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Measurement of triclabendazole and its metabolites in liver flukes: method development and full validation

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Abstract

The trematode parasite *Fasciola hepatica* is still the cause of considerable loss in livestock production all over the world. The knowledge of the pharmacological properties of the available flukicidal drugs is critical to control this parasite. Triclabendazole is an halogenated benzimidazole anthelmintic with potent flukicidal activity. A simple reversed-phase high-performance liquid chromatographic analytical method has been developed, validated and applied for the quantitative determination of the flukicidal drug triclabendazole and its sulpho- and hydroxy-metabolites in the liver fluke, *F. hepatica*. Triclabendazole, triclabendazole sulphoxide, triclabendazole sulphone, hydroxy-triclabendazole, hydroxy-triclabendazole sulphoxide and hydroxy-triclabendazole sulphone were measured simultaneously in this trematode parasite. Linearity, resolution, precision, accuracy, recovery, limits of detection and quantification of the method were determined. Drug extraction from the parasite's tissue homogenate was effectively performed using liquid extraction (acetonitrile), avoiding clean up by solid phase extraction, prior to analysis by reversed-phase high-performance liquid chromatography. The resolution of all the halogenated benzimidazole thiol 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 detection at 300 nm. Regression analyses were linear over the concentration range examined (from 0.272 to 16.331 nmol/100 mg trematode protein) and the correlation coefficients of the calibration curves ranged between 0.996 and 1.000. The calculated limits of detection of the proposed method for the parent drug and its metabolites ranged between 0.007 and 0.079 nmol/100 mg trematode protein. The extraction efficiency for the different analytes from the parasite material was greater than 71%. The results obtained indicated that the developed chromatographic method was selective, accurate and easy to reproduce. The developed procedure was successfully applied to quantify triclabendazole/metabolites in *F. hepatica* incubated under ex vivo conditions, demonstrating to be efficient for the determination of the most extensively used flukicidal drug available for veterinary medicine, and its metabolites. The analytical method described here is an useful tool for the measurement of this flukicidal compound in different studies addresses to evaluate drug influx/eflux and metabolism in its main target parasite.

Keywords: Reversed-phase high-performance liquid chromatography; Flukicidal drugs; Triclabendazole; Parasite material; *Fasciola hepatica*

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1. Introduction

Benzimidazole (BZD) anthelmintics are widely used as antiparasitic drugs in veterinary and human medicine [1,2]. The BZD methylcarbamate compounds (albendazole, fenbendazole, etc.) are broad spectrum anthelmintics with an excellent nematodicidal activity. Triclabendazole (6-chloro-5(2-3 dichlorophenoxy)-2-methyl thio-benzimidazole) (TCBZ) is an halogenated BZD thiol derivative with specific activity against *Fasciola hepatica* infections in cattle and sheep [3]. Fascioliasis, which is caused by the liver fluke, *F. hepatica*, is still the cause of considerable loss in sheep and cattle production all over the world. The main strategy for the effective control of fascioliasis is still based on the use of antiparasitic drugs. Most of the available fasciolicidal compounds, at their recommended therapeutic doses, have good activity against the mature stage of the liver fluke, but are not sufficiently effective against immature stages. Interestingly, TCBZ, the most widely used fasciolicide, shows excellent efficacy against both the mature and immature stages of the liver fluke in sheep and cattle and requires fewer doses to achieve the same fluke kill as other actives [3,4]. Despite this, the drug does not show clinical efficacy against nematodes, cestodes and other trematode parasites.

TCBZ parent drug is not detected in plasma after its administration, indicating it was completely removed from portal blood by the liver following absorption, providing evidence of first pass hepatic metabolism [5]. TCBZ is oxidised to form the sulphoxide and sulphone metabolites, triclabendazole sulphoxide (TCBZSO) and triclabendazole sulphone ($TCBZSO_2$), respectively [5]. Hydroxylation of TCBZ occurred at the 4′ position of the dichlorophenoxy ring, forming the corresponding hydroxylated metabolites, hydroxy-TCBZ (OH-TCBZ), hydroxy-TCBZSO (OH-TCBZSO) and hydroxy-$TCBZSO_2$ (OH-$TCBZSO_2$) (Fig. 1). Extremely low amounts of TCBZ were detected in bile and major metabolites identified were TCBZSO, $TCBZSO_2$ and the hydroxy metabolites. Hydroxylated TCBZ metabolites have not been detected in plasma, indicating that they did not exchange with the plasma pool, being TCBZSO and $TCBZSO_2$ the only metabolites found in plasma [5]. These halogenated molecules reach the target parasite *F. hepatica* to interact with their pharmacological receptor. BZD nematodicidal activity is based on its binding to parasite ß-tubulin at the colchicine-binding site [6–8] which inhibits polymerisation into microtubules [9]. Thus, all the functions ascribed to microtubules at the cellular level are altered [7]. Interestingly, TCBZ does not bind to colchicine in *F. hepatica* homogenates [10]. It is likely that a different site of action is involved on the flukicidal activity of TCBZ, which could also explain its lack of efficacy against other helminth parasites [11,12]. The exact mode of action of TCBZ and the flukicidal potency of the parent drug and its metabolites await elucidation.

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 few publications describing the determination and quantification of TCBZ/metabolites concentrations by high-performance liquid chromatography (HPLC) in plasma [13–17] and in biological fluids/tissues of different animal species [18–20]. 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 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 (studies using intact living parasites and a closed perfusion system) 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 the increasing anthelmintic resistance to this flukicidal drug, very limited information is available on the extraction and HPLC analysis of TCBZ and metabolites molecules from *F. hepatica*, where high solvent volumes (30 ml of ethyl acetate) [21] and/or clean up by solid phase extraction [21,22] are required. The aim of the present work was to develop, validate and apply a simple method to extract, analyse and quantify the therapeutically most important flukicidal molecule, TCBZ, and its sulpho-

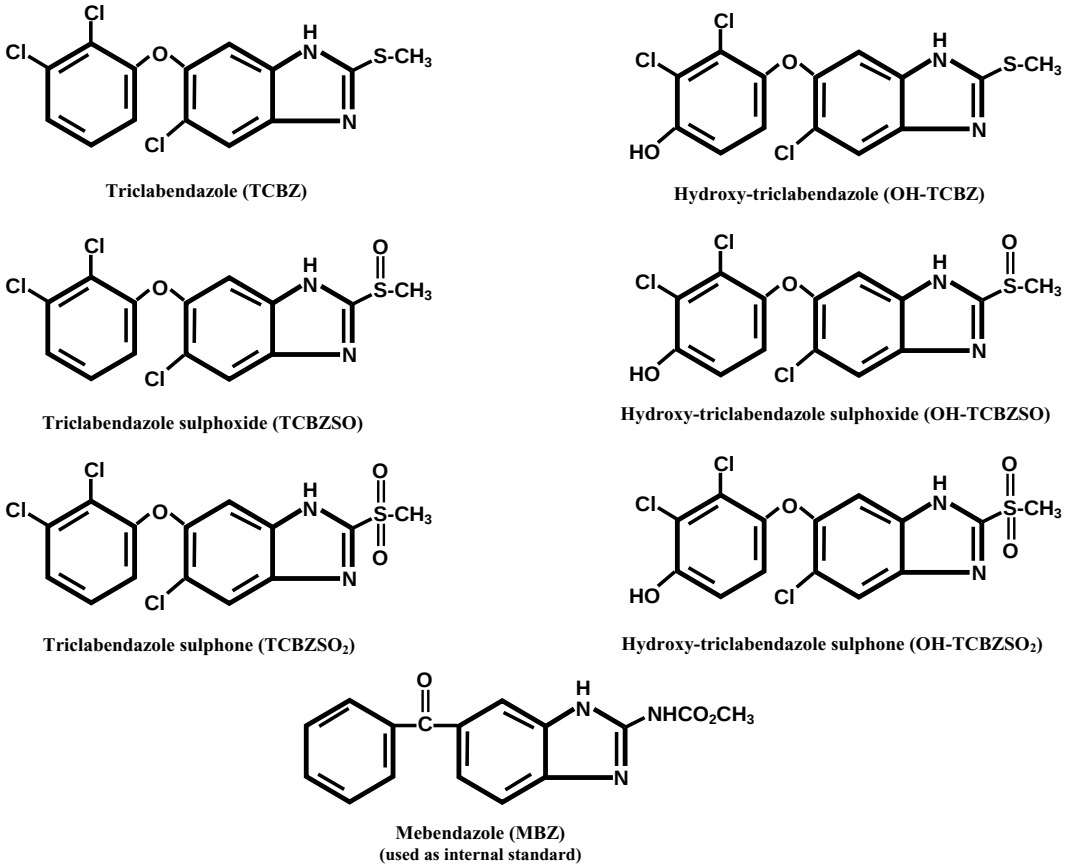

Fig. 1. Chemical structures of halogenated benzimidazole thiol molecules assayed in the current work. Mebendazole (a benzimidazole methylcarbamate compound) was used as internal standard.

and hydroxy-metabolites, in its target parasite *F. hepatica*. The developed methodology includes the chromatographic determination of TCBZ, TCBZSO, $TCBZSO_2$ including the hydroxylated metabolites, OH-TCBZ, OH-TCBZSO and OH-$TCBZSO_2$.

2. Experimental

2.1. Chemicals

Pure reference standards of TCBZ (99.5%, purity), TCBZSO (97%), $TCBZSO_2$ (99%), OH-TCBZ (90%), OH-TCBZSO (94%), OH-$TCBZSO_2$ (94%) kindly supplied by Novartis Animal Health Inc. (Basel, Switzerland) and mebendazole (MBZ) (99%) (Schering Plough, Kenilworth, New Jersey, USA) were used for the validation of the analytical methodology. The solvents (acetonitrile, methanol) used during the extraction and drug analysis were HPLC grade and purchased from Sintorgan® (Buenos Aires, Argentina). Saline solution (NaCl) (99%) and ammonium acetate (98%) were purchased from Anedra (Buenos Aires, Argentina) and Merck (Haar, Germany), respectively. Water was double distilled and deionized using a water purification system (Simplicity®, Millipore, Sao Paulo, Brazil).

2.2. HPLC system and conditions

Chromatography was performed on 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 data 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 dimensions of the separation column were 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 the mobile phase. During the initial 12 min of the chromatographic run, the elution was performed with 35:65 acetonitrile:ammonium acetate buffer. Then, it was linearly changed to 60:40 in 2 min, maintained for 6 min and modified to 35:65 in 2 min, which was then maintained over 3 min. The detection of TCBZ and its metabolites was done at a wavelength of 300 nm.

2.3. Preparation of standard solutions

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

2.4. Sample preparation and extraction procedures

To recover adult specimens of *F. hepatica* from the liver of artificially infected Corriedale sheep (200 metacercariae/sheep), common bile ducts and the gall-bladder of each sheep were opened. The specimens were rinsed extensively with NaCl (0.9%) (37 °C) to remove bile and/or adhering materials. Drug concentrations are expressed as nmol/100 mg trematode protein. The determination of parasite protein concentrations was carried out according to the methodology described by Smith et al. [23]. Protein concentration in *F. hepatica* ($n = 5$) was 183.7 ± 20 mg/g of trematode parasite. Drug-free *F. hepatica* material (0.1 g) was spiked with internal standard (IS) compound, mebendazole (MBZ) (10 μl, stock solution of 500 μM) and each molecule (TCBZ, TCBZSO, $TCBZSO_2$, OH-TCBZ, OH-TCBZSO, $OH\text{-}TCBZSO_2$) to reach the following final concentrations: 0.272, 0.544, 1.361, 2.722, 5.444, 10.887, 16.331 nmol/100 mg trematode protein. After 10 min, the parasite material was homogenised (15 s, at 4 °C) (Ultraturrax®, T 25, Ika Works Inc., Labortechnik, Wilmington, NC, USA). The liver fluke homogenate was mixed with 1.5 ml of acetonitrile (three times) and shaken (multi-tube vortexer, VWR Scientific Products, West Chester, PA, USA) over 5 min to extract the drug analyte(s) present in the sample, and then centrifuged (Jouan®, BR 4i Centrifuge, Saint Herblain, France) to allow phase separation (2000 × *g*, 10 min, 10 °C). The final collected acetonitrile phase (4.5 ml) was concentrated to dryness in a vacuum concentrator (Speed-Vac®, Savant, Los Angeles, CA, USA), and then reconstituted with 150 μl of mobile phase. Fifty μl 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. Complete validation of the analytical method

The absolute recovery of each compound was assessed at three concentration levels (0.544, 2.722, 5.444 nmol/100 mg protein) by quintuple analysis. The extraction efficiency of the six 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 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 0.272 to 16.331 nmol/100 mg trematode protein ($n = 3$). Three standard curves were prepared on three separate days and appropriate regression statistics were determined. 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.

Precision and accuracy (intra-day and inter-day) of the method were determined by evaluation of replicates of drug-free parasite material fortified ($n = 5$) with each compound at three different concentrations

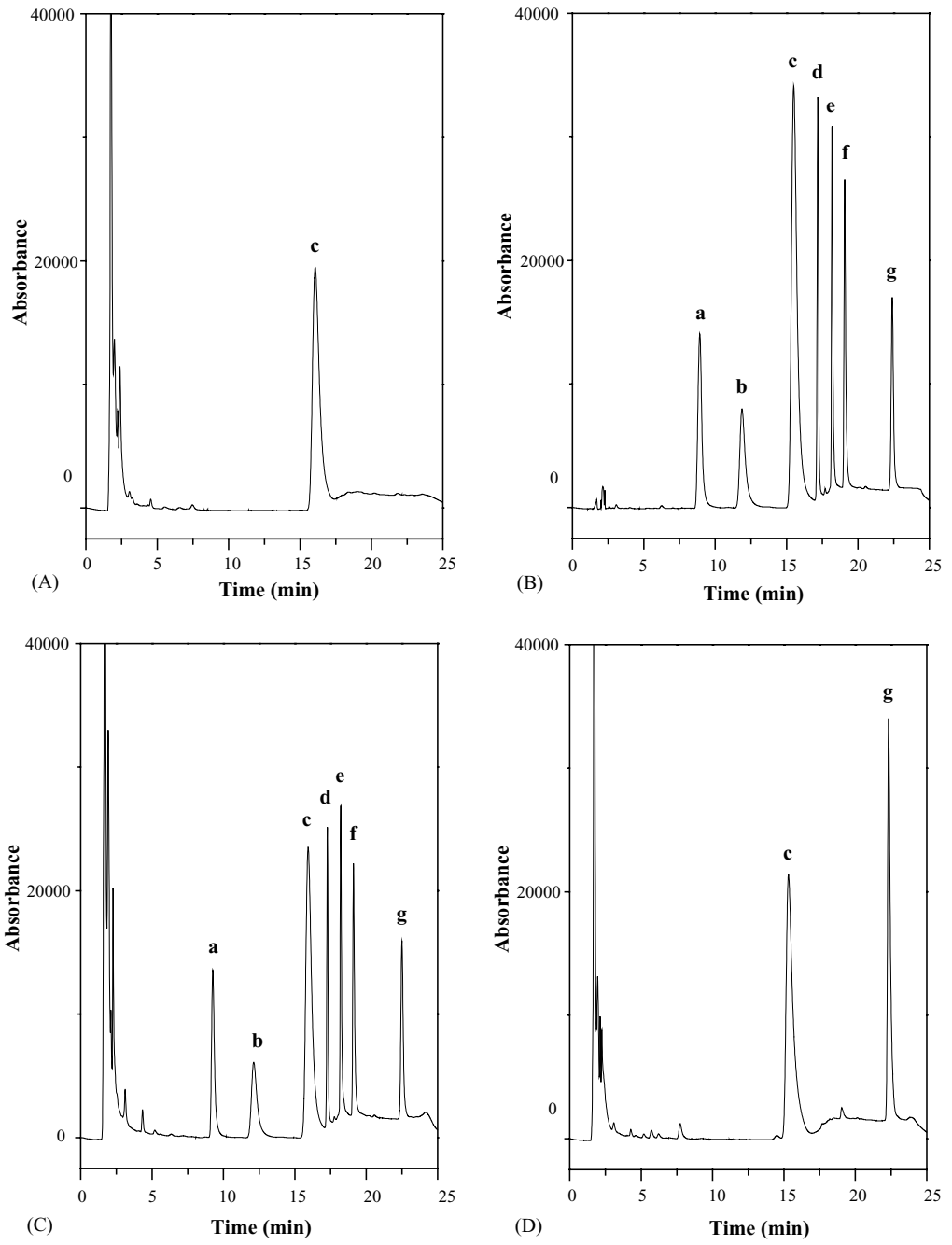

Fig. 2. Chromatograms obtained from (A) drug-free *Fasciola hepatica* parasite sample spiked with mebendazole (c) (as internal standard); (B) mobile phase and (C) *F. hepatica*, both spiked with hydroxy-triclabendazole sulphone; (a) (9.2 min), hydroxy-triclabendazole sulphoxide (b) (12.2 min), mebendazole (c) (15.9 min), triclabendazole sulphone (d) (17.3 min), hydroxy-triclabendazole (e) (18.2 min), triclabendazole sulphoxide (f) (19.1 min) and triclabendazole (g) (22.5 min); (D) *F. hepatica* incubated with triclabendazole (g) during 180 min [mebendazole (c) as internal standard].

(0.544, 2.722, 5.444 nmol/100 mg protein). The evaluation of the intra-day precision involved five ($n = 5$) measurements of the parasite material samples 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 over a 10 day period for a parasite sample at the three concentrations mentioned above. Precision was expressed as coefficient of variation (%CV). Accuracy of the method was measured by the differences between observed and calculated concentration results obtained intra-day and inter-day (5 consecutive working days), and expressed as the relative error (%R.E.).

The theoretical limit of detection (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 limit of quantification (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 [24].

3. Results and discussion

3.1. Analytical method development

3.1.1. Chromatographic separation

The work reported here describes the development and complete validation of a precise, reliable and simple reversed-phase HPLC method to quantify TCBZ and its sulpho- and hydroxy-metabolites in *F. hepatica*. The HPLC separation and detection of the analytes were obtained with a gradient of 0.025 M ammonium acetate (pH 6.6) and acetonitrile and photodiode array detection (wavelength 300 nm). Under the described chromatographic conditions, the retention times were 9.2 (OH-TCBZSO$_2$), 12.2 (OH-TCBZSO), 15.9 (MBZ), 17.3 (TCBZSO$_2$), 18.2 (OH-TCBZ), 19.1 (TCBZSO) and 22.5 (TCBZ) min. The total run time for the method was 25 min. The procedure employed for the extraction of the six molecules from parasite material was simple and highly efficient. Representative chromatograms are shown in Fig. 2. Those chromatograms were obtained after analysis of *F. hepatica* blank samples spiked with the IS, MBZ (Fig. 2A), mobile phase (Fig. 2B) and fluke material (Fig. 2C) fortified with TCBZ and its metabolites (the spiked concentration for each molecule was 5.444 nmol/100 mg trematode protein) and ex vivo incubated with TCBZ over 180 min in Kreb's Ringer Tris buffer incubation medium (Fig. 2D). The *F. hepatica* blank was free of interferences in the time regions of analytical interest. No major endogenous chromatographic peaks, which could interfere with the resolution of TCBZ and its metabolites, were observed. Peaks of interest were well separated from other sample components and also from neighboring peaks; besides, good peak shape was obtained for the compounds.

3.1.2. Peak purity

The purity of the halogenated BZD peaks was confirmed by analysing experimental parasite samples using alternative chromatographic conditions (isocratic elution, 50:50 acetonitrile:ammonium acetate buffer, flow rate 1.2 ml/min, during 20 min, at 300 nm UV) which did not show the appearance of hidden interferent peaks. The resolution obtained for each band was 3.1 ± 0.2 (TCBZSO$_2$), 4.3 ± 0.1 (TCBZSO), 5.1 ± 0.2 (OH-TCBZ), 5.3 ± 0.1 (MBZ), 5.5 ± 0.3 (OH-TCBZSO), 12.7 ± 0.1 (TCBZ) and 15.5 ± 2.3 (OH-TCBZSO$_2$). The resolution obtained with the developed method is in accordance with standard analytical parameters as resolution ≥ 2 is a desirable target for method development [24].

3.1.3. Drug extraction and clean up procedures: absolute analyte recoveries

The methods for extraction and isolation of the drug are main factors influencing the overall analytical method. The intent is to isolate the drug residue with adequate (>60%) recovery, relatively free of interfering co-extractants that could complicate the analysis to be performed for the detection, identification or quantitation of the residue [25]. Mean absolute recoveries assessed at three concentrations levels (0.544, 2.722, 5.444 nmol/100 mg protein) (quintuple determinations) for all the molecules assayed are shown in Table 1. High recoveries were obtained using this

Table 1
Mean (± S.D.) values of absolute recoveries for triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZSO), triclabendazole sulphone ($TCBZSO_2$), hydroxy-triclabendazole (OH-TCBZ), hydroxy-triclabendazole sulphoxide (OH-TCBZSO) and hydroxy-triclabendazole sulphone (OH-$TCBZSO_2$) in spiked *Fasciola hepatica* samples

Molecule	Absolute recovery (%) ($n = 5$)		
	Drug concentration spiked (nmol/100 mg trematode protein)		
	0.544	2.722	5.444
TCBZ	77 ± 4.4	78 ± 10.2	83 ± 6.6
TCBZSO	76 ± 10.8	82 ± 4.1	90 ± 4.5
$TCBZSO_2$	74 ± 7.8	73 ± 9.3	81 ± 4.4
OH-TCBZ	74 ± 9.7	84 ± 9.1	86 ± 8.2
OH-TCBZSO	90 ± 8.5	84 ± 4.1	88 ± 3.3
OH-$TCBZSO_2$	71 ± 11.2	81 ± 6.9	83 ± 2.4
MBZ (IS)			86 ± 2.0

MBZ: mebendazole, used as internal standard (IS) (5.444 nmol/100 mg trematode protein).

Table 2
Evaluation of the linearity of the method: regression analysis of the calibration curves of TCBZ/metabolites in *Fasciola hepatica* obtained on 3 different days ($y = ax + b$)

Molecule	Day	Slope (a)	Intercept (b) (peak area ratio)[a]	Correlation coefficient (r)
TCBZ	1	0.0297	−0.0019	0.998
	2	0.0286	0.0009	0.999
	3	0.0272	−0.0123	0.997
TCBZSO	1	0.0318	−0.0038	0.998
	2	0.0286	0.0035	0.999
	3	0.0284	−0.0052	0.999
$TCBZSO_2$	1	0.0255	−0.0059	0.998
	2	0.0249	−0.0007	0.998
	3	0.0246	−0.0098	0.998
OH-TCBZ	1	0.0300	−0.0006	0.998
	2	0.0300	0.0024	0.999
	3	0.0259	−0.0043	0.997
OH-TCBZSO	1	0.0273	−0.0006	0.999
	2	0.0274	−0.0068	0.999
	3	0.0267	−0.0059	1.000
OH-$TCBZSO_2$	1	0.0315	−0.0018	0.996
	2	0.0307	0.0004	0.999
	3	0.0313	−0.0038	0.999

Concentration range: 0.272–16.331 nmol/100 mg trematode protein.

[a] Peak area ratio of the analyte to the internal standard.

Table 3
Analytical intra- and inter-day precision and accuracy of the determination of triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZSO), triclabendazole sulphone ($TCBZSO_2$), hydroxy-triclabendazole (OH-TCBZ), hydroxy-triclabendazole sulphoxide (OH-TCBZSO) and hydroxy-triclabendazole sulphone (OH-$TCBZSO_2$) in spiked *Fasciola hepatica* samples

		Concentration added (nmol/100 mg trematode protein)																			
		TCBZ			TCBZSO			$TCBZSO_2$			OH-TCBZ			OH-TCBZSO			OH-$TCBZSO_2$				
		0.544	2.722	5.444	0.544	2.722	5.444	0.544	2.722	5.444	0.544	2.722	5.444	0.544	2.722	5.444	0.544	2.722	5.444		
Intra-day precision (%CV)		7	5	4	4	4	3	6	3	6	5	6	4	7	4	1	4	6	4		
Inter-day precision (%CV)		12	6	5	5	4	10	9	2	10	9	8	9	7	5	5	9	4	4		
Intra-day accuracy	Concentration obtained (nmol/100 mg trematode protein)	0.603	2.729	5.293	0.612	2.700	5.300	0.573	2.730	5.420	0.626	2.675	5.323	0.531	2.864	5.397	0.462	2.586	5.798		
	(%R.E.)	10.8	0.2	−2.8	12.5	−0.8	−2.7	5.4	0.3	−0.5	15.1	−1.7	−2.2	−2.4	5.2	−0.9	−15.0	−5.0	6.5		
Inter-day accuracy	Concentration obtained (nmol/100 mg trematode protein)	0.479	2.433	5.215	0.544	2.548	5.259	0.500	2.646	4.938	0.560	2.439	5.052	0.522	2.608	5.215	0.490	2.673	5.330		
	(%R.E.)	−12.0	−10.6	−4.2	0.0	−6.4	−3.4	−8.0	−2.8	−9.3	3.0	−10.4	−7.2	−4.0	−4.2	−4.2	−10.0	−1.8	−2.1		

R.E. (relative error) = 100 [(predicted concentration–nominal concentration)/nominal concentration].

Table 4
Limits of detection (LOD) and quantification (LOQ) of the method developed in spiked *Fasciola hepatica* samples

Molecule	LOD		LOQ		
	nmol/100 mg trematode protein	Precision (%CV)	nmol/100 mg trematode protein	Precision (%CV)	Accuracy (%R.E.)
TCBZ	0.007	7	0.272	9	−8.3
TCBZSO	0.079	9	0.272	16	−5.5
$TCBZSO_2$	0.041	12	0.272	5	2.1
OH-TCBZ	0.035	11	0.272	14	−2.9
OH-TCBZSO	0.030	9	0.272	9	−7.3
$OH\text{-}TCBZSO_2$	0.017	12	0.272	8	1.3

CV: coefficient of variation, R.E. (relative error) = 100 [(predicted concentration–nominal concentration)/nominal concentration].

simple method based on liquid to liquid extraction with low acetonitrile volumes (3 × 1.5 ml); the mean recoveries calculated for the compounds assayed were within 73 and 90%, with CV < 9%. Ethylacetate and methanol, as extraction solvents for liquid extraction, were also assayed during the development of this analytical method. Changing the extraction solvent for ethylacetate raised variability between samples and extraction recoveries were lower than those obtained with acetonitrile. When methanol was used as extraction solvent, the evaporation of the recovered organic phase was slower than that obtained with acetonitrile, besides, solid phase extraction was required to obtain cleaner sample extracts, delaying drug extraction process. On the other hand, supplementary parasite material clean up by solid phase extraction, prior to analysis by reversed-phase HPLC, influenced the analyte recovery unfavourably as the recoveries obtained for TCBZ ranged between 42 and 75%. Therefore, as parasite samples were clean enough (acetonitrile precipitates trematode proteins allowing fast recovering of the organic phase after shaking) and resolution of TCBZ and its metabolites were well performed using only liquid extraction, solid phase extraction was avoid.

3.1.4. Linearity of standard curves

The standard calibration curves for the parent drug and its metabolites in the liver fluke were obtained using the linear least squares regression procedure. The linearity of the method was proved with seven calibration points (0.272, 0.544, 1.361, 2.722, 5.444, 10.887, 16.331 nmol/100 mg protein). The regression analyses were linear over the concentrations examined and the correlation coefficients (r) of the calibration curves ranged between 0.996 and 1.000. The correlation coefficients and the equations for the straight lines are shown in Table 2.

3.1.5. Precision and accuracy

The analysis of low (0.544 nmol/100 mg protein), middle (2.722 nmol/100 mg protein) and high (5.444 nmol/100 mg protein) drug concentration values was used to determine intra- and inter-day precision and accuracy. As the precision and accuracy results in Table 3 show, the method exhibited a high degree of intra- and inter-day precision and accuracy as demonstrated by low CV (≤12%) and R.E. (from −15.0 to 15.1%).

3.1.6. Limits of detection and quantification

The LOD in *F. hepatica*, giving a peak area three times the baseline noise of the blank extracted sample, were between 0.007 and 0.079 nmol/100 mg protein. LOD and LOQ values are shown in Table 4. The LOQ were low and accurate enough (0.272 nmol/100 mg protein) for the application of the method to study the comparative diffusion pattern of TCBZ and its oxidised metabolites into mature *F. hepatica* under ex vivo conditions.

3.2. Conclusion: application of the developed method

The analytical procedure developed here was successfully applied to quantify triclabendazole/metabolites in *F. hepatica* incubated under ex vivo conditions, demonstrating to be efficient for the determination of the most extensively used flukicidal drug available for veterinary medicine. Recovered liver flukes, from artificially infected sheep, were incubated from 15 to

180 min in a Kreb's Ringer Tris buffer [26] containing 5 nmol/ml of either TCBZ, TCBZSO, $TCBZSO_2$, OH-TCBZ, OH-TCBZSO or OH-$TCBZSO_2$. After the incubation time elapsed, the parasites were rinsed with saline solution, blotted on coarse filter paper, homogenised and prepared for HPLC analysis. TCBZ and all its metabolic products were recovered from *F. hepatica* after the ex vivo incubations, but the diffusion of the hydroxy-derivatives was significantly lower than that observed for TCBZ, TCBZSO and $TCBZSO_2$ (unpublished results). Understanding the relative ability of the parent drug and its metabolites to reach the target parasite may be critical to optimise TCBZ flukicidal activity, particularly when TCBZ resistant flukes have been already isolated, and TCBZSO and $TCBZSO_2$ are the main unconjugated analytes recovered in the bloodstream and bile of treated animals. To illustrate the application of this methodology, the diffusion of TCBZ into the liver fluke after 180 min of incubation is shown in Fig. 2D, where the concentration reached by TCBZ was 11.5 nmol/100 mg trematode protein (MBZ was used as IS).

The work reported here describes the development, validation and then application of a simple, well-defined, reproducible, precise, accurate and easy to perform method for the determination of one of the main flukicidal drugs available on the veterinary market, and its sulpho- and hydroxy-metabolites, in its target helminth parasite, *F. hepatica*.

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