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ORIGINAL RESEARCH PAPER

Development and validation of a rapid selective high-throughput LC-MS/MS method for the determination of triclabendazole sulfoxide concentrations in sheep plasma and its use in bioequivalence studies

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ABSTRACT

Triclabendazole is one of the main drugs used to treat liver fluke in livestock. A rapid LC-MS/MS method was developed and validated to determine ovine plasma levels of triclabendazole sulfoxide.

A Gemini NX-C18 column was used to achieve analytical separation, with gradient elution of a mobile phase composed of 0.1% formic acid in acetonitril and 0.1% formic acid in water at flow rate of 0.6 mL/min. MRM with positive ESI ionization was used for the detection of triclabendazole sulfoxide (m/z 360.10 from m/z 376.97). Fenbendazole was used as internal standard. Plasma protein precipitation with acetonitrile was used for sample processing.

The method was validated with regards to selectivity, linearity ($r > 0.9939$), within run and between run precision ($CV < 8.9\%$) and accuracy (bias $< 8.9\%$) over the concentration range 1–100 $\mu\text{g/mL}$ plasma.

The method developed is simple, selective and can be applied in bioequivalence and bioavailability studies.

KEYWORDS

triclabendazole, fenbendazole, LC/MS, ovine, plasma

INTRODUCTION

Hepatic dystomatosis, or fasciolosis, is a parasitic liver disease, following infection with *Fasciola hepatica* and *Fasciola gigantica*. The disease is common for sheep and cattle in areas with wet grass and rare for humans. These trematodes are responsible for wide-spread morbidity and mortality in sheep and determine the diminishing of wool growth, weight loss and liver disease [1].

Fasciolosis is “an emerging animal disease”, a “zoonosis of major global and regional importance” [1, 2]. *F. hepatica* and *F. gigantica* are found in tropics and sub-tropics, but also in temperate areas and in cooler areas of high altitude [1]. The infection is now widespread all over the world, in Africa and Asia both species are incriminated, in Europe and America it seems that only *F. hepatica* is transmitted. In Europe there are cases in France and the United

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Kingdom as a consequence of eating contaminated fresh salad [3]. Fasciolosis is common in poor people living in cattle-raising areas which are infected through the consumption of fresh vegetables, drinking water contaminated with the larval stage metacercariae [4]. Generally a minor concern for public health in most countries, globally it is estimated that 180 million humans are at risk of infection with 17 million already being infected with the these parasites [5].

Triclabendazole is a benzimidazole anthelmintic used for the control of liver fluke, it kills early immature and adult *Fasciola* species, in sheep and other livestock [6]. Unfortunately, the parasites can develop resistance to triclabendazole and that compromises fluke control in livestock and humans, too [7].

The success of triclabendazole against livestock infections with *Fasciola* is dated in the early 1980s [8]. After oral administration, the absorbed triclabendazole cannot be detected in the plasma due to its fast clearing by the liver where it is metabolised to triclabendazole sulfoxide and triclabendazole sulphone [8]. The metabolites appears in plasma in 2 h and persisted about 170 h maybe due to the fact that they are bound to plasma proteins [8]. Triclabendazole and its two main metabolites are transformed in hydroxylated chemical species in liver, which are fully excreted in bile as free and conjugated compounds [8]. Triclabendazole sulfoxide and triclabendazole sulphone are present in bile, too [8]. Triclabendazole is mainly eliminated in conjugated form, about 50%, through bile and less than 10% is excreted in the urine [8]. The success of its activity could be explained by the long residence in blood of active triclabendazole metabolites, their action being now considered as an additive effect [8]. Even though triclabendazole sulfoxide is not the only active metabolite and other metabolites, such as triclabendazole sulphone, have been shown to have some activity against the liver fluke, triclabendazole sulfoxide is still the metabolite most active and effective against the fluke and is the main metabolite detected in the hosts [8].

According to the Joint FAO/WHO Expert Committee on Food Additives triclabendazole is administered in oral doses of 12 mg/kg body weight for cattle and for sheep and goats the dose is 10 mg/kg body weight [9]. If the treatment is unsuccessful, triclabendazole is readministered in 10 mg/kg dose followed by another equal dose after 12–24 h, for a total dose of 20 mg/kg [2].

Barrera et al. (2012) showed that triclabendazole and its metabolites could have a potential “role as ABCG2 inhibitors to participate in drug interactions and modulate ABCG2-mediated pharmacokinetic processes”, the strongest inhibition being observed for triclabendazole, probable to the fact that is the most hydrophobic between them [6].

The determination of drug concentrations in plasma or other biological samples through liquid chromatography coupled with mass spectrometry (LC-MS/MS) methods is now considered a technological prerequisite for highly specific and sensitive analysis. Determination of triclabendazole metabolites from different types of biological matrices in

animals has been described in several studies using different analytical methods [10–16]. Several methods reported in literature use high performance liquid chromatography (HPLC) to determine triclabendazole and its metabolites in the plasma of different species [10, 13, 14]. In recent years, several LC-MS/MS methods have been described in literature for the determination of triclabendazole and its metabolites from bovine milk [15, 16], and fat, muscle, liver and kidney tissues of cattle and goat [11, 12]. No methods have yet been described in literature for the determination of triclabendazole or its metabolites from ovine plasma using a validated LC-MS method.

One of the earliest analytical methods for the determination of triclabendazole from biological samples was report by Bull and Shume [10]. This method used HPLC for analyte separation coupled with UV detection, a relatively new technique at that time. The total runtime for each sample was more than 40 min and for sample purification solid phase extraction was used in this methodology. A research team lead by Chengyuan Cai developed LC-MS methodologies used to determine triclabendazole and its metabolites from different types of bovine and goat matrices, such as muscle tissues and internal organs (kidney, liver) [11], as well as fat tissue [12]. Muscle, kidney and liver samples were purified using extraction of interfering compounds with hexane. Analytical separation was performed on an XTerra MS C18 HPLC column with mobile phase consisting of acetonitrile and aqueous formic acid solution in gradient elution, while detection was carried out after positive electrospray ionization, having a total runtime of 20 min for each sample [11]. For analysis of triclabendazole and its metabolites from fat tissues Cai and his research team optimized the previous method, simplifying the sample purification of samples. This method uses simpler liquid-liquid extraction for the purification of biological samples, while chromatographic conditions are the same as with the previous method described by the research team, with analytical separation performed on an Xterra C18 analytical column using aqueous formic and acetonitrile as eluents, positive ESI ionization and a total runtime of 20 min per sample [12]. Cañas-Müller and his research team reported an analytical methodology for the quantification of triclabendazole and its metabolites from the plasma of cattle which uses rotating disk sorptive extraction (RDSE) for sample extraction and clean up of the compounds from the plasma. Analysis was performed with HPLC coupled with UV detection using acetonitrile and water as mobile phase in isocratic elution and a C18 HPLC column. Under these chromatographic conditions the total runtime is 13 min for one sample [13]. Ceballos et al. developed an analytical method to measure triclabendazole and its metabolites from sheep plasma for pharmacokinetic studies [14]. The method uses protein precipitation for plasma sample purification, after which analytes are chromatographically separated on a Selectosil C18 analytical column and detected using a UV detector. The total runtime was over 11 min for each sample [14]. Research teams of Takeba and Kinsella both developed analytical methods for the determination of triclabendazole

and its metabolites from bovine milk [15, 16]. Takeba et al. described an HPLC method using reverse phase chromatography to separate analytes on a Capcell Pak C18 column with acetonitrile and aqueous ammonium acetate in isocratic elution, with detection being performed with both UV and MS detectors [16]. Milk samples were homogenized with anhydrous sodium sulfate and acetonitrile, centrifuged, supernatant rinsed with hexane saturated with acetonitrile, and ultimately evaporated [16]. Total analysis time was 30 min when using UV detection and 15 min when using MS detection coupled with ESI negative ionization. Comparatively the LC-MS method developed by Kinsella et al. can be used to measure a wider array of compounds and uses a sample purification method comparable to the one described by Takeba et al. but with an added step which uses solid-phase extraction (SPE). Reverse phase chromatography for the separation of compound used a C18 type analytical column and mobile phase consisting of aqueous ammonium formate and ammonium formate in acetonitrile in gradient elution, the final runtime amounting to 15.5 min per sample. Detection was carried out in MS/MS mode after negative ESI ionization of triclabendazole sulfoxide [15]. Currently there are no methods reported in literature which describe analytical methodologies for the measurement of triclabendazole sulfoxide from ovine plasma which use LC-MS.

The aim of our study was to develop a new analytical method to be used for the quantification of triclabendazole sulfoxide from sheep plasma samples. The study was conducted in order to propose a method suitable for high-throughput bioavailability and bioequivalence studies using a simple, fast and inexpensive sample preparation method, short analytical runtime and validated performance parameters according to current guidelines [17, 18].

EXPERIMENTAL

Reagents

HPLC grade acetonitrile and formic acid 98–100% of analytical grade were manufactured by VWR International (Radnor, USA). A Milli-Q Integral 3 Water purification system (Millipore - Milford, USA) was used to obtain ultrapurified water. Ovine blank plasma was supplied by the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (Romania) and was obtained from the healthy animals. Triclabendazole sulfoxide and fenbendazole Vetranal analytical standards were purchased from Sigma-Aldrich (St. Louis, USA).

Apparatus and equipment

Thermo Fisher Scientific (Waltham, USA) LC-MS system composed of Accela HPLC system (Accela 600 quaternary pump with inline degasser, PAL HTS-xt auto-sampler, Accela VIM 2303TX column compartment) coupled with Thermo Fisher Scientific TSQ Quantum Access Max mass spectrometer was used for analytical separation and detection. Other equipment used: Centurion Scientific (Stoughton, United

Kingdom) K3 centrifuges; Mettler-Toledo (Greifensee, Switzerland) XS204 analytical balance; Velp Scientifica (Usmate Velate, Italy) Vortex mixer; Elma Transsonic (Singen, Germany) ultrasonic bath; Mettler-Toledo Rainin series and Eppendorf Reference 2 automatic pipettes.

LC-MS parameters

Chromatographic separation of triclabendazole sulfoxide and fenbendazole (internal standard – IS) was performed using a Gemini NX-18 2.0 × 50 mm, 3 µm column (Phenomenex Inc) thermostated at 30 °C with a mobile phase consisting of 0.1% formic acid in acetonitrile (mobile phase A) and 0.1% formic acid in water (mobile phase B) in gradient elution, the mobile phase gradient was as follows: 0–1 min 35% mobile phase A, 1–2.5 min 55% mobile phase A, 2.5–4 min 35% mobile phase A. The flow rate was constant at 0.6 mL/min. Detection was carried out in multiple reaction monitoring (MRM) mode for triclabendazole sulfoxide and fenbendazole, monitoring transition m/z 360.10 derived from m/z 376.97 ion at collision energy of 19 V for triclabendazole sulfoxide and m/z 268.08 derived from m/z 300.07 ion at collision energy of 23 V for fenbendazole (IS). Ionization of samples was performed using heated electrospray ion source in positive ionization mode. The following ionization parameters were used for the ionization source: Spray voltage: 3800 V, vaporizer temperature: 300 °C, sheath gas pressure: 25 psi, Ion Sweep Gas Pressure: 3.0 psi, Aux Gas Pressure: 7 psi, capillary temperature: 300 °C, capillary offset: 5, Tube Lens Offset: 45, Skimmer Offset: –0, Collision pressure: 1.6 psi. Sample run-time was 4 min for each sample.

Standard solutions

Two stock solutions of triclabendazole sulfoxide in plasma were prepared having concentrations of 100 and 500 µg/mL respectively. These stock solutions were used to obtain the calibration working solutions and reference working solutions. An IS solution having a concentration of 2 µg/mL of fenbendazole in acetonitrile was prepared. Plasma calibration standards for the calibration curve with concentrations of 1 (lower limit of quantification – LLOQ), 2, 5, 10, 25, 50, 75 and 100 µg/mL, and quality control (QC) plasma samples with concentrations of 3 µg/mL (lower), 40 µg/mL (medium) and 80 µg/mL (higher) were prepared using the working solutions and IS solution. 200 µL plasma working solution was spiked with 100 µL IS solution and deproteinized with 500 µL acetonitrile, vortexed for 2 min and centrifuged for 3 min at 10,000 rpm. Volumes of 100 µL of supernatant was diluted with 900 µL of a 50:50 mixture of acetonitrile and 0.1% aqueous formic acid solution in a chromatographic vial and inserted into the auto-sampler. Sample volumes of 5 µL were injected into the LC-MS system. The concentration of internal standard in the final solution was 250 ng/mL of fenbendazole.

Sample preparation and analyte extraction

In order to analyze plasma samples 200 µL plasma sample was spiked with 100 µL IS solution and deproteinized with

500 μL acetonitril, vortexed for 2 min and centrifuged for 3 min at 10,000 rpm. Volumes of 100 μL of supernatant was diluted with 900 μL of a 50:50 mixture of acetonitrile and 0.1% aqueous formic acid solution in a chromatographic vial and inserted into the auto-sampler. Sample volumes of 5 μL were injected into the LC-MS system.

Method validation

Selectivity of the method was tested. For selectivity testing chromatograms of plasma samples spiked with triclabendazole sulfoxide and IS were compared to blank plasma sample chromatograms. Six different sources of appropriate blank matrix were analyzed and evaluated for interference against a sample spiked at the lower limit of quantification.

The method was tested for carryover effect by injecting a blank sample after a high concentration plasma calibration standard sample (100 $\mu\text{g}/\text{mL}$) in each run of the validation process.

Concentrations were calculated automatically by the instrument data system using the internal standard method. Calibration curves were linear and constructed from single calibration standards, using $1/y^2$ weighting factor.

Within run and between run accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) and precision (expressed as coefficient of variation, CV, %) were determined by analyzing five samples ($n = 5$) of LLOQ and each quality control plasma sample (lower, medium and higher QC, respectively) in the same analytical run and in different analytical runs, respectively.

Extensive stability testing was carried out to determine the stability of triclabendazole sulfoxide and fenbendazole in different conditions, using quality control plasma samples (lower and higher QC samples). Long term stability, short term stability, auto-sampler stability, freeze-thaw stability and stability of stock, working and IS solutions was studied. Stock solution stability was tested for 72 h at 5 $^{\circ}\text{C}$, long term stability of plasma samples at -20°C was tested for 3 months, while short term stability of plasma samples at room temperature was tested for 4 h. Auto-sampler stability of plasma samples was tested for 24 h (kept in the auto-sampler at 5 $^{\circ}\text{C}$), while freeze-thaw stability (from -20°C to room temperature, then back to -20°C) was tested for 3 cycles.

Validation of dilution integrity in case of diluting samples was studied by preparing samples with the concentration above ULOQ (100 $\mu\text{g}/\text{mL}$) having a concentration of 200 $\mu\text{g}/\text{mL}$ which were then diluted, with dilution factor of 10, with blank plasma to a concentration of 20 $\mu\text{g}/\text{mL}$, a value within the calibration curve. Between-run and within-run accuracy and precision of diluted samples was calculated in order to determine dilution integrity.

Matrix effect and recovery were also investigated by analyzing four lower QC and four high QC samples prepared in plasma, and one lower QC and one high QC sample prepared in ultrapurified water. The recovery of the analyte and IS was determined by calculating the ratio of peak areas in the presence of plasma and in absence of plasma. In order

to determine the matrix effect matrix factor (ratio between area in presence of matrix and in the absence of matrix, MF) of both the analyte and the IS was calculated, and then the CV of the IS normalized matrix factor (MF of the analyte divided by the MF of the IS) was calculated for the four lower QC and four high QC samples prepared in plasma.

RESULTS AND DISCUSSION

Detection was optimized for triclabendazole sulfoxide with the optimal results being obtained using heated electrospray positive ionization (HESI) mode. Both electrospray and atmospheric pressure chemical ionization (APCI) were tested, as well as positive and negative ionization mode. Intensity of signals for triclabendazole sulfoxide was higher and more stable using electrospray ionization compared to APCI ionization. Due to its chemical structure triclabendazole sulfoxide is easily ionized in positive ionization mode, while negative ionization is extremely poor. In positive ionization mode triclabendazole sulfoxide accepts a proton due to the acidic mobile phase forming the molecular ion $[\text{M}+\text{H}]^{+}$ with a mass to charge ratio m/z 376.97 which is then fragmented to fragment m/z 360.10 at a collision energy of 19 V. Fenbendazole ionizes well under the same conditions as triclabendazole sulfoxide and forms the protonated molecular ion m/z 300.07 ion which is fragmented to m/z 268.08 at a collision energy of 23 V.

The chromatographic column was selected in order to achieve optimal peak separation of analyte and IS peaks as well as good shape of chromatographic peaks. After testing analytical separation on Zorbax SB-C18 100×3 mm, Gemini NX-C18 100×3 mm, Gemini NX-C18 50×2 mm and Hypersil Gold C18 50×2 mm columns and with various types of mobile phases consisting combinations of acetonitrile, acetonitrile with 0.1% formic acid, methanol and 0.1% aqueous formic acid the best chromatographic results were achieved using Phenomenex Gemini NX-C18 2.0×50 mm (3 μm) column and a mobile phase consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in water in gradient flow, at a flow rate of 0.6 mL/min, with the column thermostated at 30 $^{\circ}\text{C}$. The shorter 50×2 mm columns (Gemini NX-C18 and Hypersil Gold C18) offered sufficient retention for the analyte and internal standard, but using the Gemini NX-C18 column resulted in better peak shapes. The mobile phase gradient was chosen in such a way as to offer a good retention of both the analyte and internal standard, a good analytical separation between them, but at the same time reduce the runtime for each sample as much as possible in order to maximize the number of sample which can be analyzed. No interfering peaks were detected at the retention times of triclabendazole sulfoxide (2.10 min) and fenbendazole (0.9 min) (Fig. 1). No carryover effect was detected for the analyte and carryover was 0.18% or less for the IS.

All calibration curves were linear, correlation coefficients for all calibration curves were higher than 0.99 (R^2 between

Fig. 1. Peaks obtained for triclabendazole sulfoxide and fenbendazole from blank sheep plasma sample (panels A and B) and sheep plasma spiked with triclabendazole sulfoxide and fenbendazole at the LLOQ (panels C and D)

0.9939–0.9987) throughout the entire concentration range (1–100 µg/mL). The calibration range was chosen in such a way as to cover the expected plasmatic concentrations. The lower limit of quantification (LLOQ) was selected in order to be at least 5% of the expected maximum plasmatic concentration (C_{max}) concentration in plasma samples. Even though the based on the signal-to-noise ratio the LLOQ could have been set at a much lower value, this was not deemed necessary as the expected concentrations were of the magnitude of tens of micrograms per milliliter of plasma. This however means that concentration lower than 1 µg/mL could be theoretically measured after adapting the method and appropriate validation. The accuracy and precision over the tested calibration range showed satisfactory performance of the method. Results for within run and between run precision and accuracy were below ±15%, and are shown in Tables 1 and 2. The stability of triclabendazole sulfoxide and

internal standard solutions was proven under various conditions (long term stability, short term stability, auto-sampler stability, freeze-thaw stability and stability of stock, working and IS solutions) and were within acceptance criteria of validation guidelines [17, 18].

Dilution integrity of plasma samples, if dilution of certain samples with blank plasma is needed, was also

Table 1. Within run accuracy and precision for triclabendazole sulfoxide

C _{nominal} (µg/mL)	Mean C _{found} (µg/mL (± SD))	Bias (%)	CV (%)
0.999 (LLOQ)	0.9567 (0.086)	−4.2	8.9
2.997 (QCA)	3.1899 (0.058)	6.4	1.8
39.96 (QCB)	43.3961 (1.082)	8.6	2.5
79.92 (QCC)	78.6608 (2.168)	−1.6	2.8

Table 2. Between run accuracy and precision for for triclabendazole sulfoxide

c_{nominal} ($\mu\text{g/mL}$)	Mean c_{found} ($\mu\text{g/mL}$ ($\pm$ SD))	Bias (%)	CV (%)
0.999 (LLOQ)	0.9956 (0.087)	−0.3	8.7
2.997 (QCA)	3.1899 (0.109)	8.9	3.3
39.96 (QCB)	42.1756 (1.192)	5.5	2.8
79.92 (QCC)	79.3730 (1.729)	−0.7	2.2

proven to be within acceptance criteria described in guidelines, as presented in Table 3. Thus, for plasma samples which yield concentrations above the 100 $\mu\text{g/mL}$ concentration limit a tenfold dilution with blank plasma can be applied in order to accurately measure the levels of triclabendazole sulfoxide.

Stability of triclabendazole sulfoxide and fenbendazole in different conditions was determined, using quality control plasma samples (lower and higher QC samples). Stock solution stability was proven for 72 h at 5 °C, long term stability of plasma samples at −20 °C was proven for at least 3 months, while short term stability of plasma samples at room temperature was proven for at least 4 h. Auto-sampler stability of plasma samples was proven for at least 24 h (kept in the autosampler at 5 °C), while freeze-thaw stability (from −20 °C to room temperature, then back to −20 °C) was proven for at least 3 cycles. Results for stability testing of triclabendazole sulfoxide are presented in Table 4.

The study of matrix effect showed that there is minimal effect of the matrix (ovine plasma) on the final results (Table 5). This means that measurements of triclabendazole

Table 3. Average accuracy, precision within and between runs for dilution integrity testing of triclabendazole sulfoxide – dilution factor of 10 from 200 $\mu\text{g/mL}$, $n = 5$

Analyte	Dilution integrity test	Avg. accuracy (% bias)	Avg. precision (% RSD)
Triclabendazole sulfoxide	Within runs	−12.3	9.8
	Between runs	−9.3	12.3

Table 4. Stability of triclabendazole sulfoxide under different conditions

Analyte	Stability test	Accuracy range (% bias)
Triclabendazole sulfoxide	Stock solution stability	2.8 $\div$ +10.9
	Freeze-thaw stability	−3.3 $\div$ +10.7
	Short-term stability	0.0 $\div$ +8.4
	Post-preparative stability	0.5 $\div$ +3.5
	Long-term stability	−1.5 $\div$ +13.5

Table 5. Matrix effect for triclabendazole sulfoxide

Analyte	IS Normalized MF	Coef. of variation (%)
Triclabendazole sulfoxide	0.978–0.983	3.08–4.39

sulfoxide levels from plasma are not influenced by the inter-individual variation of the plasma from different subjects.

The method was used to measure triclabendazole sulfoxide plasma levels from samples collected in a bioequivalence study of two oral triclabendazole formulations [19]. The study protocol was reviewed and approved by the National Ethics Committee and the Romanian National Agency for Medicines and Medical Devices. Chromatograms for study samples obtained from ovine subject plasma samples collected predose and around the maximum plasma concentration (C_{max}) are presented in Fig. 2, a typical plasma curve obtained for an ovine subject is presented in Fig. 3.

Method comparison

Various methods for the determination of triclabendazole and its metabolites in biological matrices of different species by HPLC or LC/MS, alone or simultaneously with other analytes [10–16] have been described in literature.

The method described by Bull and Shume uses HPLC-UV to measure triclabendazole and its metabolites from sheep and cattle plasma, a relatively new technique at the time it was developed, but which has inferior selectivity and sensitivity compared to LC-MS. The total runtime for each sample was more than 40 min which makes it impractical for analyzing the large number of samples which are required to be analyzed in pharmacokinetic studies. The method also uses solid phase extraction for sample cleanup which is time consuming and can be expensive when applied to a large number of samples due to the cost of SPE cartridges [10]. Comparatively protein precipitation is simple, fast and very cost-effective, being ideal for processing large numbers of plasma samples.

Cai et al. developed two very similar LC-MS methodologies used for measuring triclabendazole and its metabolites from bovine and goat muscle tissues and internal organ tissues (kidney, liver) [11], as well as fat tissue [12]. The two methods are different only in sample clean up procedure which is adapted to the tissues which need to be analyzed. Analytical separation for both methodologies is performed on XTerra MS C18 HPLC columns (100 $\times$ 2.1 mm) with mobile phase consisting of acetonitrile and 0.1% aqueous formic acid solution in gradient elution. Total runtime was 20 min for each sample [11–12]. Comparatively the method described in the current manuscript has a total runtime of only 4 min which enables the analysis of a larger number of samples during the same time period and use of smaller quantities of mobile phase for analysis.

Cañas-Müller et al. developed a method for the quantification of triclabendazole and its metabolites from the

Fig. 2. Peaks obtained for tricloabendazole sulfoxide and fenbendazole from study sample collected from an ovine subject at predose (panels A and B) and around the C_{max} value (panels C and D)

Fig. 3. Typical plasma profile of tricloabendazole sulfoxide after oral administration of single dose 30 mg/kg bodyweight of suspension, to a healthy subject

plasma of cattle which uses rotating disk sorptive extraction (RDSE) for sample extraction and clean up of the compounds from the plasma. This method however can be much more expensive and time consuming compared to protein precipitation which is a much simpler and faster technique. This method uses HPLC coupled with UV detection which is inferior with regards to selectivity and sensitivity compared to mass spectrometric detection. The separation is also performed on a very large HPLC column with a length of 250 mm and diameter of 4.6 mm which results in a total runtime of 13 min for each sample, compared to the much shorter runtime achieved using the smaller Gemini NX-C18, 50 × 2 mm, analytical column [13].

The method developed by Ceballos et al. to measure tricloabendazole and its metabolites from sheep plasma also uses protein precipitation for plasma sample purification,

however chromatographic separation is performed using a Selectosil C18 analytical column (250 mm × 4.6 mm) and UV detection. The total runtime for this method was over 11 min per sample [14]. Compared to this method the runtime of our method is much shorter and uses mass spectrometry for detection, which make it more suitable for the analysis of larger number of samples from different subjects.

The LC-MS method developed by Kinsella et al. can be used to measure a wider array of compounds from bovine milk. The final runtime of this method is 15.5 min per sample, with triclabendazole sulfoxide having a retention time of 10.6 min [15]. Comparatively our method has a total runtime of 4 min and a retention time for triclabendazole sulfoxide of only 2.10 min. This allows for a much faster analysis and larger numbers of samples.

Takeba et al. described an HPLC method for analysis of triclabendazole and its metabolites from bovine milk with both UV and MS detection. Total analysis time was 30 min when using UV detection and 15 min when using MS detection, much longer than our method which has a runtime of only 4 min per sample [16].

Compared to existing methods described in literature for the measurement of triclabendazole sulfoxide from biological samples our method has a shorter runtime for one sample which combined with the very simple sample preparation method offers the optimal conditions for simple, inexpensive, fast, high-throughput measurement. The use of mass spectrometry for measuring triclabendazole sulfoxide from plasma offers good selectivity and sensitivity which reduces the risk of interference from coeluting peaks from plasma compounds. However no LC-MS methods have yet been described for the determination of triclabendazole sulfoxide in ovine plasma, the presented method being the first described in literature. Such a method, being fully validated, can be used in various bioavailability, bioequivalence or other types of pharmacokinetic studies involving triclabendazole.

The method developed and validated is simple, rapid and selective, needs small amounts of ovine plasma samples (200 µL) and the samples processing technique is inexpensive, simple and rapid. The large range of the calibration curve (1–100 µg/mL) allows samples in a wide concentration range to be analyzed giving the method a wide usability and the short run-time makes it ideal for bioavailability and bioequivalence studies with large numbers of sample.

Due to validation being audited by the Romanian National Agency for Medicines and Medical Devices the method validation was carried out in total accordance with current international guidelines [17, 18].

The calibration curves were linear and the method is sensitive, selective, as well as accurate and precise over the entire concentration range studied. Requirements for accuracy and precision described in international guidelines were also met (Tables 1 and 2). The matrix effect testing on six different types of ovine plasma shows no major influence of plasma components on sensitivity, selectivity or accuracy, the method is robust and can be used for any type of ovine plasma.

By demonstrating the stability of the various solutions in different types of conditions the current method can also aid when planning for the analysis of triclabendazole sulfoxide samples from either the same matrix as the presented study (plasma), or in some cases from other matrices. The fact that dilution integrity of samples after dilution with blank plasma was proven to be within acceptance criteria means that the method could be applied to samples obtained from studies which may yield plasma concentrations of triclabendazole sulfoxide outside of the interval studied in this research.

CONCLUSIONS

We succeeded in achieving the main aim of our study, which was to develop and validate a new rapid, simple, inexpensive, sensitive and selective LC-MS/MS method for the measurement of ovine plasma concentration levels of triclabendazole sulfoxide to be used bioavailability and bioequivalence studies or other types pharmacokinetic studies (e.g. drug-drug interaction studies) of triclabendazole formulations. The method was successfully used in a bioequivalence study of two oral triclabendazole formulations.

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