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# Development and validation of a liquid chromatography/mass spectrometry method for pharmacokinetic studies of OZ78, a fasciocidal drug candidate

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ABSTRACT

Fascioliasis is a zoonotic disease of considerable public health and great veterinary significance and new drugs are needed. OZ78 is a promising fasciocidal drug candidate. In order to support the development of OZ78, including pharmacokinetic (PK) studies an accurate, precise, and selective liquid chromatography/mass spectrometry (LC/MS) method for OZ78 was developed for sheep plasma and validated in accordance with the US Food and Drug Administration Guidance on Bioanalytical Method Validation. Protein precipitation was used for sample clean up. Separation was performed through a Phenomenex C8(2) analytical column (50.0 mm × 2.0 mm, 5 μm) with a mobile phase of acetonitrile (buffer B) and 5 mM ammonium formate (buffer A) at a flow-rate of 0.3 mL/min and a gradient from 20% to 95% acetonitrile. The mass spectrometer was operated under selected ion monitoring, and orifice voltage set to −4.1 kV and ion spray temperature to 400 °C. Nitrogen was used as a nebulizer, curtain, and collision gas. OZ78 was monitored at 321.4 *m/z* (deprotonated parent compound, M-). The validated linear dynamic range was between 156.25 ng/mL and 5 μg/mL and the achieved correlation coefficient ($r^2$) was greater than 0.99. The validation results demonstrated that the developed LC/MS method is precise, accurate, and selective for the determination of OZ78 in sheep plasma. The method was successfully applied to the evaluation of the PK profile of OZ78 in sheep.



## 1. Introduction

The secondary ozonide, 1,2,4-trioxolane OZ78 belongs to a new class of synthetic peroxide antimalarials [1]. In contrast to the semisynthetic artemisinins, currently the most important antimalarials available [2], 1,2,4-trioxolanes are characterized by excellent biopharmaceutical properties and low toxicities [1]. OZ277 combined with piperaquine is undergoing phase III clinical testing [3]. Another trioxolane antimalarial candidate, OZ439, is currently in phase IIa trials [4].

The 1,2,4-trioxolanes do not only possess antiplasmodial but also trematocidal activities, since hemoglobin metabolism is common in plasmodia and several trematodes including *Fasciola* spp. [3]. A recent structure-activity study evaluating 26 OZ derivatives in the *Fasciola hepatica* rat model, showed OZ78 to have an optimized ozonide structure for efficacy against *F. hepatica*. At a single oral dose of 100 mg/kg, OZ78 cured acute (immature flukes) and chronic (adult flukes) *F. hepatica* infections in rats, including infections with resistant isolates [5]. Addition of a new drug in the current drug armamentarium for the treatment of trematode infections, which are neglected tropical diseases [6,7], would be most welcome. Currently the treatment of fascioliasis in humans relies on one single drug, triclabendazole. Though triclabendazole resistance has not been shown in humans, resistance to this drug is widely spreading in sheep and cattle [8,9]. In veterinary medicine additional drugs are commonly used in the treatment of infections with *Fasciola* spp. including albendazole, closantel, hexachlorophene, mebendazole, nitroxynil, rafoxanide, and triclabendazole [9]. Different analytical methods have been established in order to quantify these drugs and their corresponding metabolites in biological fluids or tissues. For example, a HPLC with fluorescence detection has recently been developed to screen closantel and rafoxanide in animal muscles [10].

An efficacy and tolerability study with OZ78 in target animals, *F. hepatica* infected sheep, was recently conducted in Australia.

*Abbreviations:* ESI, electrospray ionization; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; LLOQ, lowest limit of quantification; OZ, ozonide; PK, pharmacokinetic; QC, quality control; RSD, relative standard deviation; USFDA, US Food and Drug Administration.

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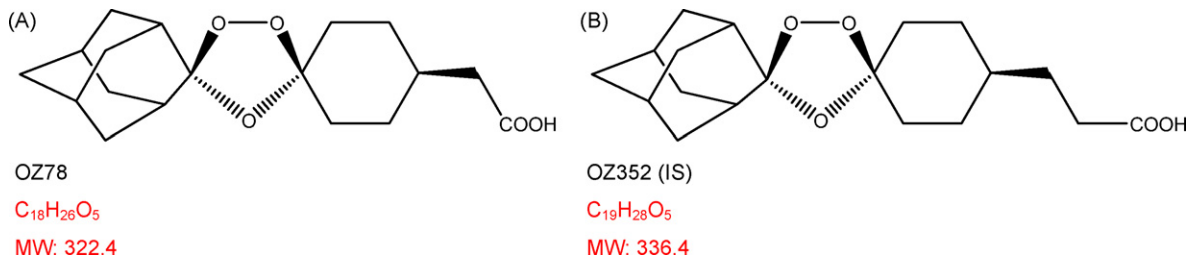


**Fig. 1.** Structures of (A) OZ78 and (B) OZ352 [internal standard (IS)].

Sheep were administered single oral or subcutaneous doses of 50 mg/kg OZ78. Tolerability, egg reduction rates, and worm burden reductions were recorded. In addition, serum samples were collected at selected time points [12]. It was the aim of the present study to develop and validate a LC/MS method to quantify OZ78 in sheep plasma to support analysis of the pharmacokinetic (PK) parameters of OZ78 of this study and future preclinical trials.

## 2. Experimental

### 2.1. Chemicals and reagents

OZ78 and OZ352 (internal standard, IS) were synthesized at the College of Pharmacy, University of Nebraska Medical Center (Omaha, USA). The chemical structures of OZ78 and OZ352 are depicted in Fig. 1.

Acetonitrile and methanol were of HPLC grade and were obtained from Bisolve (Valkenswaard, Netherlands) and J.T. Baker (Deventer, Netherlands), respectively. Ammonium formate was purchased from Fluka Analytical (Buchs, Switzerland). Ultrapure water was obtained from a Millipore Milli-Q water purification system and applied for the preparation of mobile phase. Blank sheep serum and plasma were obtained from Novartis Animal Health (Kemps Creek, Australia) and the local slaughterhouse (Basel, Switzerland).

### 2.2. LC/MS/MS system and conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), and an online DG-3310 degasser (Sanwa Tsusho, Tokyo, Japan). Chromatographic separation was performed through a Phenomenex C8(2) (50.0 mm × 2.0 mm, 5 μm, Brechbühler AG, Schlieren, Switzerland) column coupled with a Phenomenex security guard used at room temperature. The following gradient was used: 0–4 min, B 20–35%; 4–7 min, B 35–65%; 7–8 min, B 65–95%; 8–10 min, 95%; 10–12 min, B 95–20%; 12–14 min B 20%, where A was 5 mM ammonium formate in ultra pure water and B was 100% acetronitrile. The flow-rate of the mobile phase was set at 0.3 mL/min.

**Table 1**
LC/MS working parameters.

| Parameter | Value |
|---|---|
| Source temperature | 400 °C |
| Nebulizer gas (NEB) | 12 L/min |
| Curtain gas (CUR) | 9 L/min |
| Declustering potential (DP) | −51 V |
| Focusing potential (FP) | −335 V |
| Entrance potential (EP) | −11 V |
| Ion spray voltage (IS) | −4.1 kV |
| Polarity of analysis | Negative |
| Detected mass for OZ78 (*m*/*z*) | 322.4/321.4 |
| Detected mass for IS (*m*/*z*) | 336.4/335.4 |

An API 365 triple–quadrupole mass spectrometer (PE Biosystems, Foster City, CA) equipped with a turbo ion spray source was operated in negative ionization mode. The major working parameters are summarized in Table 1. Instrument control and data analyses were performed using the Analyst 1.4.2. software package (PE Biosystems, Foster City, CA).

### 2.3. Standard, QC and IS preparation

Stock solutions (OZ78 150 μg/mL, IS 150 μg/mL) were prepared in methanol. Appropriate volumes of stock solutions were serially

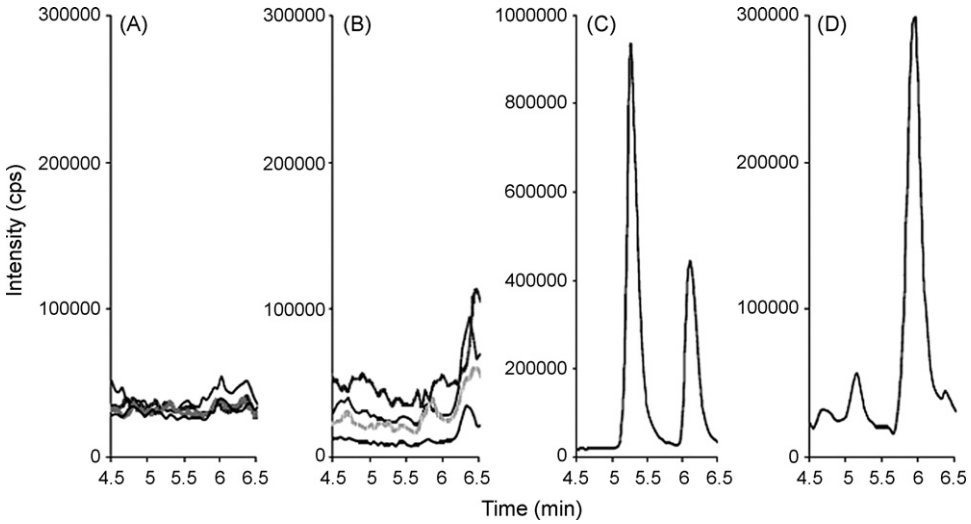


**Fig. 2.** Chromatograms of OZ78 and the internal standard OZ352 covering a retention time of 4.5–6.5 min: (A) blank sheep serum ($n = 4$), (B) blank sheep plasma ($n = 4$), (C) mobile phase spiked with OZ78 5 μg/mL and OZ352 (IS) 2 μg/mL, and (D) spiked plasma at concentrations of 0.3125 μg/mL (OZ78) and 2 μg/mL (OZ352).

**Table 2**
Intra- and inter-assay accuracy and precision.

| Nominal concentration (μg/mL) | Intra-assay ($n = 5$) | | | Inter-assay ($n = 20$) | | |
|---|---|---|---|---|---|---|
| | Mean concentration (μg/mL) | RSD (%) | Accuracy (%) | Mean concentration (μg/mL) | RSD (%) | Accuracy (%) |
| 0.3125 | 0.30 | 11.07 | 95.52 | 0.29 | 6.27 | 92.68 |
| | 0.29 | 3.87 | 91.54 | | | |
| | 0.28 | 4.66 | 91.04 | | | |
| | 0.29 | 5.29 | 93.19 | | | |
| 1.25 | 1.28 | 3.71 | 102.11 | 1.23 | 5.99 | 98.48 |
| | 1.21 | 6.00 | 96.79 | | | |
| | 1.17 | 6.77 | 93.91 | | | |
| | 1.26 | 4.63 | 101.13 | | | |
| 5 | 5.24 | 3.54 | 104.73 | 4.79 | 8.09 | 95.90 |
| | 4.65 | 4.78 | 93.06 | | | |
| | 4.51 | 5.06 | 90.15 | | | |
| | 4.87 | 9.59 | 97.43 | | | |

diluted with methanol to obtain OZ78 working solutions from 4.7 to 150 μg/mL or a working IS solution at 60 μg/mL.

Calibration samples were freshly prepared and included in each analytical run by the dilution of working solutions with blank sheep plasma, resulting in concentrations of 5000, 2500, 1250, 625, 312.5, and 156.25 ng/mL.

Blank sheep plasma was used for method development and the preparation of calibrators and quality control (QC) samples.

### 2.4. Plasma sample extraction procedure

A 300 μL plasma aliquot was vortex-mixed with 10 μL of IS working solution. For protein precipitation 900 μL of ice-cooled methanol was added to each sample and vortex-mixed for 1 min. The samples were cooled on ice for 10 min and subsequently centrifuged for 15 min at 16,100 × *g* (Eppendorf centrifuge 5415 R, Hamburg, Germany) at 4 °C. The supernatant was transferred to a 1.5 mL microtube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 50 μL methanol and 150 μL acetonitrile in water (30:70, v/v), vortex-mixed, and then transferred to an auto-sampler vial. The auto-sampler rack was cooled to 6 °C. Finally, a 20 μL aliquot of each sample was injected into the LC/MS/MS system for analysis.

### 2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, recovery, and stability according to the US Food and Drug Administration (USFDA) guidelines [11].

#### 2.5.1. Selectivity

Frozen blank sheep serum and plasma were examined for potential interferences with endogenous substances using the proposed extraction procedure but without adding any internal standard working solution.

#### 2.5.2. Calibration curves

Four calibration curves were established using the Internal Standard method, plotting the peak-area ratios of OZ78 to the IS vs. the concentration of OZ78. Six point standard calibration curves, covering the range of concentrations expected in sheep (156.25, 312.5, 625, 1250, 2500, and 5000 ng/mL) were calculated and fitted by a linear regression.

#### 2.5.3. Lowest limit of quantification (LLOQ)

The lowest limit of quantification (LLOQ) was chosen as the minimum concentration in plasma samples which could be determined with a standard deviation (SD) below 15%.

#### 2.5.4. Accuracy and precision

Intra- and inter-day assay precisions were calculated using the relative standard deviation (RSD%). Intra- and inter-day assay accuracies were calculated as the ratio of the measured concentration to the nominal concentration multiplied by 100%.

Data from replicate analysis of QC samples at three different concentrations (312.5, 1250, and 5000 ng/mL, $n = 5$) on four different days were used to calculate the intra-day precision and accuracy as well as the inter-assay precision and accuracy. The USFDA recommended acceptance criterion (percentage of deviation between theoretical and back-calculated concentrations less than ±15%) was used in our study [11].

#### 2.5.5. Recovery

Absolute recovery of OZ78 was determined by comparing the absolute peak areas obtained from QC samples with those of OZ78 spiked plasma after extraction at three concentrations (312.5, 1250, and 5000 ng/mL, $n = 4$). The recovery value of the IS was determined at a single concentration of 2000 ng/mL.

#### 2.5.6. Stability

Stability studies included autosampler stability, bench-top stability, and freeze-thaw stability. For the autosampler stability QC samples (1.25 and 5 μg/mL, $n = 3$) were measured over a period of 30 h.

Freeze–thaw stability was tested by the analysis of 1.25 and 5 μg/mL of QC samples ($n = 3$) in two freeze and thaw cycles (1 h RT/1 h −80 °C/1 h RT/1 h −80 °C).

Bench-top stability was determined by analyzing QC samples (1.25 and 5 μg/mL, $n = 3$) stored over a time period of 4 h at RT. Quality control samples of 1.25 and 5 μg/mL (injected immediately after preparation) served as control.

The solutions were accepted as stable with a deviation of not more than ±15%.

**Table 3**
Absolute recovery (ARE) of OZ78 and IS.

| Analyte | Nominal concentration (μg/mL) | ARE ($n = 4$) | |
|---|---|---|---|
| | | Mean (%) | RSD (%) |
| OZ78 | 0.3125 | 84.36 | 15.33 |
| | 1.25 | 69.58 | 7.93 |
| | 5 | 78.57 | 7.71 |
| IS | 2 | 85.84 | 6.90 |

**Table 4**
Stability analysis of quality control samples for OZ78 in sheep plasma ($n = 3$).

| Nominal concentration (μg/mL) | Calculated concentration (μg/mL) | RSD (%) | Accuracy (%) |
|---|---|---|---|
| Autosampler stability (6 °C for 30 h after processing) | | | |
| 1.25 | 1.21 | 6.22 | 97.01 |
| 5 | 5.43 | 9.96 | 108.67 |
| Bench-top stability (room temperature for 4 h) | | | |
| 1.25 | 1.29 | 11.44 | 103.25 |
| 5 | 5.46 | 6.93 | 109.22 |
| Freeze–thaw stability (two cycles) | | | |
| 1.25 | 1.27 | 8.51 | 101.85 |
| 5 | 5.39 | 3.18 | 107.87 |

### 2.6. Pharmacokinetic study

Details of the pharmacokinetic study have been presented elsewhere [12]. Briefly, serum samples were collected at Yarrandoo R & D Centre, Novartis Animal Health, Kemps Creek, NSW 2178, Australia. Twelve sheep were selected from the Yarrandoo Merino lamb mob (3 months of age at commencement, weights 20.8–30.2 kg). On day −2, all study animals were bled for pre-treatment pharmacokinetic data. On day 0, blood samples were withdrawn from the jugular vein into vacutainers from all sheep of the group treated with 50 mg/kg OZ78 subcutaneously and from all sheep of the group treated with 50 mg/kg OZ78 orally at 0.5, 1, 2, 4, 8, 24, 36, and 168 h post-treatment.

## 3. Results and discussion

### 3.1. Method development

OZ78 is a novel fasciocidal drug candidate, for which to date no analytical method has been developed and validated. In the framework of developing the antimalarial drug development candidate OZ277, levels of OZ78 were quantified in rat plasma using negative electrospray ionization (ESI), however only few details of the analytical method had been presented [1]. A recently developed analytical method for the related molecule OZ277, characterized by an amide functional group and its polar metabolites was based on hydrophilic interaction chromatography in combination with mass spectrometry using positive ESI [13]. However, this method is not suitable for the determination of OZ78 in biofluids, due to its acidic functional group.

In a first step, an IS had to be chosen. A number of OZs sharing structural similarities with OZ78 were evaluated as possible IS. Best results were obtained with OZ352. As depicted in Fig. 1, the only difference between OZ352 and OZ78 is an extension of the connecting alkyl link of the carboxylic acid functional group in OZ352.

After the IS had been selected, the MS conditions were optimized. MS optimization was performed by directly infusing solutions of OZ78 and OZ352 (IS) (10 μg/mL in methanol) into the electrospray injection unit of a mass spectrometer at a constant flow-rate of 10 μL/min. Quadrupole full scans (Q1 scans) were carried out in negative ion detection mode to optimize ESI conditions. As expected, no peak was detected with acceptable sensitivity in the positive mode. The mass spectra of OZ78 and OZ352 revealed base peaks at $m/z$ 321.4 and 335.4, respectively, corresponding to the deprotonated parent molecules.

The MS parameters were set to maximize the amounts of deprotonated parent ions produced (Table 1).

In a second step, the LC conditions were refined. Different tests were done with varying concentrations of ammonium formate, and hence different pH values. Best results were obtained with 5 mM ammonium formate at a pH of $6.0 \pm 0.3$, resulting in symmetric peaks with a good ratio of height to width. An increase in mobile phase polarity with time was selected to achieve baseline separation of the OZs.

Protein precipitation is one of the easiest and fastest methods for processing biological samples such as plasma [14]. For the separation of analytes from endogenous components different solvents were evaluated. Finally, methanol at a ratio of 3:1 (precipitant to plasma) was chosen. To get a better response (higher sensitivity) the samples were evaporated to dryness under a stream of nitrogen and resuspended in a smaller volume of methanol and acetonitrile in water (30:70, v/v). In order to minimize contamination of the source of the MS, eluents were diverted to the instrument with a limited time window (4.5–6.5 min).

### 3.2. Method validation

#### 3.2.1. Selectivity

In the majority of chromatograms of blank plasma and serum samples, no visible interferences were seen (Fig. 2(A) and (B)). Thus, no endogenous peaks were detected at the retention time of OZ78 ($5.2 \pm 0.25$ min) or OZ352 ($6.1 \pm 0.3$ min) as presented in Fig. 2(C). In some plasma from European sheep, there were contaminating signals near the IS peak (Fig. 2(B)). However, these signals did not interfere with IS peak integration and data evaluation (Fig. 2(D)).

#### 3.2.2. Linearity and LLOQ

The calibration curve was linear over a concentration range from 156.25 to 5000 ng/mL in sheep plasma with a coefficient of correlations ($r^2$) above 0.994. A signal-to-noise ratio of 3 at 156.25 ng/mL was reached. This concentration was defined as LLOQ since the standard deviation at this concentration was lower than 15% and the calibration line was still linear. It should be noted that the sensitivity of the used API 365 mass spectrometer is suboptimal when the instrument is operated in the negative mode. However, to evaluate plasma samples in the present project the obtained LLOQ was considered to be acceptable.

#### 3.2.3. Accuracy and precision

Four batches of QC samples at three concentrations were analyzed in terms of accuracy and precision. The intra- and inter-assay deviations are shown in Table 2. Intra- and inter-batch precisions were below 11.1% and 8.1%, respectively, with accuracy ranging from 90.2% to 104.7%. These values were within the suggested range of 15% for accurate and precise methods.

#### 3.2.4. Extraction recovery

Extraction recoveries of three concentrations of OZ78 and IS were 84.4, 69.6, 78.6 and 85.8%, respectively. The recovery data are shown in detail in Table 3.

#### 3.2.5. Stability

The results obtained from autosampler, freeze–thaw, and bench-top stability studies indicate that samples were stable under

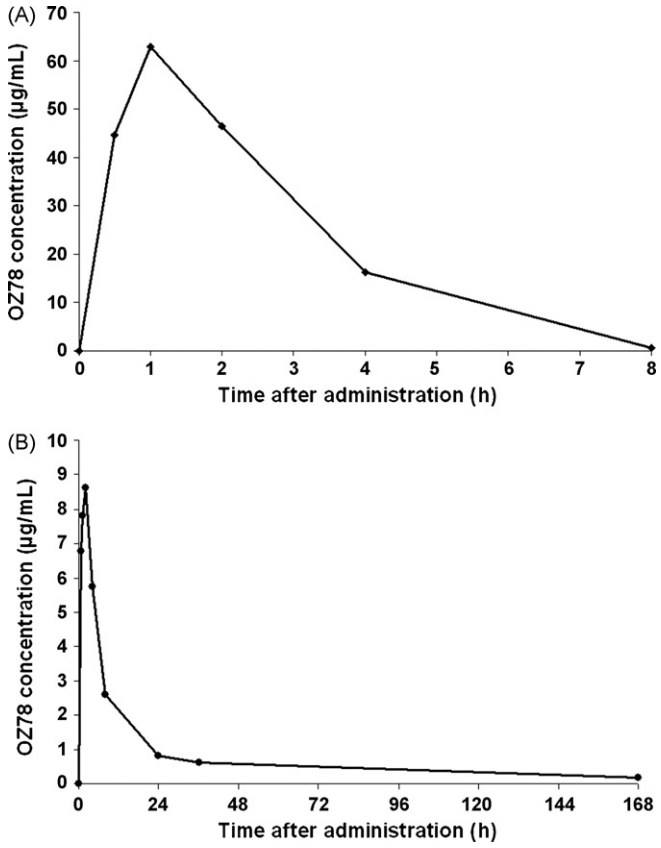


**Fig. 3.** Serum concentration–time profile of OZ78 in sheep, (A) following oral administration of 50 mg/kg OZ78 and (B) following subcutaneous administration of 50 mg/kg OZ78.

the different conditions (Table 4). Accuracy of all stability samples was within 109.2% with precision below 11.4%.

### 3.3. Application of the method

The validated method was used to analyze serum samples from ongoing PK studies with sheep receiving oral or subcutanous OZ78 (50 mg/kg). The usefulness of the method could be demonstrated. It was possible to measure the concentration vs. time profile of all animals. PK parameters were calculated, which have been presented elsewhere [12]. In Fig. 3(A) and (B) we present a representative serum concentration vs. time profile of an oral and a subcutaneous treated sheep.

## 4. Conclusions

This analytical LC/MS method has proven to be accurate, precise, simple, and sensitive. It was possible to monitor concentrations of OZ78 as low as 156.25 ng/mL in sheep plasma or serum. The validated method was successfully used to support a PK study in *F. hepatica* infected sheep [12]. Further work with this method is currently ongoing. With minor variations, the method will be validated, for example for the determination of OZ78 in bile fluids and rat plasma and adapted for potential novel fasciocidal OZ derivatives.

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