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RESEARCH ARTICLE

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Control of a sulfadoxine/trimethoprim combination in the competition horse: Elimination, metabolism and detection following an intravenous administration

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Abstract

The combination of sulfadoxine (SDO) with trimethoprim (TMP) is widely used in veterinarian medicine. The aim of the present study was to compare excretion profiles and detection time windows of SDO and TMP in plasma and urine by means of a validated quantitative method.

Eight horses received a single intravenous (i.v.) dose of 2.7 mg TMP and 13.4 mg SDO per kg bodyweight. Plasma and urine samples were collected up to 15 and 70 days post-administration, respectively.

While urine samples underwent an enzymatic hydrolysis, plasma samples were proteolysed before further analysis. After solid-phase extraction, samples were analysed by liquid chromatography/electrospray ionisation tandem mass spectrometry in positive ionisation mode. The applied multiple reaction monitoring (MRM) method allowed the detection of SDO and TMP with a lower limit of detection of 0.03 ng/mL in plasma and 0.2 (SDO) and 0.4 ng/mL (TMP) in urine, respectively. In the present study, detection times for SDO were 15 days in plasma and 49 days in urine, respectively. TMP was detected for up to 7 days in plasma and up to 50 days in urine, respectively. The detection via the TMP metabolite 3-desmethyl-trimethoprim was possible for 70 days in urine. Detection times of the other confirmed metabolites N_4-acetylated sulfadoxine, hydroxytrimethoprim, trimethoprim-1-oxide and trimethoprim-3-oxide were significantly lower.

In order to postulate reasonable screening limits (SLs) to control specific withdrawal times, a Monte Carlo simulation was performed for SDO. The proposed SL of 10 ng/mL SDO in blood and 300 ng/mL urine corresponds to a detection time of 4 days.

KEYWORDS
detection time, horse, LC/MS/MS, sulfadoxine, trimethoprim

1 | INTRODUCTION

Antimicrobial drugs (AMD) are used in the treatment of many infectious conditions in horses, and their use is important in maintaining health and welfare.[1] More specifically, a large use of AMDs in competing horses has been confirmed by a survey of the European Horserace Scientific Liaison Committee (EHSLC): In doping control samples, which were monitored for the presence of AMD by EHSLC laboratories, 17.8% of the urine and 11.1% of the plasma samples returned positive screening findings for at least one AMD. Furthermore, 'it was also observed that there were more AMD findings in post-race than in training samples and that post-race samples contained higher concentrations of AMD than samples from horses in training'.[2] Thus, AMD application to sport horses coinciding with competitions seems to be common practice. One claimed justification is the prophylactic administration of AMD before competitions in order to prevent transport stress-related shipping fever.[3]

As antimicrobial resistance (AMR) has become a global threat that has gained scientific attention, the notion of prudent use of AMD in competing horses has been raised and is now considered as an objective by racing authorities. In terms of doping or controlled medication, an exception rule applies for AMD, which are not part of the 'Prohibited Substances' according to the guidelines of the International Federation of Horseracing Authorities (IFHA)[4] and the '2022 Equine Prohibited Substance List' of the Fédération Équestre Internationale (FEI).[5] In contrast, in Europe, some national federations have been pioneers in this matter, for example the German Equestrian Federation has classified AMD as prohibited in competition,[6] mainly because of animal welfare reasons: Horses with infectious diseases should be given a recovery break before the next competition, and other horses need to be protected from possible infection. Very recently, the German Trotting Association has added AMD to their list of prohibited substances.[7] A broad discussion within the EHSLC resulted in a recommendation of the EHSLC Board to reinstate AMD as a prohibited substances in Article 6a and to implement a 'Minimum 7 day Stand Down after any AMD treatment'.[8] These recommendations have been transposed into the national law of racing federations. For example, 'France Galop' and 'Deutscher Galopp' have prescribed that a horse must not compete four days after an AMD treatment.[9,10]

In order to ensure effective control of AMD, several conditions must be met: Detection methods need to be implemented by doping control laboratories and pharmacokinetic (PK) studies must be conducted in order to implement appropriate screening limits to ensure that adverse analytical findings reflect the respective doping control regulations. To implement its new policy, EHSLC had to select the AMD to be studied as a priority and for that took into account the AMD classification of the European Medicine Agency (EMA). EMA ranked antibiotics by considering both the risk that their use in animals causes to public health through the possible development of AMR and the need to use them in veterinary medicine.[11] The classification comprises four categories, ranging from most to least critical, that is from A to D: Avoid, Restrict, Caution and Prudence. Category D or 'Prudence' includes antibiotics that should be used as first-line treatments, whenever possible. For horses, this includes penicillin, amoxicillin, tetracyclines and sulfonamides in combination or not with trimethoprim (TMP).

The TMP/sulfonamide combinations are widely used by equine veterinarians because of their broad-spectrum activity,[12] the convenience of oral administration, the lack of apparent toxic effects and their low costs.[13] Different sulfonamides have been associated with TMP in licensed products for horses mainly sulfadiazine, which is a short-acting drug, and sulfadoxine (SDO), which is a rather long-acting drug, especially in humans with a half-life of 7.4 days[14] but apparently much shorter in horses where the average elimination half-lives of SDO and TMP were reported to 7.94 and 1.35 h.[13] Actually, there is no recent PK investigation in horses describing the fate of TMP combined with SDO using sensitive and selective analytical methods, which are required for the setting of screening limits. For example, TMP (2.5 mg/kg) and SDO (12.5 mg/kg body weight [BW]) were administered by intravenous (i.v.) route to six pony mares,[15] but plasma concentrations were assayed either with a microbiological analytical method (TMP) or using a colorimetric method (SDO) that is no longer accepted for PK investigations due to their lack of specificity. Using a higher dose of 40 mg/kg, plasma disposition of SDO in horses was investigated also with a non-specific colorimetric analytical method and a half-life of 14.1 h was reported.[16] In the same trial, the concentration of ^{14}C-TMP (plus metabolites) was measured in plasma and urine and the half-life was reported to be 2.7 h. TMP disposition in horses was reported in many publications for TMP administered in combination with different sulfonamides with a plasma half-life between 2 and 3 h.[13] In all previous studies, the investigated time periods were rather short and therefore the requirements for analytical sensitivity were correspondingly low. As the focus of our study was on the detection of TMP and SDO in post-competition samples, the investigated time windows were longer, the lower limits of quantification (LLOQs) of the method were lower and urinary excretion was determined as well.

For doping control, the metabolic profile needs to be studied in order to find target analytes for control purposes. In horses, sulfonamides were described to be acetylated at the para-amino-group (N_4) and hydroxylated at the methyl-groups or the pyrimidine-ring.[13] Gelsa (1979) calculated concentrations of N_4-acetylated SDO (AcSDO) in the horse by an indirect method without detection of the metabolite itself.[17] For TMP, extensive metabolism was depicted for humans[18] and other species.[13,19,20] Ring-N oxidation, α-hydroxylation and O-demethylation with subsequent conjugation were reported. Alexander and Collett (1975) described that only a minor proportion of the injected TMP was excreted unchanged in horse urine.[21] However, to the author's knowledge there have been no studies describing TMP metabolites in the horse.

Although many of the earlier papers reported on the pharmacokinetics of sulfonamides or TMP using fluorimetric assays,[15,16,21] in recent papers, the state-of-the-art technology has been liquid chromatography/tandem mass spectrometry (LC/MS/MS), resulting in considerably lower limits of detection between 10 and 100 ng/mL.[22–24]

The aim of the present study was to compare metabolism and detection time windows of SDO, TMP and metabolites in plasma and urine after a single intravenous dose of 2.7 mg/kg TMP and 13.4 mg/kg SDO with a sensitive LC/MS/MS method in order to implement a reasonable detection method to control the misuse of these antibiotics in sport horses.

2 | EXPERIMENTAL

2.1 | Study design

Eight horses—two Spanish, two French Trotter, one Arabian, one Anglo-Arabian and two mixed breed horses (398–560 kg, 10–16 years, all geldings)—housed at the horse center of the National Veterinary School of Toulouse were used for this study. Horses passed a physical examination by a qualified veterinarian and were found healthy prior to the investigation. The study was declared to the CEEA (Ethical Committee for Animal Experimentation), and it was undertaken with regards to the Directive 2010/63/EU and with the approval of the regional administration of the governmental body (registration no. 10250_2017061615123788, Toulouse, France).

The administered preparation was Borgal 24% solution® for injection containing 200 mg SDO and 40 mg TMP (Virbac). Depending on the weight, horses received a single i.v. infusion of 27–37 mL into the right jugular vein corresponding to a recommended theoretical dose of 2.7 mg TMP and 13.4 mg SDO per kg BW. To minimize adverse reactions, the i.v. bolus was administered slowly over a period of 2 to 3 min. Blood samples were taken at 0, 0.08, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72, 96, 120, 168, 216, 264, 312 and 360 h post-administration (p.a.) by venipuncture of the left jugular vein. The Li-heparin tubes were immediately centrifuged, and plasma was separated. Urine was collected close to scheduled time points at 0, 4, 8, 12, 24, 48, 72, 96, 120, 168, 216, 264 ($n = 6$), 288 (2), 312 (6), 360, 432 (2), 456 (6), 504 (2), 552 (6), 600 (2), 648 (6), 696 (2), 768 (6), 792 (2), 888, 984 (2), 1,008 (6), 1,080 (2), 1,176 (6), 1,200 (2), 1,344 (5), 1,512 (5) and 1680 (5) h p.a. after spontaneous voiding. Accurate time points were recorded. All samples were stored at −20°C prior to analysis.

2.2 | Materials and chemicals

Ammonium acetate, sodium dihydrogenphosphate monohydrate and sodium acetate were of *pro analysi* quality and obtained from Merck (Darmstadt, Germany). Acetonitrile (analytical grade), methanol, acetic acid (*pro analysi*) and di-sodium hydrogenphosphate were received from VWR Prolabo (Darmstadt, Germany). Arylsulfatase/β-glucuronidase from *helix pomatia* and β-glucuronidase from *E. coli* were purchased from Roche (Mannheim, Germany). Buffers and solutions were made with ultrapure water made by the Barnstead GenPure xCAD Plus from Thermo Scientific (Schwerte, Germany). Chromabond® HLB SPE cartridges (60 mg, 3 mL) were purchased from Macherey & Nagel (Düren, Germany). Isotonic NaCl 0.9% was obtained from Serumwerk Bernburg AG (Bernburg, Germany). Protease from bovine pancreas, TMP and d_9-TMP were obtained from Sigma-Aldrich (Steinheim, Germany). SDO and d_3-SDO were purchased from Ehrenstorfer (Augsburg, Germany); 3-desmethyltrimethoprim ($_3$DMT), 4-desmethyltrimethoprim ($_4$DMT) and α-hydroxytrimethoprim (OHT) were provided by TRC (Toronto, Canada), trimethoprim-3-oxide (TP_3O) by Haoyuan Chem Express (Shanghai, China), trimethoprim-1-oxide (TP_1O) by Pharmaffiliates (Haryana, India) and Ac-SDO by Combi-Blocks (San Diego, USA). Stock and working solutions were prepared in methanol.

2.3 | Sample preparation

Aliquots of 0.5 mL plasma were fortified with 20 and 2 ng/mL of the internal standards d_9-TMP and d_3-SDO, respectively. Samples with expected concentrations above 100 ng/mL aliquots were diluted with deionised water accordingly. Subsequently, proteolysis was achieved by the addition of 10 µL of protease solution (5 mg/mL) and incubation of samples for 1 h at 50°C.

Aliquots of 1 mL urine were spiked with 50 ng/mL d_9-TMP and 10 ng/mL d_3-SDO, respectively. Subsequently, samples were adjusted to pH 5.2 with 0.1 mL 4 M sodium acetate buffer and potential conjugates were hydrolysed with β-glucuronidase/arylsulfatase from *Helix pomatia* at 50°C for 1 h. In the context of method development, six p.-a. urine samples of different horses and p.a. time points were analysed without and after hydrolysis with *Helix pomatia* (pH 5.2), ß-Glucuronidase from *E.coli* (pH 7.0) and arylsulfatase from *Pseudomonas aeruginosa* (pH 7.5) in order to obtain information on phase-II-metabolism of the analytes.

Afterwards, a solid-phase extraction (SPE) of urine and plasma samples was conducted by means of Oasis® HLB SPE cartridges (60 mg, 3 mL), which were conditioned with 1 mL of methanol and 1 mL of ultrapure water. After the application of the samples, a washing step followed with 1 mL of a water/methanol mixture (90:10, v/v). The cartridges were eluted with 1 mL methanol, according to the manufacturer's recommendation. The methanolic eluate was evaporated to dryness by means of a rotary evaporator under vacuum. The dry residue was reconstituted in 100 µL of an ammonium acetate (containing 1% acetic acid)/acetonitrile mixture (60:40, v/v) for analysis; 3 µL of the solution was injected into the LC/MS/MS system. Fresh calibration curves and negative control samples were analysed with each batch of samples.

2.4 | LC/MS/MS

LC/MS/MS analyses were performed on an Agilent Series 1260 liquid chromatograph (Waldbronn, Germany) coupled to a 5500 QTrap triple-quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an electrospray ionisation (ESI) interface. The column was a Gemini C_6-Phenyl 110 Å column with dimensions of

4.6 × 150 mm and a particle size of 3 µm from Phenomenex (Aschaffenburg, Germany). Gradient elution was carried out with ammonium acetate buffer (5 mM ammonium acetate, 0.1% acetic acid in ultrapure water and pH 5) as solvent A and acetonitrile as solvent B. The gradient flow rate was adjusted to 500 µL/min, starting with 100% A for 1 min. From 1 to 7 min, the aqueous phase was decreased to 0% and kept constant for 2 min. Subsequently, a re-equilibration at 100% A was performed for 4.5 min in order to return to the initial conditions.

Samples were measured at an interface temperature of 450°C with an ion spray voltage (ISV) of +5500 V. Diagnostic ions of the analytes were generated by collision-induced dissociation (CID) with nitrogen at a collision gas pressure of 2.3×10^{-3} Pa. Multiple reaction monitoring (MRM) experiments were performed on the most abundant ion transitions, which were optimised by support of the software Analyst 1.6 after infusion of the corresponding reference solutions. Selected MRM transitions are listed in Table 1.

For quantitation purposes, the ion transitions at *m/z* 311-156, *m/z* 291-261, *m/z* 277-261, *m/z* 307-243 and *m/z* 307-275 were monitored for SDO, TMP, $_3$DMT, OHT and TP_3O, respectively. Quantitation was done by means of the internal standards d_3-SDO (*m/z* 324-156) and d_9-TMP (*m/z* 300-264). A calibration curve was analysed with each batch of samples. Relative peak area ratios and concentrations were calculated by the Analyst software (version 1.6). Additionally, semiquantitative analysis was done for AcSDO (*m/z* 353-198) and trimethoprim-1-oxide (TP_1O, *m/z* 307-275).

2.5 | Method validation

The method was fully validated for quantitative analysis of SDO, TMP, $_3$DMT, OHT and TP_3O in horse plasma and urine considering the parameters specificity, recovery, linearity, precision, accuracy, LLOQ and lower limit of detection (LLOD), stability and ion suppression/enhancement effects.

For specificity, product ion scans were compared with spectra from literature, where available. Three diagnostic product ions were chosen for each analyte and implemented in the acquisition method. Ten blank samples of each specimen were prepared as described above in order to probe for interfering peaks in the selected reaction chromatograms at the expected retention time of the analytes.

Recovery was calculated from the ratio of sets A and B, in which A is the mean of six samples spiked with each analyte at a concentration of 1 ng/mL at the beginning and B is the mean of six blanks spiked at the end of the sample preparation. The internal standard was added to both sets of samples at the end of sample preparation.

Linearity of signal response in plasma was tested over a range of 0.05 to 100 ng/mL for SDO and 0.1 to 100 ng/mL for TMP, $_3$DMT, OHT and TP_3O, respectively, by fortifying the blank plasma matrix with the equivalent amount of the reference material. Calibration curves in urine were constructed covering concentration ranges from 0.3 to 300 ng/mL for SDO, 0.5 to 300 for TMP and $_3$DMT and 0.5 to 100 ng/mL for OHT and TP_3O, considering their expected concentrations in p.a. samples. Area ratios of analyte and internal standard

TABLE 1 Method validation results in plasma and urine (qualitative parameters).

	Ion transitions $[M+H]^+$ (*m/z*)	RT (min)	Selectivity ($n = 10$)		Recovery ($n = 6/6$) (%)		Matrix effect ($n = 6$) (%)		LLOD (ng/mL)	
			Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine
TMP	311–156 311–92 311–108	7.52	√	√	64	86	−23.4	−52.8	0.03	0.4
OHT	307–243 307–259 307–289	7.38	√	√	65	75	−5.8	−48.5	0.03	0.2
$_3$DMT	307–261 307–123 307–187	7.42	√	√	64	79	−2.2	−52.7	0.1	0.2
TP_1O	307–275 307–290 307–259	7.77	√	√	n.d.	n.d.	n.d.	n.d.	0.03	2
TP_3O	307–275 307–290 307–259	8.01	√	√	69	74	−49.3	−72.3	0.03	0.5
SDO	311–156 311–92 311–108	8.96	√	√	69	81	−21.0	−51.2	0.03	0.2
AcSDO	353–198 353–154 353–140	8.91		√	n.d.	n.d.	n.d.	n.d.	0.03	0.2

Abbreviations: AcSDO, N_4-acetylated SDO; $_3$DMT, 3-desmethyltrimethoprim; LLOD, lower limit of detection; n.d., not determined; OHT, hydroxytrimethoprim; RT, retention time; SDO, sulfadoxine; TMP, trimethoprim; TP_1O, trimethoprim-1-oxide; TP_3O, trimethoprim-3-oxide.

(y) were plotted against the nominal concentration (x) and a calibration curve ($y = ax + b$) was generated by linear least square regression with a weighting factor of $1/x$ with x as the analyte concentration.

The LLOD was estimated as the lowest concentration which could be detected at a signal-to-noise ratio ≥ 3 for the quantifier ion. The LLOQ was postulated as the lowest concentration detectable with a signal-to-noise ratio ≥ 9 and verified by a sixfold determination of the estimated level in order to obtain the respective precision. The criterion for acceptance of the LLOQ was a coefficient of variation (CV) below 20%.

Precisions of SDO, TMP, $_3$DMT, OHT and TP$_3$O were determined using 18 quality control (QC) samples, which were spiked at low, medium and high concentrations quantified within one day ($n = 6$) and on three separate occasions ($n = 6 + 6 + 6$). In order to include the dilution step in the validation, high concentration levels were diluted 1:100 with isotonic NaCl (0.9%) prior to the sample preparation. The CV was determined by six (intraday precision) and 18 (interday precision) different samples. Respective concentrations of the QC samples are listed in Table 2. For determination of accuracy, QC samples ($n = 6 + 6 + 6$) each spiked at low, medium and high concentrations were quantified by means of a calibration curve. The means of calculated values were compared with the theoretical values.

The stability was checked by means of 12 plasma and urine samples, each fortified with 20 and 10 ng/mL SDO, TMP, $_3$DMT, OHT and TP$_3$O, respectively. Samples were stored at −20°C degrees and quantified after 28 ($n = 6$) and 56 ($n = 6$) days, respectively, using freshly prepared calibrators. For evaluation of ion suppression or enhancement effects, analyte intensities were compared in solutions with and without urine or plasma matrix.

2.6 | Data modelling and Monte Carlo simulations

Data analysis was carried out using Phoenix®WinNonlin®8.3 (Certara USA, Inc., Princeton, NJ, USA). SDO was the exposure marker of the TMP/SDO combination. Plasma concentrations collected in the eight horses were analysed simultaneously using a non-linear mixed effect model (NLME), that is a population model. Based on the AIC criterion,[25] a three-compartment model with Michaelis–Menten elimination process was selected for SDO. Parametrization was in terms of plasma CL (as described by a Michaelis–Menten process), intercompartmental clearance(s) (Cld) and volume(s) of distribution (V). Concentrations lower than the LLOQ were considered as censored and managed with the M3 approach.[26] The between-subject variability (BSV) was modelled using an exponential model. It was assumed that random effects were independent for all parameters but *Vmax* and *Km*. For *Vmax* and *Km*, a covariance term was included in the random model largely improving the estimability of *Vmax* and *Km* and post hoc estimates of individual *Vmax* and *Km*. The residual model was an additive plus a multiplicative (proportional) model. The primary estimated parameters were reported as typical values (tv) with their confidence interval, as a measure of the precision as estimated using the bootstrap tool of Phoenix (50 resampling). The steady-state urine-to-plasma (U/P) ratio (Rss) concentration was estimated by simply adding to the plasma population model an equation expressing the urinary concentration as being proportional to the plasma concentration. Rss, the factor of proportionality, was obtained from the best fit of both plasma and urinary SDO concentrations. For the computation of Rss, only urinary data collected at 24 h post-administration up to the last urine sample collected after the last quantifiable plasma concentration were considered to ensure both pseudodistribution equilibrium and parallelism of plasma and urinary concentrations.

Secondary parameters including slopes and half-lives were computed from estimate parameters of this three-compartmental model.[27] Optimisation was carried out with the Phoenix likelihood Laplacian engine that is appropriate for censored data.

Using the fitted population model, Monte Carlo simulation[28] was used to generate SDO plasma and urine concentrations of a virtual population of 5,000 horses using individual predictions (IPRED). Single (13.4 mg/kg) or multiple doses (13.4 mg/kg at 24 h intervals for 5 days) of SDO were simulated. From this simulated population, the distribution of the 5,000 plasma and urinary SDO concentrations, from 1 to 10 (14) days p.a., was considered. Using the Phoenix statistical tool the quantiles of interest (90th and 95th) were computed to estimate the corresponding critical plasma or urinary concentrations, that is the concentrations for which 90% or 95% of the horses of the metapopulation fell below these critical concentrations.

3 | RESULTS AND DISCUSSION

3.1 | Method development and validation

For the detection and quantification of the investigated analytes, a previously published method[29] based on LC/MS/MS was adopted for the detection of SDO and TMP.

Different hydrolysis methods to cleave potential phase-II metabolites in urine samples were tested. Since $_3$DMT seems to be excreted mainly conjugated as sulfate and glucuronide, hydrolysis of urine samples via *Helix pomatia* was carried out in order to quantify the combined fraction of conjugated and free analytes.

Depending on plasma concentration, about 51% of TMP and 14%–72% of SDO are bound to plasma proteins.[16] Thus, a proteolysis of plasma samples via bovine protease was performed in order to liberate protein-bound analytes.

Unambiguous identity of SDO, AcSDO, TMP, $_3$DMT, OHT and TP$_3$O was shown by comparison of the three diagnostic ion transitions in a reference standard mixture with the ion transitions obtained from the analysis of a plasma *p.a.* or urine *p.a.* sample, respectively. Optimised ion transitions were in accordance with generated product ion scans of the precursor ions (Figures 1 and 2) as well as with data from the literature, where available.[30]

Regarding specificity, no interfering signals above the LOD for the optimised diagnostic ion transitions of the investigated analytes were observed in ten blank plasma as well as urine samples at the

TABLE 2 Method validation results in plasma and urine (quantitative parameters)

	Matrix	LLOQ ($n = 6$) (ng/mL)	Linearity ($n > 6$) Slope intercept R^2	Precision ($n = 18/18/18$) Levels (ng/mL)	CV(%)	Accuracy ($n = 18/18/18$) CV(%)	Stability ($n = 6/6$) Decrease 28d/56d (%)
TMP	Plasma	0.2	0.0453	1	6.5	110.5	<10
		18	1.91×10^{-7}	50	4.6	105.7	
		109.3	0.9996	2,000[a]	10.5	103.7	
	Urine	1.0	0.021	1	18.3	96.2	<10
		18.3	9.78×10^{-8}	50	12.3	106.7	
		96.2	0.9983	100,000[b]	11.7	101.0	
OHT	Plasma	0.1	0.043	1	13.1	93.9	<10
		9.9	1.58×10^{-7}	50	11.1	94.6	
		105.2	0.9985	2,000[a]	11.6	88.6	
	Urine	0.3	0.017	1	10.2	110.1	<10
		5.6	5.32×10^{-8}	50	12.3	100.3	
		105.6	0.9982	100,000[b]	15.7	104.9	
$_3$DMT	Plasma	0.2	0.024	1	16.4	89.4	<10
		9.3	9×10^{-4}	50	9.7	96.1	
		93.8	0.9987	2,000[a]	6.5	94.5	
	Urine	0.3	0.015	1	7.9	109.8	<10
		15.6	9.27×10^{-4}	50	10.6	100.9	
		105.6	0.9996	100,000[b]	11.2	98.0	
TP_3O	Plasma	0.1	0.036	1	12.3	90.9	<10
		12.7	3×10^{-4}	50	11.2	90.7	
		101.8	0.9998	2,000[a]	12.8	90.5	
	Urine	0.5	0.010	1	12.2	94.5	<10
		8.4	6.21×10^{-4}	50	12.6	97.3	
		97.7	0.9994	100,000[b]	15.6	n.d.	
SDO	Plasma	0.05	0.219	1	6.5	110.5	<10
		6.9	1.03×10^{-2}	50	3.0	105.2	
		105.8	0.9992	2,000[a]	5.7	99.8	
	Urine	0.3	0.035	1	13.0	102.2	<10
		14.4	1.13×10^{-2}	50	9.9	106.4	
		84.2	0.9964	100,000[b]	6.0	95.0	
AcSDO	Plasma	n.d.	0.051	-	n.d.	n.d.	n.d.
			1.1×10^{-3}				
			0.9987				
	Urine	n.d.	0.028	-	n.d.	n.d.	n.d.
			5.3×10^{-3}				
			0.9992				

Abbreviations: AcSDO, N_4-acetylated SDO; CV, coefficient of variation; $_3$DMT, 3-desmethyltrimethoprim; LLOQ, lower limit of quantification; n.d., not determined; OHT, hydroxytrimethoprim; SDO, sulfadoxine; TMP, trimethoprim; TP_3O, trimethoprim-3-oxide.

[a]Samples have been diluted 1:100 with NaCl 0.9% before analysis.

[b]Samples have been diluted 1:1000 with deionised water before analysis.

respective retention time (RT). Validation parameters regarding qualitative identification are summarised in Table 1.

For quantitation purposes, d_3-SDO and d_9-TMP were added as internal standards. Following method development, the method was fully validated for quantitative analysis of SDO, TMP, $_3$DMT, OHT and TP_3O in horse plasma and urine considering the parameters recovery, linearity, precision, accuracy, LLOQ and LLOD, stability and ion suppression/enhancement effects. The method allowed precise quantification of the respective analytes in a concentration range of up to 10^7 ng/mL. LLOQs were determined between 0.3 and 1 ng/mL in

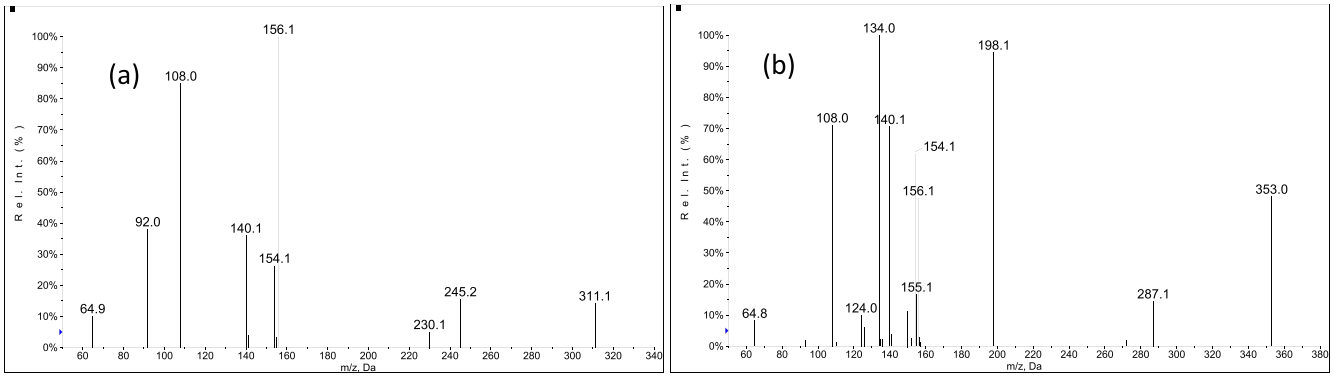


FIGURE 1 Product ion mass spectra of protonated molecules of (a) sulfadoxine (*m/z 311*) and (b) N_4-acetylated sulfadoxine (*m/z 353*). [Colour figure can be viewed at wileyonlinelibrary.com]

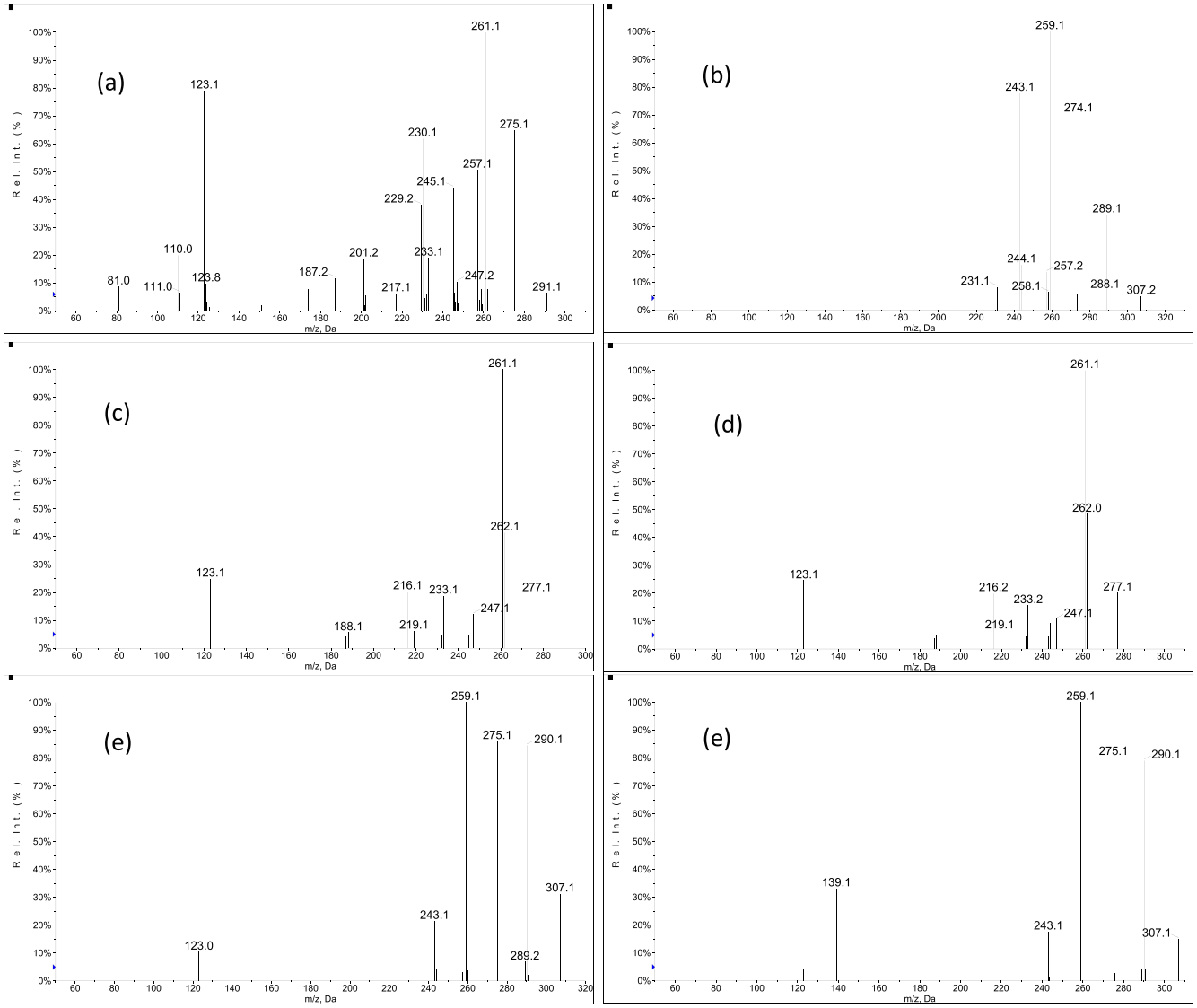
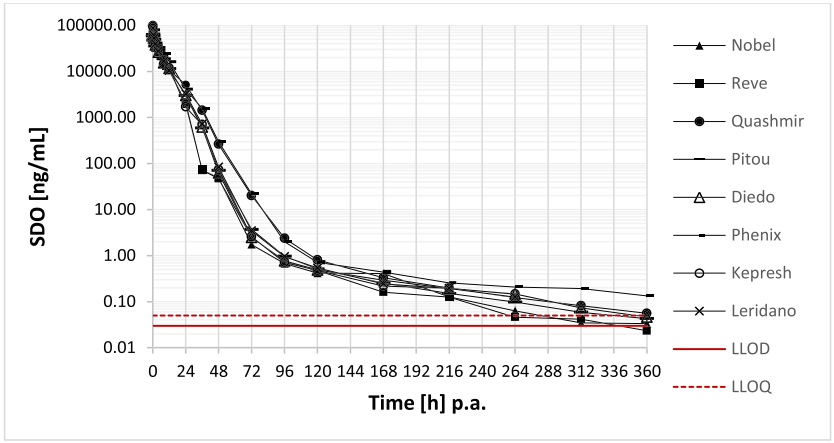


FIGURE 2 Product ion mass spectra of protonated molecules of (a) trimethoprim (*m/z 291*), (b) hydroxytrimethoprim (*m/z 307*), (c) 3-desmethyltrimethoprim (*m/z 277*), (d) 4-desmethyltrimethoprim (*m/z 277*), (e) trimethoprim-1-oxide (*m/z 307*) and (f) trimethoprim-3-oxide (*m/z 307*). [Colour figure can be viewed at wileyonlinelibrary.com]



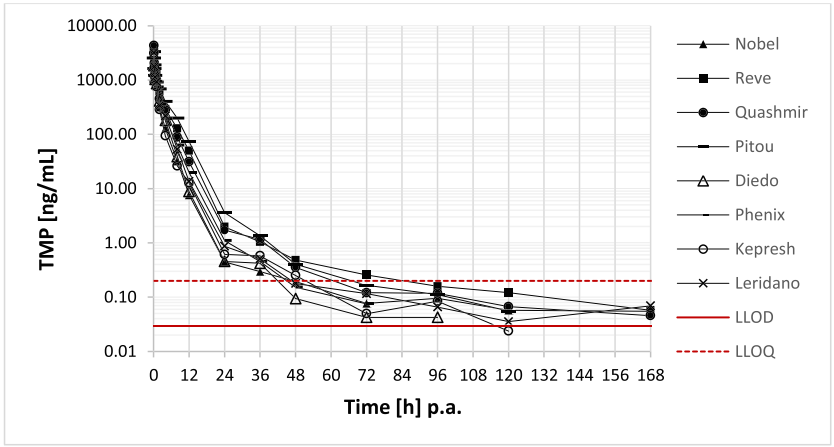


FIGURE 3 Plasma disposition of SDO (a) and TMP (b) following intravenous administration of SDO at a dose of 13.4 mg/kg and TMP at a dose of 2.7 mg/kg in eight horses. Abbreviations: LLOD, lower level of detection; LLOQ, lower level of quantification; SDO, sulfadoxine; TMP, trimethoprim. [Colour figure can be viewed at wileyonlinelibrary.com]

both matrices. Samples with plasma levels above 100 ng/mL required a dilution with NaCl 0.9%. Urine samples above 300 ng/mL were diluted with deionised water in advance of the sample preparation. The dilution step was included in the evaluation of the high precision and accuracy values. Detailed results of the quantitative method validation are summarised in Table 2.

3.2 | Excretion profiles

Plasma levels of SDO and TMP obtained after a single *i.v.* administration of SDO at a dose of 13.4 mg/kg and TMP at a dose of 2.7 mg/kg are shown in Figure 3. As expected, after *i.v.* application, *Cmax* was measured at 0.08 h (*Tmax*) after the administration (p.a.). SDO *Cmax* values varied between 62.6 and 98.0 µg/mL (mean 83.6 µg/mL). According to visual inspection, concentrations over time curves followed a polyphasic elimination profile, and after 5 days, the concentrations had decreased by a factor of 10^5 resulting in levels of SDO below 1 ng/mL. However, SDO could be detected up to the last collection time point (15 days p.a.) in five of the six horses with plasma levels in the range of LLOQ. Plasma TMP concentrations were significantly lower with maximum levels ranging from 2.55 to 3.86 µg/mL (mean 3.2 µg/mL). Five days p.a. plasma TMP levels of all horses fell below the LLOQ of 0.2 ng/mL. The longest detection time was determined at 168 h in four of eight horses. A comparison of SDO and TMP elimination curves (Figure 4) shows that TMP is eliminated significantly faster during the first 24 h, which indicates a shorter initial half-life.

Figure 5 shows the urinary elimination of SDO and TMP. SDO *Cmax* values appeared 4 to 8 h p.a. and ranged between 514 and 1,370 µg/mL. After 15 days, concentrations of all the collected urine samples were below 5 ng/mL, lower by a factor of 10^5 compared to the initial values. Maximal detection times varied between 23 and 49 days p.a. At day 56 p.a. SDO was not found in any horse urine. Four urine samples (18, 21, 29 and 37 days p.a.) of one horse ('Leridano') showed unexpectedly high concentrations between 10 and 15 ng/mL. These results were confirmed by analysis of a second aliquot of the samples and can thus be declared as outliers. A possible explanation would be a cross-contamination via other samples or other horses of the same study. As the concentration difference between the LLOD and early time points is extremely high (10^6), it was crucial to be aware of the danger of cross-contamination during the whole study process (medication, sample collection, stable hygiene, sample transportation, storage and aliquoting).

The Rss of SDO was estimated by the population model at a value of 29.6 (median) with a 95% confidence interval of 22.7%–35.9%. The variability between horses for Rss was large with a BSV of 32% and individual values ranging from 19.9 to 63.4.

Maximum TMP urine concentrations were detected at 4–8 h p.a. and ranged between 11.1 and 35.9 µg/mL (mean 21.3 µg/mL). Similar to blood, the decline of urinary TMP concentrations was rapid

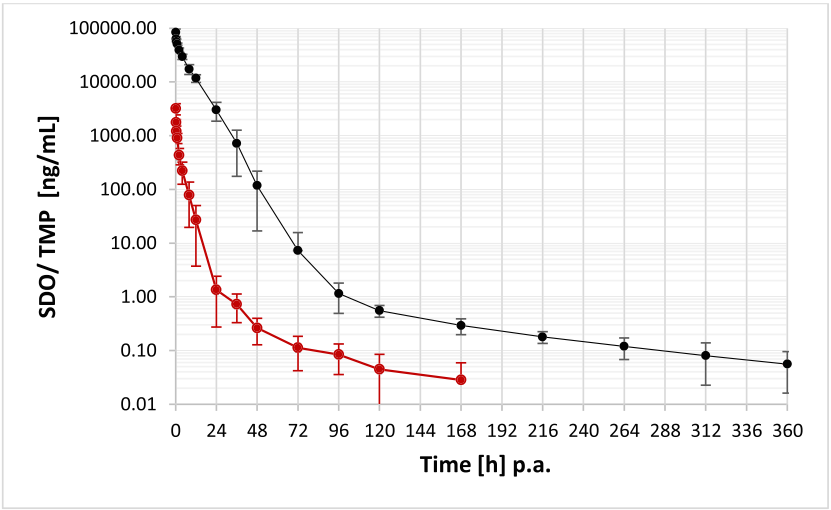
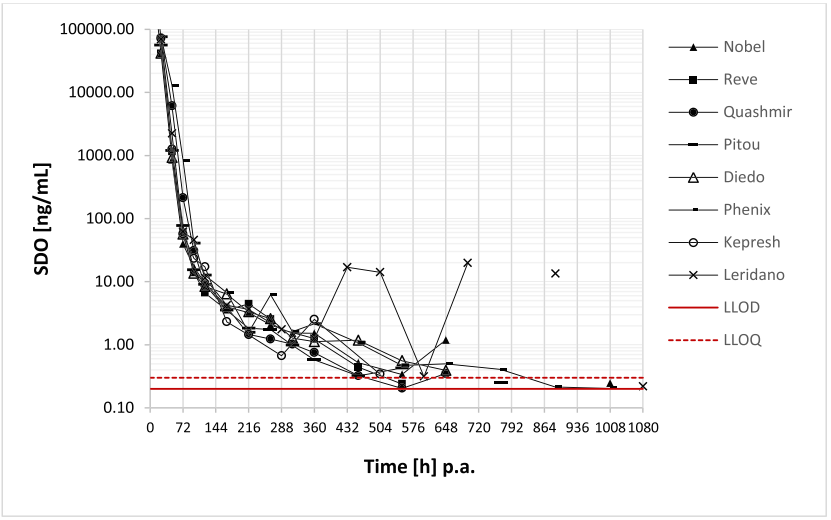


FIGURE 4 Plasma disposition of SDO (black) and TMP (red) following intravenous administration of SDO at a dose of 13.4 mg/kg and TMP at a dose of 2.7 mg/kg. Values are expressed as means (±sd) of three to eight horses. Abbreviations: sd, standard deviation; SDO, sulfadoxine; TMP, trimethoprim. [Colour figure can be viewed at wileyonlinelibrary.com]



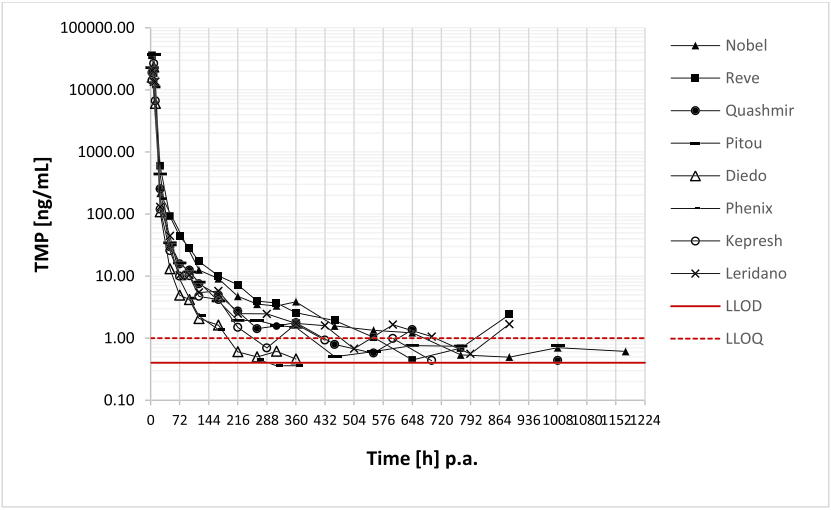


FIGURE 5 Urine disposition of SDO (a) and TMP (b) following intravenous administration of SDO at a dose of 13.4 mg/kg and TMP at a dose of 2.7 mg/kg in eight horses. Abbreviations: LLOD, lower level of detection; LLOQ, lower level of quantification; SDO, sulfadoxine; TMP, trimethoprim. [Colour figure can be viewed at wileyonlinelibrary.com]

during the first 3 days followed by one or two slow elimination phases. Last detection times varied between 15 and 50 days p.a. At 8 weeks p.a., TMP could not be found in any horse. A summary of the results can be found in Table 3.

The comparison of urinary SDO and TMP concentrations (Figure 6) shows that in contrast to the first 72 h, where TMP values were clearly lower than the corresponding SDO concentrations, the two curves approach and finally cross each other indicating slightly

TABLE 3 Descriptive statistics for the plasma and urine disposition of SDO and TMP after a single intravenous administration of SDO at 13.4 mg/kg and TMP at 2.7 mg/kg in eight horses.

Matrix		Unit	Analytes	
			SDO	TMP
Plasma	*Cmax* (range)	(µg/mL)	62.6–98.0	2.55–3.86
	Tmax (range)	(h)	0.08	0.08
	Concentration at 4 days p.a.	(ng/mL)	0.67–2.37	<LLOD–0.16
	Last detection time for a concentration > LLOD	(days)	13–≥15	3–7
Urine	*Cmax* (range)	(µg/mL)	514–1,370	11.1–35.9
	Tmax (range)	(h)	4–8	4–8
	Concentration at 4 days p.a.	(ng/mL)	13.5–45.6	4.2–28.2
	Last detection time for a concentration > LLOD	(days)	23–49	15–50

Abbreviations: LLOD, lower limit of detection; SDO, sulfadoxine; TMP, trimethoprim.

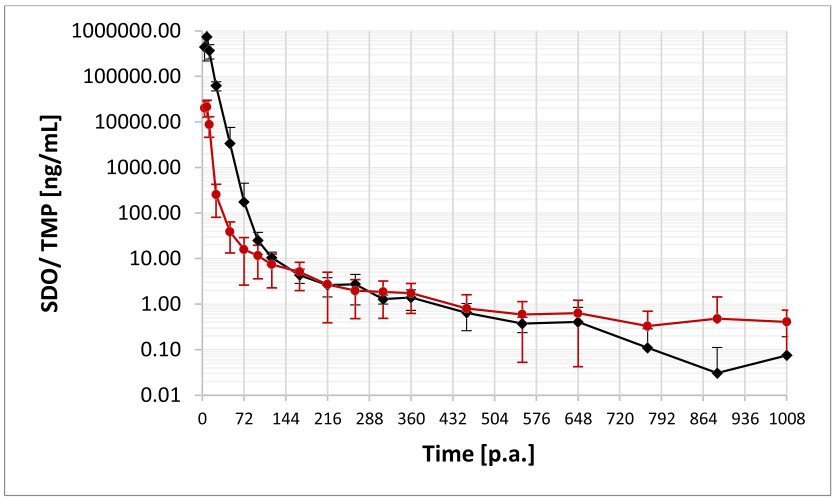


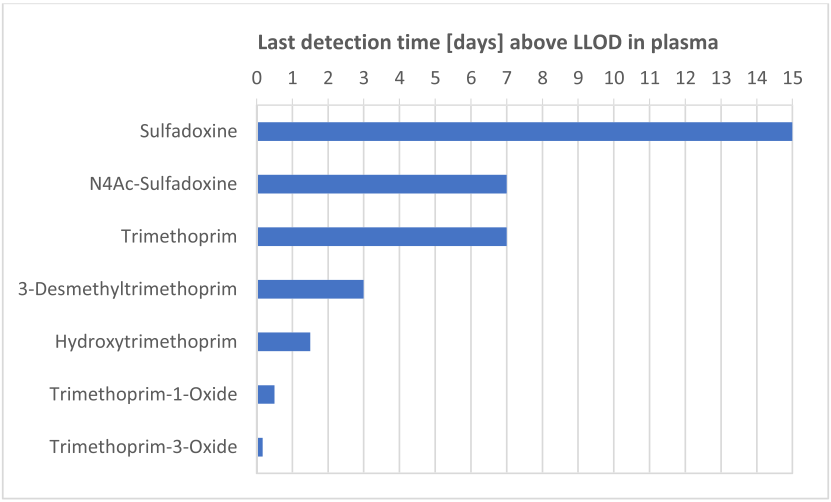
FIGURE 6 Urine disposition of SDO (black) and TMP (red) following intravenous administration of SDO at a dose of 13.4 mg/kg and TMP at a dose of 2.7 mg/kg. Values are expressed as means (±sd) of three to eight horses. Abbreviations: SDO, sulfadoxine; sd, standard deviation; TMP, trimethoprim. [Colour figure can be viewed at wileyonlinelibrary.com]

higher TMP concentrations after 23 days p.a. Thus, a long terminal elimination half-life of TMP in urine can be assumed.

3.3 | Metabolism

We tested p.a. urine and plasma samples for the presence of five metabolites of TMP, which were described in the literature and for which reference material was available. O-demethylation was observed at the meta-position of the benzyl ring, yielding the 3-desmethyl-metabolite, which has been already described for humans[18] and rats[19] and which is well known from equine doping controls.

In contrast, the 4-desmethyl-metabolite could not be detected in either plasma or urine p.a. samples, which is different from the reports on metabolism in rats and humans where both metabolites were eliminated to a similar degree.[18–20] $_3$DMT is the main TMP metabolite that can be found in horses and it is more prominent in urine than the intact drug. In fact, $_3$DMT was detected in urine up to 10 weeks p.a. (Figure 7). A minor portion of TMP passes through α-hydroxylation at the methylene bridge, yielding in OHT, which could be detected in plasma and urine. Peak urine concentrations of OHT ranged between 993 and 4,550 ng/mL and were detected 4 to 12 h p.a. Twenty-four hours p.a., OHT was only detectable in one horse. In plasma, OHT could be detected up to 36 h (six of eight horses) and *Cmax* of 4–12 ng/mL was reached within the first hour indicating a very fast metabolism of TMP. OHT has already been reported in humans, rats, dogs and piglets.[20,31] The same authors reported N_1-oxidation of TMP and the generated TP_1O could be detected 24 h in plasma samples of our study up to a concentration of 12.5 ng/mL. In urine, TP_1O was only detected in the 24-h-p.a. sample at low concentrations, ranging from 0.5 to 4.4 ng/mL. During the first 4 h p.a., low amounts of TP_3O could be observed in plasma (*Cmax*, 0.8–2.8 ng/mL), which implies oxidation at N_3. Interestingly, urinary TP_3O concentrations are considerably higher with peak concentrations between 2.9 and 11.5 µg/mL (mean ~ 5.2 µg/mL) 4–12 h p.a. This significant difference between urinary TP_1O and TP_3O concentrations could be an indication for a selective renal excretion of the two stereoisomers (Figures 8 and 9). The TP_3O metabolite had already been identified in humans.[18]



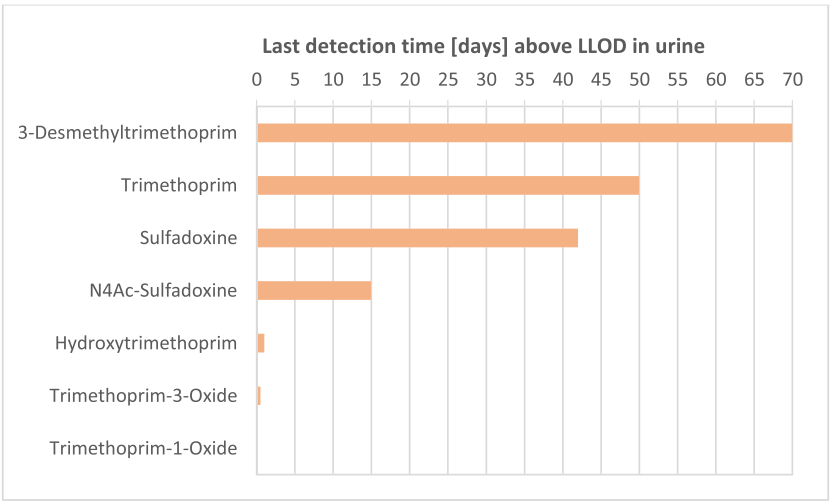


FIGURE 7 Maximal detection times of trimethoprim, sulfadoxine and the corresponding metabolites. Abbreviation: LLOD, lower limit of detection. [Colour figure can be viewed at wileyonlinelibrary.com]

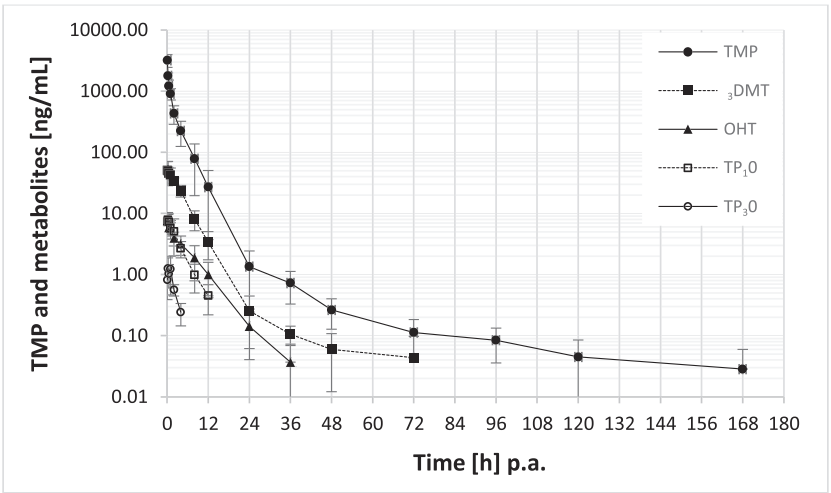


FIGURE 8 Plasma disposition of TMP, $_3$DMT, OHT, TP_1O and TP_3O following intravenous administration of TMP at a dose of 2.7 mg/kg. Values are expressed as means (±sd) of eight horses. Abbreviations: $_3$DMT, 3-desmethyltrimethoprim; OHT, hydroxytrimethoprim; TMP, trimethoprim; sd, standard deviation; TP_1O, rimethoprim-1-oxide, TP_3O, trimethoprim-3-oxide.

Regarding SDO, also N_4-acetyl metabolisation, which is typical of several sulfonamides (Figure 10), was investigated. AcSDO could be detected in horse plasma and urine. By comparing the excretion curves, the relative amount of the metabolite was estimated to be approximately 10% of the intact drug (Figures 11 and 12). This result is in line with the paper of Gelsa who determined the AcSDO concentrations by an indirect method.[17] In plasma, *Cmax* between 264 and 1830 ng/mL were observed 2 h p.a. Highest urine

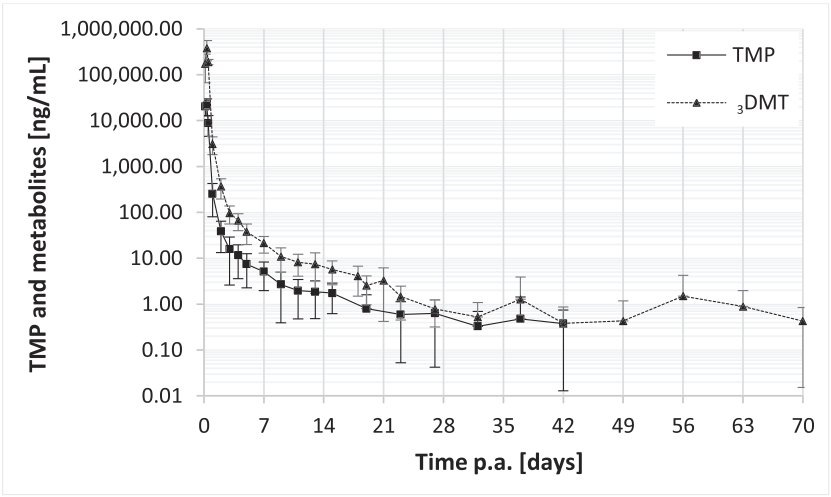


FIGURE 9 Urine disposition of TMP and $_3$DMT following intravenous administration of TMP at a dose of 2.7 mg/kg. Values are expressed as means (±sd) of eight horses. Abbreviations: $_3$DMT, 3-desmethyltrimethoprim; sd, standard deviation; TMP, trimethoprim.

FIGURE 10 Structures of sulfadoxine (left) and N_4-acylsulfadoxine (right), a confirmed metabolite in horse plasma and urine. [Colour figure can be viewed at wileyonlinelibrary.com]

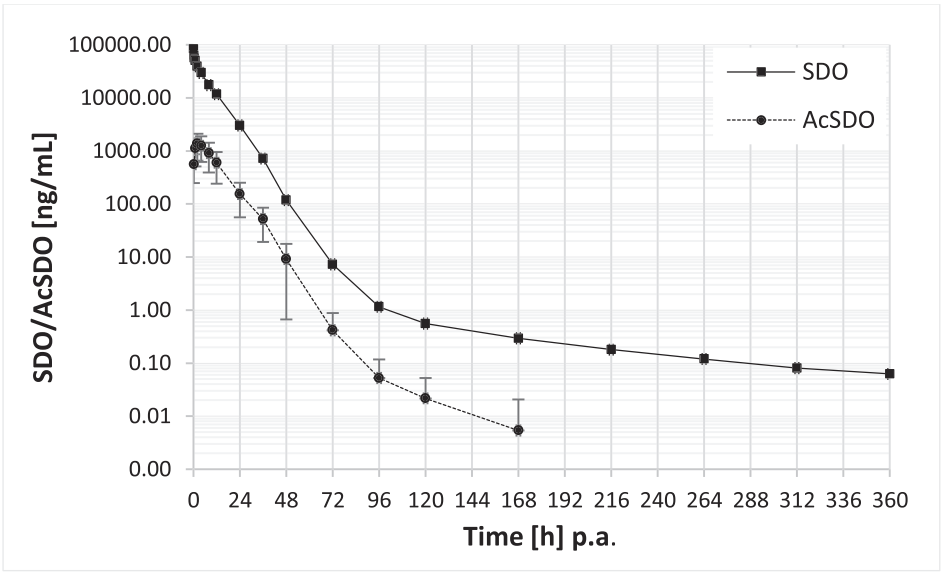


FIGURE 11 Plasma disposition of SDO and AcSDO following intravenous administration of SDO at a dose of 13.4 mg/kg. Values are expressed as means (±sd) of eight horses. Abbreviations: AcSDO, N_4-acetylated SDO; sd, standard deviation; SDO, sulfadoxine.

concentrations were calculated to be between 99 and 192 µg/mL at 8 to 12 h p.a.

Different hydrolysis methods to cleave potential phase-II metabolites in urine samples were tested. For SDO, N_4AcSDO and TMP, no significant difference between hydrolysis with arylsulfatase and ß-glucuronidase compared to non-hydrolyzed samples could be observed. This indicates that these analytes do not undergo a significant phase-II metabolism. This is in accordance with Rieder (1973), who postulated an unconjugated excretion of TMP, OHT and TP_1O in humans and rats.[20] In contrast, only about 7% of $_3$DMT was excreted as its free form, whereas the major part is either bound to sulphate or glucuronic acid (Table 4).

As further evidence, non-hydrolyzed p.a. samples were analysed for the protonated precursor ions of $_3$DMT-glucuronic acid (*m/z* 357 $[M + H]^+$) and $_3$DMT-sulphate (*m/z* 453 $[M + H]^+$). Signals matching both analytes were detected in different p.a. samples showing typical product ions for $_3$DMT (Figure 2). The obtained MS data clearly indicate that both glucuronic acid and sulphate conjugates of $_3$DMT can be formed in the horse.

These results are in accordance with the studies in humans, rats and pigs[20,31] where the predominant glucuronidation of desmethylmetabolites has been described. Sulfatation has not been reported before, but it is very typical for the metabolism in horses and has been reported in several equine studies before.[32] A

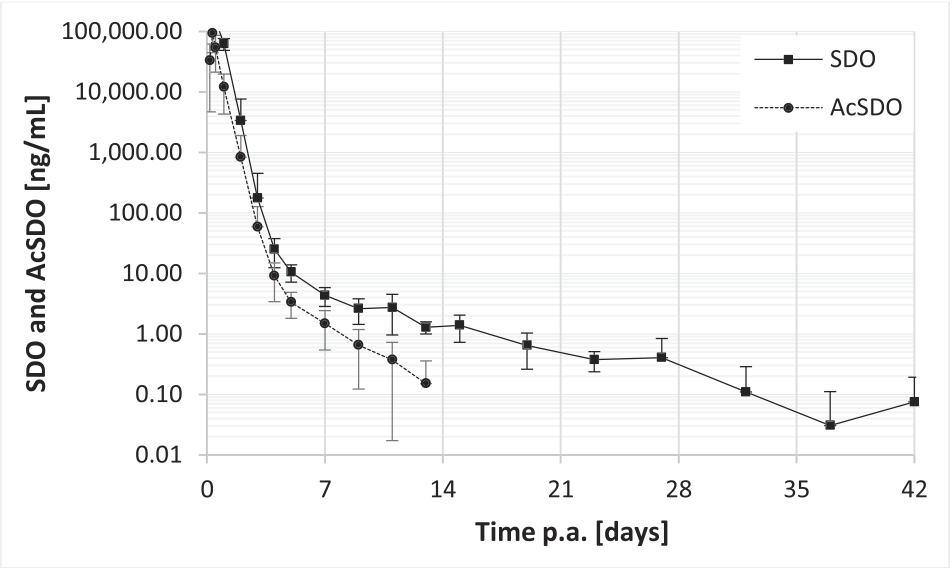


FIGURE 12 Urine disposition of SDO and AcSDO following intravenous administration of SDO at a dose of 13.4 mg/kg. Values are expressed as means (±sd) of eight horses. Abbreviations: AcSDO: N_4-acetylated sulfadoxine; sd, standard deviation; SDO, sulfadoxine.

TABLE 4 Recoveries of SDO, AcSDO, TMP and $_3$DMT after different hydrolysis options in relation to hydrolysis with '*Helix pomatia*'.

		Escherichia coli		*Pseudomonas aeruginosa*		No hydrolysis	
	H. pomatia	%	(sd)	%	(sd)	%	(sd)
SDO	100	92	(±11)	83	(±8)	90	(±10)
AcSDO	100	106	(±26)	118	(±39)	104	(±18)
TMP	100	118	(±8)	113	(±8)	117	(±8)
$_3$DMT	100	30	(±11)	44	(±8)	7	(±3)

Abbreviations: AcSDO, N_4-acetylated SDO; $_3$DMT, 3-desmethyltrimethoprim; sd, standard deviation; SDO, sulfadoxine; TMP, trimethoprim.

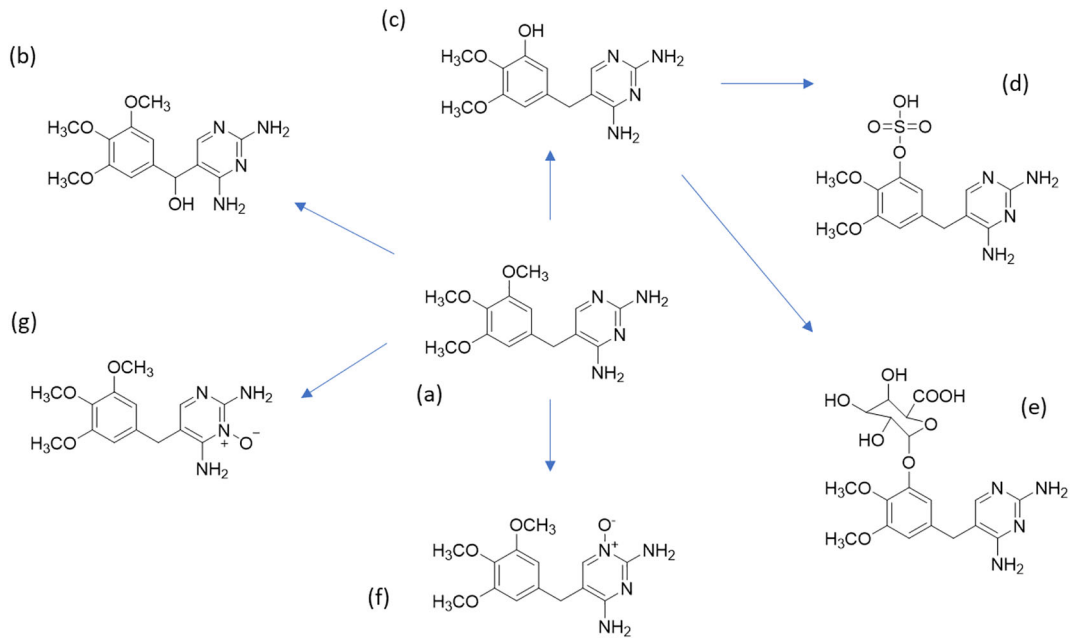


FIGURE 13 Structures of (a) trimethoprim and its confirmed metabolites in horse plasma and/or urine: (b) hydroxytrimethoprim, (c) 3-Desmethyltrimethoprim, (d) 3-Desmethyltrimethoprim sulphate, (e) 3-desmethyltrimethoprim glucuronic acid, (f) trimethoprim-3-oxide and (g) trimethoprim-1-oxide. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 5 Critical values of plasma and urinary SDO concentrations for which 90% and 95% of horses show concentrations below at the specified time points

Specified time points		Plasma (ng/mL)		Urine (ng/mL)	
(days) p.a.	(h) p.a.	P90	P95	P90	P95
0	0	66,317.01	66,447.87	3,127,646.6	3,575,388.5
1	24	5,076.73	5,750.05	177,734.2	216,797.6
2	48	304.79	397.45	9,792.3	13,259.0
3	72	16.61	24.04	521.0	784.7
4	**96**	**1.66**	**2.28**	**54.7**	**76.3**
5	120	0.76	0.89	26.5	32.4
6	144	0.57	0.66	20.1	24.3
7	**168**	**0.46**	**0.53**	**16.3**	**19.6**
8	192	0.38	0.44	13.3	16.3
9	216	0.32	0.37	11.1	13.7
10	240	0.27	0.31	9.3	11.8
11	264	0.23	0.27	8.1	10.1
12	288	0.20	0.23	6.9	8.6
13	312	0.17	0.21	6.0	7.4
14	336	0.15	0.18	5.3	6.5

Note: Monte Carlo simulation ($n = 5{,}000$) of a single i.v. administration of SDO at a dose of 13.4 mg/kg. Numbers in bold highlight the calculated concentrations for a detection time of 4 or 7 days.
Abbreviations: p.a., post-administration; SDO, sulfadoxine.

TABLE 6 Critical values of plasma and urinary SDO concentrations for which 90 and 95% of horses show concentrations below at the specified time points

specified time points			Plasma [ng/mL]		Urine [ng/mL]	
[days]	[h]	post [x] administration	P90	P95	P90	P95
	0	1st	66317.01	66447.87	3127646.6	3575388.5
	24	1st	5076.73	5750.05	177734.2	216797.6
	24	2nd	7545.67	8778.77	258478.9	314250.6
	24	3rd	8849.34	10523.84	301626.3	369844.1
0	24	4th	9579.22	11593.12	323470.6	402759.4
1	24	5th	9985.86	12249.65	337727.1	421852.4
2	48	5th	725.76	1101.03	22552.2	35951.2
3	72	5th	41.69	69.29	1284.9	2261.5
4	**96**	5th	**5.48**	**7.92**	**183.9**	**271.2**
5	120	5th	3.10	3.75	107.1	135.4
6	144	5th	2.47	2.88	84.6	105.0
7	**168**	5th	**2.02**	**2.42**	**69.4**	**87.0**
8	192	5th	1.71	2.03	58.3	74.1
9	216	5th	1.45	1.73	49.4	63.2
10	240	5th	1.24	1.49	42.6	54.4
11	264	5th	1.07	1.30	36.8	46.6
12	288	5th	0.93	1.13	31.9	40.2
13	312	5th	0.82	1.00	27.7	35.3
14	336	5th	0.72	0.88	24.3	31.2

Note: Monte Carlo simulation (n=5000) of 5 i.v. daily administrations of SDO at a dose of 13.4 mg/Kg. Numbers in bold highlight the calculated concentrations for a detection time of 4 or 7 days.
Abbreviations: p.a., post administration; SDO, sulfadoxine.

summary of the proposed metabolism of TMP in the horse is depicted in Figure 13.

3.4 | Implications for medication control

As visible in Figure 7, SDO, TMP and $_3$DMT all display remarkably long detection times (DT) after a single intravenous infusion of Borgal 24%® at the recommended dose. In particular, $_3$DMT could be detected up to 70 days p.a. in urine. After adding an appropriate safety margin of factor two or three, it is obvious that the preparation cannot be used in sports horses without risking an adverse analytical finding. This is a consequence of the increasing sensitivity of analytical methods that detect the substance in urine and blood at concentrations where no pharmacological effect is measurable. Yet, even if it is strongly recommended to reduce the overall use of antibiotics, they must remain applicable if therapeutically necessary. Thus, screening limits (SLs) have to be determined to enable fair and reasonable medication control of AMDs. SLs are already in place for a variety of licensed veterinary drugs.[4] Most of them have been specified according to the Toutain-Model, where, based on an effective plasma concentration (EPC) ineffective urine and plasma levels are calculated.[33] However, this approach is not applicable to antibiotics, as the drug targets the bacterium. Hence, the EPC is depending on the respective bacterial strain. In addition, some federations have already communicated stand-down periods requiring a different approach. Taking a stand-down recommendation of 4 days as a basis, SDO plasma concentrations at 4 days p.a. range between 0.67 and 2.37 ng/mL (mean 1.15 ng/mL). Corresponding TMP concentrations are below the LLOQ (0.2 ng/mL) or even below the LLOD and can be neglected just like other metabolites. Installing an appropriate SL for SDO requires the consideration of the population distribution, which can be estimated using a population model allowing Monte Carlo simulations. The aim was to calculate critical plasma and urine values in a virtual population of 5,000 horses. At least 90% or 95% of the horses were supposed to have plasma or urinary concentrations below this critical value after periods of 1 to 10 days after i.v. administration of SDO at a dose of 13.4 mg/kg (Table 5). A scenario with five administrations of SDO at 13.4 mg/kg at a fixed interval of 24 h was also simulated to see the consequences of the accumulation of the terminal phase of the disposal of SDO (Table 6).

4 days after a single SDO administration, it is expected that 95% of horses are below 2.28 and 76.3 ng/mL for plasma and urine concentrations, respectively.

Inspection of Table 6 shows that according to the Monte Carlo simulation plasma and urine concentrations increase progressively up

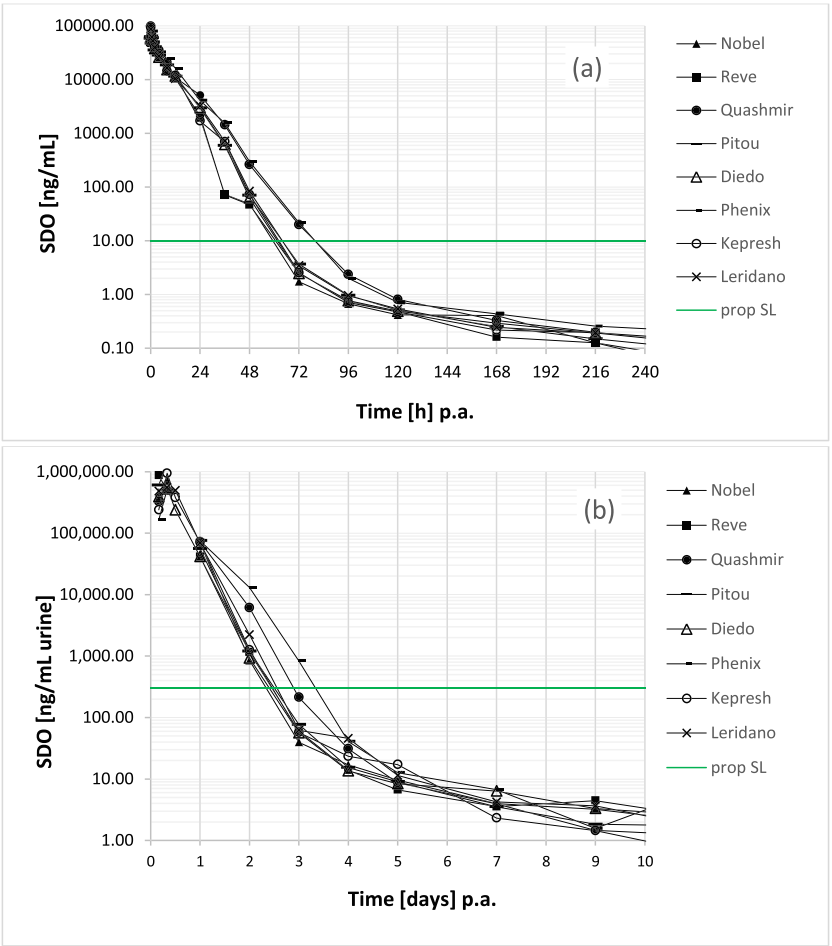


FIGURE 14 Plasma (a) and urinary (b) disposition of SDO following intravenous administration of SDO at a dose of 13.4 mg/kg in eight horses with regard to the proposed SLs. Abbreviations: SDO, sulfadoxine; SL, screening limit. [Colour figure can be viewed at wileyonlinelibrary.com]

to 5 days after initiation of the treatment, then decrease. Comparison of Tables 5 and 6 shows that the delay to achieve plasma concentrations of less than about 2.28 ng/mL in 95% of horses is between 7 and 8 days for multiple i.v. administrations in contrast to 4 days for a single application. According to the manufacturer's recommendation, Borgal®24% should be administered for a period of 3–5 days. Thus, the modulation with five administrations was taken as a basis for a respective SL. For safety reasons, critical values that were calculated based on the 95% prediction interval were used. Thus, given that the desired detection period is 4 days, a provisional plasma SL of 7.92, rounded up to 10 ng/mL and a respective urinary SL of 271.2, rounded up to 300 ng/mL can be postulated. Figure 14 illustrates that concentrations of all eight horses are clearly below these values 4 days p.a. Of course, longer withdrawal times go along with lower SLs, which can be similarly derived from Table 5.

Monte Carlo simulation of TMP values will be part of a second paper. But inspection of Figure 4 already indicates that 4 days p.-a. TMP plasma concentrations are 10-fold lower than corresponding SDO concentrations which would lead to an SL of 1 ng/mL for TMP in plasma. Four days p.a. mean urinary TMP concentrations are at 11.6 (±8) ng/mL, whereas the corresponding SDO mean values are at 25.1 (±12.7) ng/mL(Figure 5). Thus, the prospective SL for TMP in urine will be between 100 and 200 ng/mL. Simultaneous concentrations of the ineffective metabolite $_3$DMT are significantly higher (mean 66.9 [±26.9] ng/mL) and should not be regarded for control of TMP in medication control samples.

4 | CONCLUSION

The generated data clearly indicate that control of SDO and TMP in competition samples is only possible with reasonable SLs. The presented data enable horse sports authorities to advise their doping control laboratories to use SLs that correspond to the desired withdrawal time. The proposed SLs for SDO of 10 ng/mL plasma and 300 ng/mL urine enable the treatment of competition horses when necessary. On the other hand, AMD application to sport horses shortly before a competition is prevented and a recovery period will be guaranteed especially if the recommended withdrawal time will be eight days. Furthermore, the prophylactic application of SDO/TMP combinations is prevented which is in line with the recommendations of WHO. The detection of SDO or TMP metabolites can give additional information but is not relevant for medication control purposes of the tested AMDs.

Finally, it is crucial to mention that the extremely high urine concentrations during 1 day p.a. go along with a potential for cross-contamination. A high degree of caution is required during the therapy of competition horses with AMDs, which includes stable hygiene and separation of treated horses from other sports horses.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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