advantages were based on the reduced solvent volume required for the extraction, high sample recoveries and cleaner sample extracts. The main disadvantages of using methanol as extraction solvent were the increased time required for evaporation and the resulting increase in the amount of N_2 required for the solvent removal. The ethyl acetate is unmiscible in water (solubility in water 8.7%, w/w), and once recovered is evaporated in a short time under a stream of N_2 at 37 °C. However, the methanol is miscible in water (solubility in water 100%, w/w), and the organic phase recovered after shaking is a mixed solution of water (from the parasite tissue material as the cestode structure consists in approximately 90% of water) and methanol. The evaporation of the recovered organic phase was slow and much more N_2 is required. The problem was solved by concentrating the collected methanol phase to dryness in a vacuum concentrator set at 43 °C. Although the time of evaporation is approximately 4 h, it does not consume N_2.

Calibration lines for each analyte in the parasite material were constructed by least squares linear regression analysis. The linearity of the method was proven with six calibration points in the concentration ranges of

Table 1
Analytical recovery, limit of detection (LOD) and limit of quantification (LOQ) of the method developed to determine mebendazole (MBZ), oxibendazole (OBZ), flubendazole (FLBZ), albendazole (ABZ), fenbendazole (FBZ), ricobendazole (RBZ) and oxfendazole (OFZ) in spiked *Moniezia benedeni* samples

Molecule	Recovery (%)	LOD		LOQ		
		(nmol/100 mg protein)	Precision (CV)	(nmol/100 mg protein)	Precision (CV)	Accuracy (RE)
MBZ	72 (7)	0.32	20	1.61	14	−13.3
OBZ	74 (17)	0.32	20	1.61	5	5.5
FLBZ	84 (11)	1.61	9	3.21	14	15.5
ABZ	70 (13)	0.19	13	1.61	2	−7.2
FBZ	79 (17)	0.32	14	1.61	8	16.4
RBZ	77 (16)	0.19	7	0.32	6	−5.7
OFZ	71 (10)	0.32	6	0.64	19	−0.7
$ABZSO_2$ (IS)	75 (8)					
$FBZSO_2$ (IS)	76 (2)					

Values in brackets are coefficients of variation (%CV).
Relative error (%RE) = 100 ((predicted concentration − nominal concentration)/nominal concentration).
$ABZSO_2$: albendazole sulphone (used as internal standard).
$FBZSO_2$: fenbendazole sulphone (used as internal standard).
For details refer to Sections 2.6.1 and 2.6.4.

1.61–64.21 nmol/100 mg protein, with the exception of FLBZ, RBZ and OFZ (as it was explained in Section 2.6.2.). As the LOQ for FLBZ was 3.21 nmol/100 mg protein, this concentration value was used as the lowest point in the regression analysis for this molecule. The regression analysis were linear over the concentrations examined and the correlation coefficients (r) of the calibration curves ranged between 0.9922 and 0.9993. The correlation coefficients and the equations for the straight lines are shown in Table 2.

Other validation criteria for the developed methodology, such as precision and accuracy, are summarized in Table 3. The analysis of low (3.21 nmol/100 mg protein), middle (16.05 nmol/100 mg protein) and high (32.10 nmol/100 mg protein) drug concentration values was used to determine intra-day and inter-day precisions and accuracy. Intra-day and inter-day precisions showed CV ≤10%. The accuracy of these estimations (expressed as RE) ranged from −8.7 to 15.4%.

In order to test the absence of endogenous interference, the IS was omitted for a blank parasite sample extract (Fig. 2a). No major endogenous chromatographic peaks, which could interfere with the resolution of the nine BZD compounds, were observed. The purity of the BZD peaks was confirmed by analysing experimental parasite samples (incubated ex vivo) using alternative chromatographic conditions, which did not show the appearance of hidden interference peaks. The LOD in *M. benedeni*, giving a peak area three times the baseline noise of the blank extracted sample, were between 0.19 and 1.61 nmol/100 mg protein. LOD and LOQ values are shown in Table 1. The LOQ were low enough (ranging between 0.32 and 3.21 nmol/100 mg protein) for the application of the method to study the diffusion of BZD molecules into *M. benedeni*. Altogether, these results indicated that the developed chromatographic method was reliable, reproducible and accurate.

The described methodology has been applied to determine the concentrations of BZD molecules in *M. benedeni*, a sheep and cattle intestinal cestode parasite, used as a model to compare the ex vivo patterns of transtegumental diffusion of different BZD anthelmintics [27]. To illustrate the application of this methodology, the comparative diffusion of three of the BZD molecules evaluated (FBZ, OBZ and OFZ) into *M. benedeni* is shown in Fig. 3. Results express drug concentrations (mean ± S.D.) (nmol/100 mg protein) into the cestode material during 120 min of incubation in a Kreb's Ringer Tris buffer incubation medium. Although

Table 2
Linearity: regression parameters of the calibration curves of different benzimidazole molecules in *Moniezia benedeni* ($y = ax + b$)

Molecule	Concentration range (nmol/100 mg protein)	Correlation coefficient (r)	Slope (a) ± S.E.M.	Intercept (b) ± S.E.M. (peak area ratio)[a]
MBZ	1.61–64.21	0.9977	0.168 ± 0.003	−0.014 ± 0.027
OBZ	1.61–64.21	0.9944	0.195 ± 0.005	−0.024 ± 0.049
FLBZ	3.21–64.21	0.9986	0.249 ± 0.004	−0.129 ± 0.038
ABZ	1.61–64.21	0.9991	0.137 ± 0.001	−0.005 ± 0.006
FBZ	1.61–64.21	0.9989	0.168 ± 0.002	−0.010 ± 0.009
RBZ	0.32–64.21	0.9922	0.223 ± 0.006	0.003 ± 0.028
OFZ	0.64–64.21	0.9993	0.206 ± 0.002	0.016 ± 0.009

S.E.M.: error standard.
[a] Peak area ratio of the analyte to the internal standard, for details refer to Section 2.6.2.

Table 3
Analytical precision and accuracy of the determination of mebendazole (MBZ), oxibendazole (OBZ), flubendazole (FLBZ), albendazole (ABZ), fenbendazole (FBZ), ricobendazole (RBZ) and oxfendazole (OFZ) in spiked *Moniezia benedeni* samples

	Concentration added (nmol/100 mg protein)																				
	MBZ			OBZ			FLBZ			ABZ			FBZ			OFZ			RBZ		
	3.21	16.05	32.10	3.21	16.05	32.10	3.21	16.05	32.10	3.21	16.05	32.10	3.21	16.05	32.10	3.21	16.05	32.10	3.21	16.05	32.10
Intra-day precision (%CV)	8	6	5	3	4	4	2	5	5	6	5	3	4	8	4	2	4	6	6	4	2
Inter-day precision (%CV)	6	3	3	8	4	6	7	7	6	10	2	5	5	2	10	6	10	9	6	3	2
Accuracy Concentration obtained (nmol/100 mg protein)	3.02	16.15	33.71	2.92	15.79	34.61	3.69	14.96	31.36	3.31	15.99	32.20	3.11	15.06	32.33	3.27	16.34	31.94	3.50	15.60	32.26
(%RE)	−5.7	0.9	5.3	−8.7	−1.3	8.1	15.4	−6.5	−2.0	3.3	-0.1	0.6	−2.7	−5.9	1.0	2.3	2.1	−0.2	9.3	−2.5	0.8

Relative error (%RE) = 100 × ((predicted concentration-nominal concentration)/nominal concentration).
For details refer to Section 2.6.3.

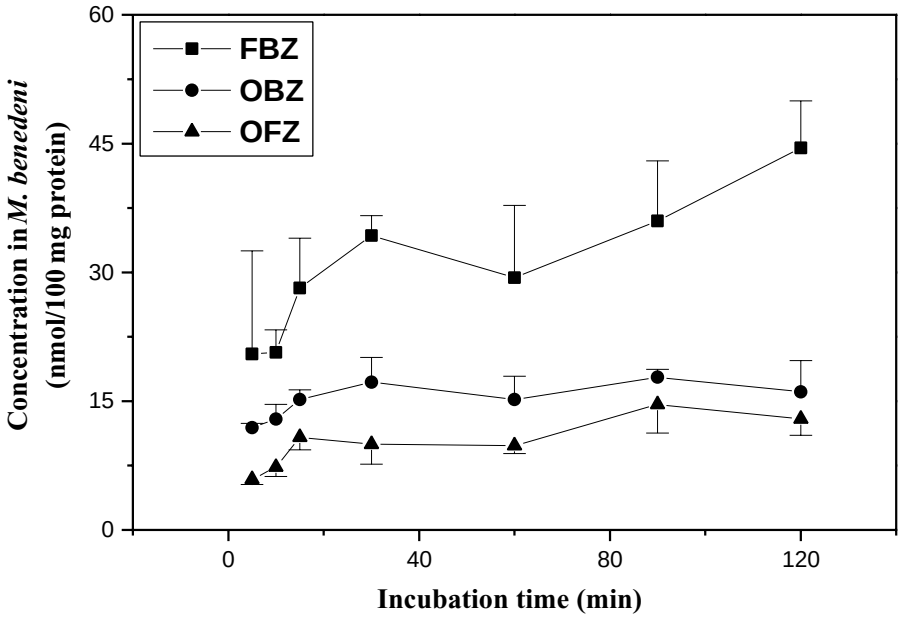

Fig. 3. Application of the developed methodology to the determination of fenbendazole (FBZ), oxibendazole (OBZ) and oxfendazole (OFZ) concentrations in *Moniezia benedeni* after their ex vivo incubation with the parasite. Results express drug concentrations (mean ± S.D.) (nmol/100 mg protein) in the cestode parasite over 120 min of incubation in a Kreb's Ringer Tris buffer incubation medium. *Data obtained from Mottier et al. [27]. FBZ concentrations at the different incubation times were significantly higher than those measured for OBZ and OFZ.

the three BZD compounds penetrated the cestode's tegument, the diffusion capacities were different. The amount of drug recovered over time increased during the incubation period up to 120 min. Diffusion of the sulphide FBZ was significantly greater ($P < 0.05$) compared to its respective sulphoxide metabolite, OFZ. The diffusion of OBZ into the cestode parasite was significantly lower than that of FBZ, but greater than that obtained for OFZ at 5, 10 and 30 min of incubation. The results shown here and those previously reported by Mottier et al. [27] demonstrate that the entry of BZD drugs into a cestode parasite is a passive diffusion process and the most lipophilic molecules reached higher concentrations inside the parasite material during the incubation times, demonstrating their greater ability to cross through the helminth tegument.

4. Conclusions

The work reported here describes for the first time the development and validation of a simple, reproducible, precise and accurate method for the determination of nine of the most common BZD methylcarbamate molecules in a target helminth parasite. The method allows a single preparation procedure for the simultaneous determination of various BZD molecules in both ex vivo and in vivo settings. The methodology permits the characterisation of the drug uptake/diffusion in target parasites, which provides useful information to optimise parasite control in both human and veterinary medicine.

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