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Short Communication

Stereoselective methadone disposition after administration of racemic methadone to anesthetized Shetland ponies assessed by capillary electrophoresis

The enantioselectivity of the pharmacokinetics of methadone was investigated in anesthetized Shetland ponies after a single intravenous (0.5 mg/kg methadone hydrochloride; $n = 6$) or constant rate infusion (0.25 mg/kg bolus followed by 0.25 mg/kg/h methadone hydrochloride; $n = 3$) administration of racemic methadone. Plasma concentrations of *l*-methadone and *d*-methadone and their major metabolites, *l*- and *d*-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), respectively, were analyzed by CE with highly sulfated γ -cyclodextrin as chiral selector and electrokinetic analyte injection from liquid/liquid extracts prepared at alkaline pH. In both trials, the *d*-methadone concentrations were lower than those of *l*-methadone and the *d*-EDDP levels were lower than those of *l*-EDDP. For the case of a single intravenous bolus injection, the plasma concentration versus time profile of methadone enantiomers was analyzed with a two-compartment pharmacokinetic model. *l*-methadone showed a slower elimination rate constant, a lower body clearance, and a smaller steady-state volume of distribution than *d*-methadone. *d*-methadone and *d*-EDDP were eliminated faster than their respective *l*-enantiomers. This is the first study that outlines that the disposition of racemic methadone administered to anesthetized equines is enantioselective.

Keywords:

Capillary electrophoresis / EDDP / Horse / Methadone enantiomers / Pharmacokinetics
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Methadone is a synthetic μ -opioid receptor agonist and an *N*-methyl-*D*-aspartate (NMDA)-receptor antagonist which has been commonly used as a perioperative analgesic in veterinary patients [1–4]. Methadone contains a single chiral carbon atom and, thus, exists as a pair of enantiomers with *l*-methadone, also known as levomethadone or *R*-methadone, and *d*-methadone, also known as dextromethadone or *S*-methadone [2]. The drug is clinically administered off-label to horses as an equal (racemic) mixture of *l*- and *d*-methadone or as a proprietary *l*-methadone/fenpipramide formulation (L-Polamivet®; [1]). The enantiomers of methadone possess different pharmacological effects. The *l*-enantiomer is responsible for most of the analgesic effects, since it has a significantly higher affinity toward the μ -opioid receptor compared with the *d*-enantiomer [5]. *d*-methadone is an antitussive opioid and acts as an *N*-methyl-*D*-aspartate receptor antagonist similar to ketamine.

The main metabolic pathways of methadone involve *N*-demethylation and cyclisation to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) followed by a second demethylation to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline [6,7]. These compounds are chiral [8,9]. In people and in Beagle dogs, the pharmacokinetics of racemic methadone has been found to be stereoselective [7,10,11]. In horses, the pharmacokinetics of racemic methadone has been evaluated using nonchiral methods [12,13]. Therefore, the present study was designed to evaluate the stereoselective pharmacokinetics of methadone enantiomers after a single IV bolus or a constant rate infusion (CRI) administration of racemic methadone to anesthetized Shetland ponies.

Enantiospecific analysis by CE has been found to be an attractive tool to investigate the stereoselectivity of drug disposition, metabolism, and pharmacokinetics [14–18]. Recently, we described an enantioselective assay for the determination

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Abbreviations: CRI, constant rate infusion; CYP, cytochrome P450; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

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of ppb enantiomer levels of methadone and EDDP in equine plasma based on CE with highly sulfated γ -cyclodextrin as chiral selector and electrokinetic analyte injection [19]. Its format is similar to that developed for the enantiomers of methadone in human plasma [20] and to that for ketamine and its metabolites in canine plasma [21]. The latter assay was successfully applied to elucidate the pharmacokinetics of ketamine in Beagle dogs [22]. The assay for methadone and EDDP was employed in a study with bolus IV application of a combination of *l*-methadone and fentanyl to anesthetized Shetland ponies that were sedated with detomidine and butorphanol and premedicated with detomidine [23]. In this article, we now report its use to determine the enantiomer levels in arterial plasma samples that were collected in a pharmacokinetic study after application of racemic methadone to Shetland ponies that were sedated and premedicated with romifidine.

The study was approved by the local ethics committee under protocol number TVV 05/15 (Landesdirektion Leipzig, Germany) and was carried out as a spin-off study of a larger trial that investigated chewing kinematics of Shetland ponies. The care and use of experimental animals complied with local animal welfare laws, guidelines, and policies. Six adult experimental Shetland ponies (four mares and two geldings; age range: 4–8 years old; weight range: 96–148 kg), that were considered clinically healthy as assessed by clinical examination and complete blood count, entered the study. The ponies were owned by the University of Leipzig (Institute of Animal Nutrition, Nutrition Diseases and Dietetics, University of Leipzig, Leipzig, Germany) for research purposes. All six ponies entered the single bolus administration trial while only three of the ponies were included in the CRI trial. There was a six-month washout interval between treatments.

The anesthetic protocols (see Fig. 1A and B) for both trials (i.e., single bolus and CRI administration) were similar with some exceptions. Only for the CRI trial, ponies received trimethoprim/sulfadiazine (5 and 25 mg/kg, respectively; Equibactin, CP-Pharma, Germany) orally and flunixin meglumine (1.1 mg/kg; Flunisolil RPS, CP-Pharma, Germany) IV, 30 min before anesthesia induction. Sedation was achieved in both trials with two IV boluses of romifidine (0.04 mg/kg IV; Sedivet, Boehringer Ingelheim, Germany) given 20 min apart. Five min later, anesthesia was induced with diazepam (0.05 mg/kg, IV; Ziapam, Ecuphar, Germany) and ketamine (2.2 mg/kg, IV; Ursotamin, Serumwerk Bernburg, Germany). Anesthesia was maintained with isoflurane (Isofluran CP, CP-Pharma, Germany) per inhalation in an oxygen/air mixture. Ponies were mechanically ventilated throughout the anesthesia and Ringer lactate solution (B. Braun, Melsungen, Germany) was infused. A 22-gauge cannula (Vasofix Braunüle, B. Braun) was introduced into the lateral digital plantar artery of the right hind limb to measure arterial blood pressure invasively and to collect blood samples. Standard monitoring (heart rate and rhythm, peripheral hemoglobin oxygen saturation) was performed (Datex Ohmeda, AS/3, GE Healthcare, Germany). The doses of racemic methadone chosen for the present study were de-

rived from clinical dose regimens [13,24]. For the single-bolus trial, 0.5 mg/kg racemic methadone hydrochloride (i.e., 0.224 mg/kg of each methadone enantiomer; Comfortan, Albrecht, Germany) was given manually IV over 1 min approximately 15 min after induction (Fig. 1A). In the case of the CRI trial, a loading dose of 0.25 mg/kg racemic methadone hydrochloride was administered instead, immediately followed by an infusion of 0.25 mg/kg/h methadone hydrochloride (Fig. 1B).

Arterial blood samples were collected 5 min before (t_{BL}) and 1, 2, 4, 8, 16, 32, and 64 (up to 32 min for the CRI trial) min after methadone administration to assess plasma concentrations of methadone and EDDP enantiomers. For sampling, 5 mL of blood was aspirated from the arterial catheter into a syringe and discarded. Thereafter, another 5 mL of arterial blood was drawn carefully into a syringe, transferred into lithium-heparin tubes, and stored on ice immediately. The samples were centrifuged for 10 min with 2875 g. Supernatant was transferred to Eppendorf tubes and stored at -80°C until further analysis. Plasma samples were analyzed as described previously [19]. Briefly, the assay involves LLE of methadone, EDDP, and the added internal standard *D*-(+)-norephedrine from 100 μL of plasma using dichloromethane at alkaline pH and electrokinetic injection of the analytes (8 kV for 20 s) from the reconstituted extract across a 50 mM phosphate buffer (pH 3.0) plug. Analyses were performed on a CE P/ACE MDQ analyzer (Beckman Coulter, Fullerton, CA, USA) equipped with a 50 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 45 cm total length (effective length 35 cm). The running buffer comprised 100 mM phosphate buffer (pH 3.0) to which 0.16% highly sulfated γ -cyclodextrin as chiral selector (Beckman Coulter) was added. A voltage of 20 kV (normal polarity) was applied and the current was about 48 μA . Sample storage and capillary cartridge temperatures were set to 25 and 15 $^{\circ}\text{C}$, respectively. Analyte detection was achieved at 200 nm (photodiode array detector). Quantification of analyte concentrations was based on five-level internal calibration using corrected peak areas. The calibration range for the enantiomers of methadone was 25–500 ng/mL and that of EDDP 5–100 ng/mL. Lower LOQ of the enantiomers of methadone and EDDP were 25 and 5 ng/mL, respectively. Intra- and interday precisions for the determined compound concentrations were <4% ($n = 6$) and <7% ($n = 6$), respectively [19].

All anesthetic episodes were uneventful and ponies recovered well from anesthesia. A sample corresponding to the minute 2 of the CRI trial was an outlier and was, thus, excluded. Therefore, a total of 48 and 20 plasma samples for the single-bolus and the CRI trial, respectively, were used for further evaluation. None of the baseline samples contained traces of methadone or EDDP enantiomers. Graphs depicting mean plasma concentration versus time are depicted in Fig. 2. For the single-bolus trial, a commercially available pharmacokinetic software (Phoenix WinNonlin 6.4, Certara, Cary, NC) was used to calculate the pharmacokinetic parameters using classic equations [25]. A two-compartment model

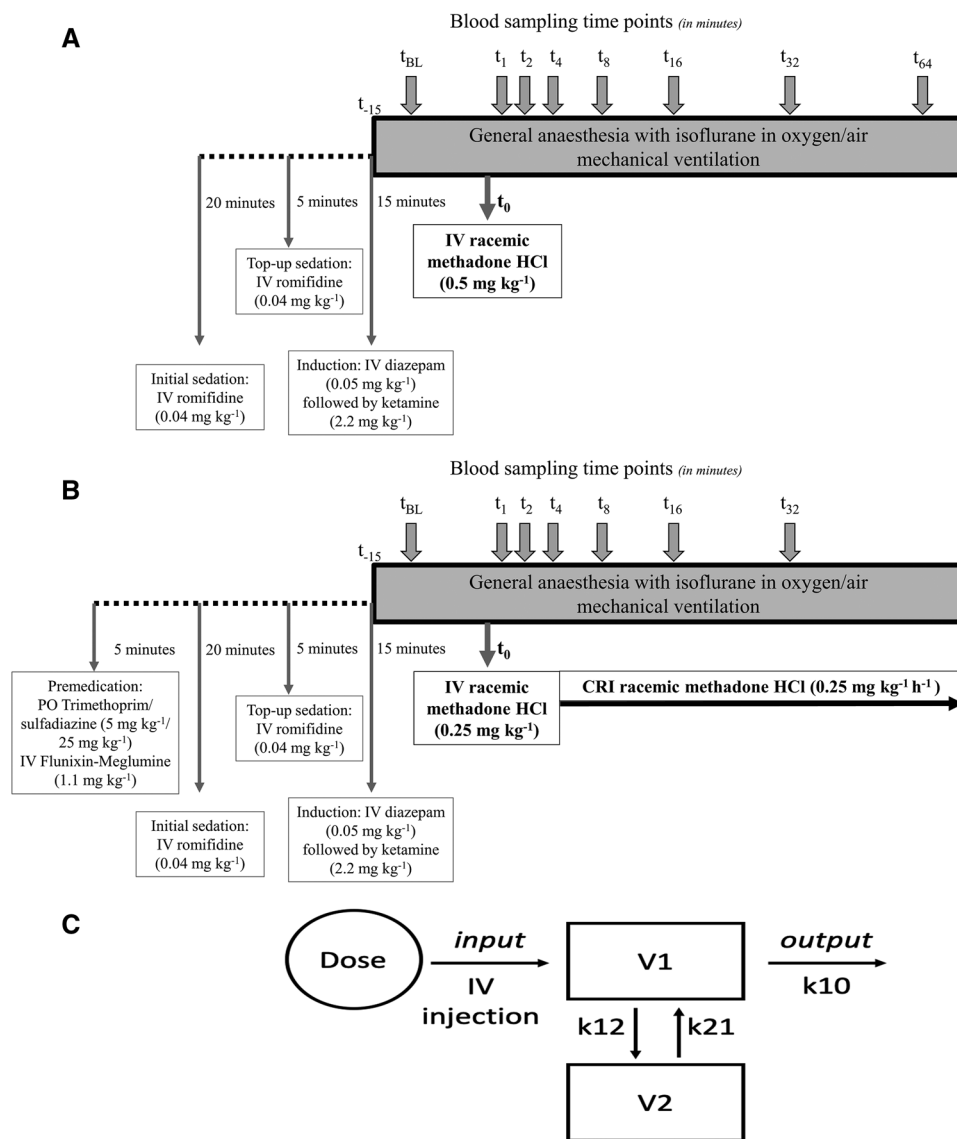


Figure 1. Time line and events for isoflurane anesthetized and artificially ventilated ponies with (A) a bolus of 0.5 mg/kg of racemic methadone hydrochloride and (B) a bolus of racemic methadone hydrochloride 0.25 mg/kg followed by a constant rate infusion of 0.25 mg/kg/h. Blood sampling time for baseline measurement (t_{BL}), the commencement of intravenous drug administration (t_0) and blood sampling at 1, 2, 4, 8, 16, 32, and 64 min thereafter (t_1 , t_2 , t_4 , t_8 , t_{16} , t_{32} , and t_{64} , respectively) are highlighted. Panel C depicts a schematic representation of the two-compartment pharmacokinetic model used to analyze the pharmacokinetic profile of methadone enantiomers. IV = intravenous; V1 and V2 = apparent volume of the central and peripheral compartments, respectively; k_{10} = elimination rate constant; k_{12} and k_{21} = transfer rate constants from compartment one to compartment two and vice versa, respectively.

(Fig. 1C) featuring a distribution and an elimination phase, as was described for the ketamine to norketamine demethylation in Beagle dogs [22], was fitted to both the individual plasma concentrations of the ponies (data not shown) and to their mean values (Fig. 2A). Akaike Information Criterion and visual inspection of the residual plots were used to determine the goodness of fit of the model [26]. The pharmacokinetic parameters for the single-bolus trial are presented in Table 1.

The results of the study provide important insights about the disposition of racemic methadone in anesthetized ponies. To the authors' knowledge, this is the first study that outlines that the disposition of racemic methadone administered as a single bolus or as a CRI to anesthetized horses is enantioselective. In both trials, the plasma concentration of methadone enantiomers showed a rapid decrease after the initial IV injection (Fig. 2). No attempts were made to calcu-

late a CRI regimen that would provide a steady-state plasma concentration. Overall, *D*-methadone plasma concentrations were lower, exhibited a larger distribution and were eliminated faster than *L*-methadone (Fig. 2 and Table 1). Similarly, *D*-EDDP plasma concentrations were lower than *L*-EDDP (Fig. 2). All of these indicate that the *D*-isoforms of methadone and EDDP are removed from plasma faster when compared with the *L*-isoforms. This is in agreement with a previous study in which higher plasma concentrations were found for *L*-methadone compared with *D*-methadone after 0.5 mg/kg IV administration of racemic methadone to unpremedicated Beagle dogs [11], or 0.2 mg/kg orally to drug-free women [27]. On the other hand, a study performed in anesthetized adolescents showed higher plasma concentrations for *D*-methadone than *L*-methadone after the administration of the racemate (0.1–0.3 mg/kg) [28]. The methadone enantiomers exhibited a considerable difference in plasma concentration already

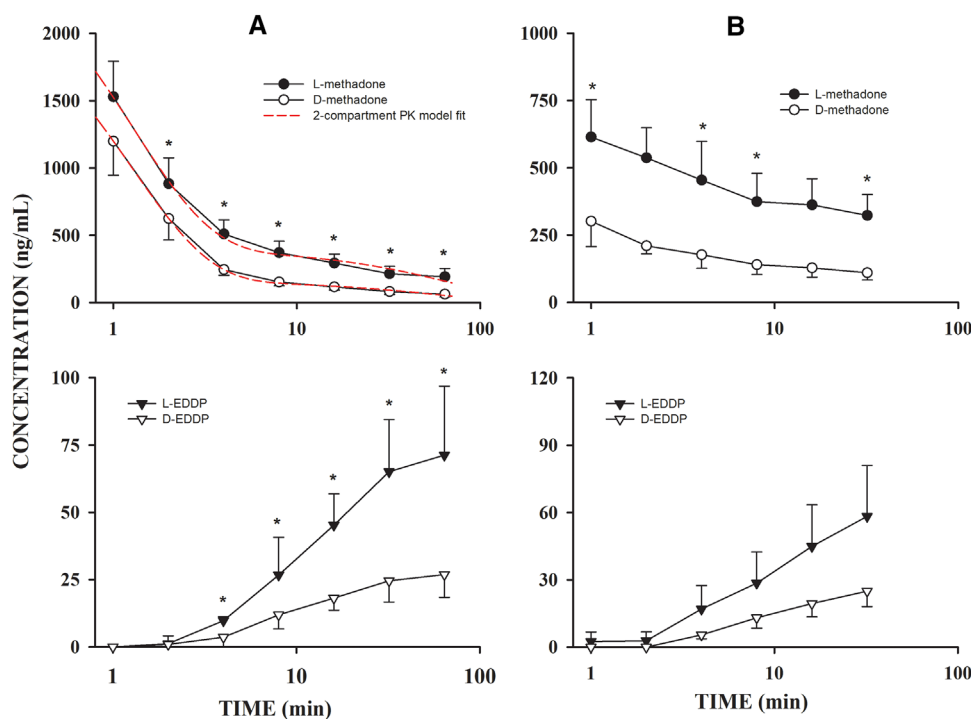


Figure 2. Mean (circles and triangles) and SD (bars) of plasma concentrations of L-methadone (filled circles), D-methadone (empty circles), and the corresponding L-EDDP (filled triangles) and D-EDDP (empty triangles) obtained over time from (A) anesthetized ponies ($n = 6$) that received an intravenous bolus of 0.5 mg/kg of racemic methadone hydrochloride and (B) anesthetized ponies ($n = 3$, except time point 2 min were $n = 2$) that received racemic methadone hydrochloride 0.25 mg/kg intravenously, followed by a constant rate infusion of 0.25 mg/kg/h. The dashed red plots in panel A represent the plasma concentration-time curves obtained through pharmacokinetic modeling of the methadone enantiomers. * = $p < 0.05$ between enantiomers of same compound assessed with the Mann-Whitney Rank Sum test.

1 min after bolus IV administration of the racemic mixture. This is unusual and cannot be caused by differences in their metabolism. Differing binding to plasma proteins, however, could be the reason for the observed result. Protein binding of methadone in humans is known to be enantioselective [29] and remains to be assessed for equines.

The pharmacokinetic parameters of the single-bolus trial for L- and D-methadone could be well described with a two-compartment model (Fig. 1C) and showed small apparent compartment volumes (V_1 and V_2), short mean residence times, and elimination half-lives of approximately 1 h, and a rapid body clearance (Cl_B), indicating limited distribution and rapid drug elimination (Table 1). These parameters bear a resemblance to those reported for racemic methadone after the IV administration of a single bolus of 0.15 mg/mL to unpremedicated horses [13]. The pharmacokinetics of L-methadone in the current study look alike to those obtained after equivalent dose administration (i.e., bolus of 0.25 mg/kg IV) of a proprietary formulation of L-methadone and fentanyl (i.e., L-Polamivet®) to anesthetized Shetland ponies that were sedated and premedicated differently [23]. In the case of the CRI trial, there was insufficient data for a pharmacokinetic analysis.

One important finding of the current study is the different pharmacokinetic profiles of L- and D-methadone. A larger steady-state volume of distribution (V_{ss}), was found for D-methadone compared with L-methadone. The same was true for the apparent volume of the central (V_1) and peripheral (V_2) compartments. The body clearance (Cl_B) was higher for

D-methadone than for the L-enantiomer. Using the Mann-Whitney Rank Sum Test, V_{ss} , V_2 , Cl_B , AUC , B and k_{21} of the pharmacokinetic parameters of the two methadone enantiomers listed in Table 1 were determined to be statistically different ($p < 0.05$). The P values for all other parameters of Table 1, including C_0 which is the maximal concentration at time 0, were much larger than 0.05. The hepatic cytochrome P450 (CYP) enzyme complex is responsible for methadone clearance, primarily via *N*-demethylation to inactive EDDP, with some urinary excretion of the unchanged drug. In humans, it has been established that CYP2B6 has an impact on methadone plasma concentrations, metabolism, and elimination [9,30]. The CYP2B6 was reported to preferentially *N*-demethylate D-methadone over L-methadone [8,31] and an equine CYP2B6 is described in the literature [32]. This could potentially explain the lower plasma concentrations and faster elimination profile observed for D-methadone compared with L-methadone in the current study. Currently, there is a lack of evidence in the scientific literature about the stereoselectivity of EDDP elimination. In the two trials plasma concentrations of L-EDDP were significantly higher than D-EDDP, suggesting that there is an enantioselective elimination of this metabolite in equines. However, the importance of evaluating the downstream metabolism of EDDP is unclear, since EDDP is considered an inactive metabolite. Interestingly, previous investigators could associate prolongations of the electrocardiogram QT-interval with the plasma concentration of EDDP in human volunteers receiving methadone [33].

Table 1. Pharmacokinetic parameters of L- and D-methadone after 0.5 mg/kg intravenous administration of racemic methadone hydrochloride to six anesthetized Shetland ponies

Parameter (unit)	L-Methadone		D-Methadone	
	Mean (SD)	Median (Range)	Mean (SD)	Median (Range)
A (ng/mL)	2936.1 (1409.4)	2455.5 (1450.5–5263.8)	2746.1 (1249.7)	2403.5 (1275.5–4683.2)
B (ng/mL)*	384.2 (92.2)	379.7 (265.8–490.9)	158.9 (48.5)	162.9 (70.74–204.1)
α (L/min)	0.860 (0.386)	0.873 (0.365–1.516)	0.887 (0.371)	0.921 (0.368–1.475)
β (L/min)	0.014 (0.004)	0.014 (0.008–0.021)	0.017 (0.009)	0.016 (0.007–0.033)
$t_{1/2\alpha}$ (min)	0.973 (0.505)	0.794 (0.457–1.901)	0.939 (0.502)	0.753 (0.470–1.885)
$t_{1/2\beta}$ (min)	53.88 (17.08)	51.41 (33.71–85.27)	50.72 (28.72)	43.53 (20.72–104.12)
k_{10} (L/min)	0.119 (0.095)	0.080 (0.048–0.306)	0.245 (0.178)	0.186 (0.096–0.587)
k_{12} (L/min)	0.641 (0.295)	0.660 (0.241–1.128)	0.591 (0.208)	0.627 (0.253–0.837)
k_{21} (L/min)*	0.114 (0.039)	0.102 (0.067–0.162)	0.067 (0.026)	0.071 (0.026–0.094)
V ₂ (L/kg)*	0.393 (0.064)	0.407 (0.317–0.467)	0.823 (0.420)	0.676 (0.460–1.644)
V ₁ (L/kg)	0.078 (0.032)	0.076 (0.040–0.131)	0.091 (0.043)	0.086 (0.046–0.166)
V _{ss} (L/kg)*	0.471 (0.078)	0.467 (0.390–0.598)	0.915 (0.460)	0.751 (0.506–1.180)
AUC _(0→∞) (min ng/L)*	33.42 (11.13)	34.94 (18.21–50.48)	13.32 (2.72)	13.87 (8.27–15.80)
MRT (min)	69.43 (24.62)	65.96 (39.49–114.88)	56.25 (32.29)	48.35 (18.68–113.92)
Cl _B (mL/min/kg) *	7.44 (2.81)	6.42 (4.44–12.30)	17.63 (4.80)	16.15 (14.18–27.08)
C ₀ (ng/mL)	3320.3 (1395.4)	2944.1 (1716.2–5566.8)	2904.9 (1269.5)	2605.8 (1346.2–4853.7)

In the applied two-compartment model, the concentration of methadone is described with the polyexponential equation $c = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$ where c and t are the concentration and time, respectively. A and $B = \gamma$ -intercepts for both exponential functions of the plasma concentration curve; α and $\beta = \gamma$ -intercepts for both exponential functions of the plasma concentration curve; α and $\beta =$ first order rate constants for the distribution and the elimination phase, respectively; $t_{1/2\alpha}$ and $t_{1/2\beta} =$ distribution and elimination half-lives, respectively; $k_{10} =$ elimination rate constant; k_{12} and $k_{21} =$ transfer rate constants from compartment one to compartment two and vice versa, respectively; V_1 and $V_2 =$ apparent volume of the central and peripheral compartments, respectively; $V_{ss} =$ steady-state volume of distribution; $AUC(0 \rightarrow \infty) =$ area under the plasma concentration–time curve with extrapolation to infinity; MRT = mean residence time; $Cl_B =$ body clearance; $C_0 =$ maximal concentration at time 0. * = $p < 0.05$ between methadone enantiomers assessed with the Mann–Whitney Rank Sum test

In conclusion, this study shows a faster elimination of D-methadone and D-EDDP compared with L-methadone and L-EDDP in arterial blood from anesthetized ponies receiving an IV bolus or a CRI of racemic methadone. Further studies are needed to assess the clinical implications of these findings. Furthermore, the study was performed with Shetland ponies and the results may, thus, not be directly transferable to the general horse population. Enantioselective CE with electrokinetic sample injection provides an attractive tool to monitor ppb levels of the stereoisomers of methadone and its metabolite in small amounts of plasma.

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The authors have declared 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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