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Pharmacokinetic profiles of the active metamizole metabolites in healthy horses

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Metamizole (MT) is an analgesic and antipyretic drug labelled for use in humans, horses, cattle, swine and dogs. MT is rapidly hydrolysed to the active primary metabolite 4-methylaminoantipyrine (MAA). MAA is formed in much larger amounts compared with other minor metabolites. Among the other secondary metabolites, 4-aminoantipyrine (AA) is also relatively active. The aim of this research was to evaluate the pharmacokinetic profiles of MAA and AA after dose of 25 mg/kg MT by intravenous (i.v.) and intramuscular (i.m.) routes in healthy horses. Six horses were randomly allocated to two equally sized treatment groups according to a 2 × 2 crossover study design. Blood was collected at predetermined times within 24 h, and plasma was analysed by a validated HPLC-UV method. No behavioural changes or alterations in health parameters were observed in the i.v. or i.m. groups of animals during or after (up to 7 days) drug administration. Plasma concentrations of MAA after i.v. and i.m. administrations of MT were detectable from 5 min to 10 h in all the horses. Plasma concentrations of AA were detectable in the same range of time, but in smaller amounts. Maximum concentration (C_{max}), time to maximum concentration (T_{max}) and $AUMC_{0\text{-last}}$ of MAA were statistically different between the i.v. and i.m. groups. The AUC_{IM}/AUC_{IV} ratio of MAA was 1.06. In contrast, $AUC_{0\text{-last}}$ of AA was statistically different between the groups ($P < 0.05$) with an AUC_{IM}/AUC_{IV} ratio of 0.54. This study suggested that the differences in the MAA and AA plasma concentrations found after i.m. and i.v. administrations of MT might have minor consequences on the pharmacodynamics of the drug.

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INTRODUCTION

Metamizole (sodium *N*-[(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazolin-4-yl)-*N*-methylamino] methanesulphonate) (MT), also known as dipyrone, is a pyrazolone derivative (Brogden, 1986) introduced to pharmacotherapy in 1922 in Germany (Hinz *et al.*, 2007). This is one of the strongest nonopioid analgesic drugs, used in both human and veterinary medicine for the treatment of pain and fever (Baumgartner *et al.*, 2009). It is a weak COX-1 and COX-2 inhibitor (Botting, 2000) but a strong COX-3 inhibitor (Chandrasekharan *et al.*, 2002). MT is on the human and veterinary market in several countries (European countries, Asia and South America) but has been withdrawn in others (Sweden, USA, Japan, UK, Australia and Iran) because of safety concerns in humans. Although MT seems to be a relatively safe drug (Bigal *et al.*, 2002; Imagawa *et al.*, 2011) compared with other nonopioid analgesics, there is some evidence, which is not unanimously accepted, suggesting that after prolonged administration, MT might cause some damage to the haematopoietic system, triggering leukopenia, agranulocytosis and even aplastic anaemia in humans (Hedenmalm & Spigset, 2002; Garcıa-Martınez *et al.*, 2003; Basak *et al.*, 2010). However, pharmacovigilance veterinary data have indicated that the incidence of adverse reactions in the target species is very low (Committee for Veterinary Medicinal Products, 2003). For veterinary use, MT is a drug labelled for use in horses, cattle, swine and dogs. It is administered parentally in the dose range of 20–50 mg/kg body weight (package leaflet, Biovetalgin, BioWet, Drwalew, Poland).

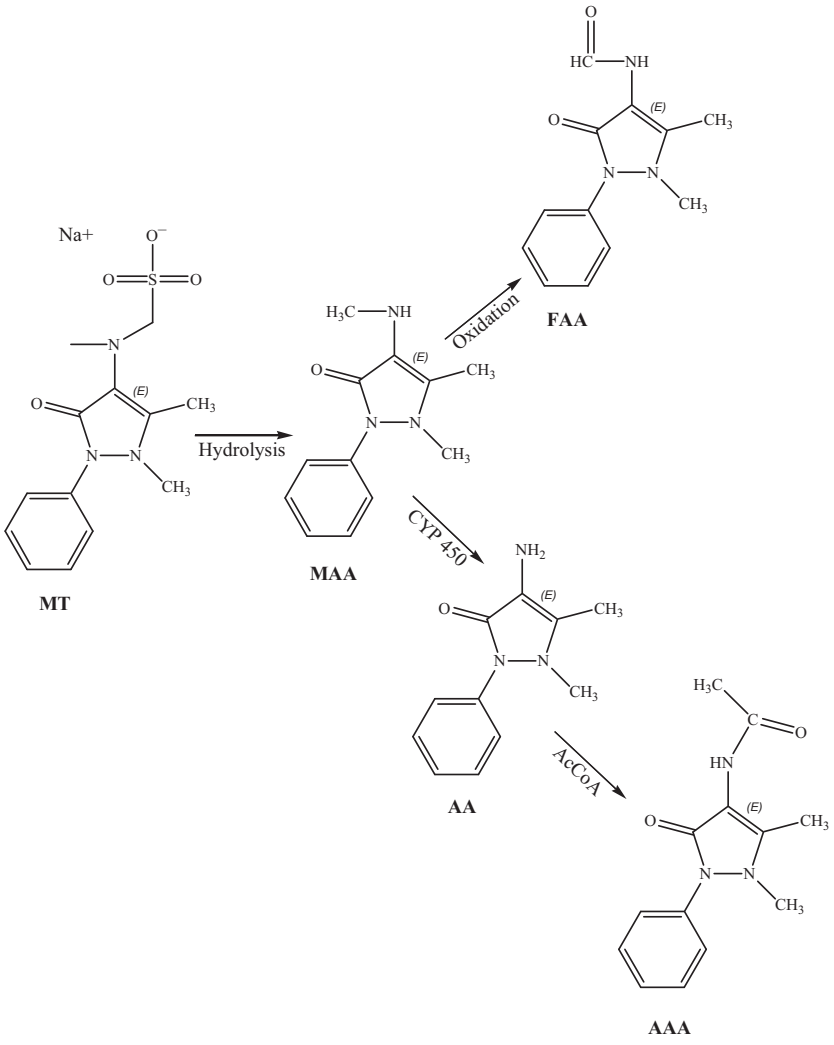

Fig. 1. Metabolic pathway of metamizole (MT) reported in humans (Geisslinger *et al.*, 1996).

There is a paucity of data on the pharmacokinetic properties of MT in animals (Klaus *et al.*, 1997), although the fate of MT administered to humans has already been described (Levy *et al.*, 1995). MT is considered a prodrug which, in an aqueous environment, undergoes spontaneous hydrolysis to numerous metabolic products (Vlahov *et al.*, 1990; Levy *et al.*, 1995). The parent drug is detectable in serum for just a few minutes after intravenous administration, but not after oral dosing. It is also not detectable in urine (Vlahov *et al.*, 1990). In humans, MT is rapidly hydrolysed to the main metabolite 4-methylaminoantipyrine (MAA) (Fig. 1). MAA is further metabolized to 4-formylaminoantipyrine (FAA), which is an end-metabolite, and to 4-aminoantypyrine (AA) (Levy *et al.*, 1995). AA is acetylated to 4-acetylaminoantipyrine (AAA) (Vlahov *et al.*, 1990; Levy *et al.*, 1995; Rogosch *et al.*, 2012). MAA and AA are active metabolites (Weithmann & Alpermann, 1985; Vlahov *et al.*, 1990). The European Medicines Agency (EMEA) document (summary report) reports that in bovine, porcine and equid species, MAA has been selected as a marker residue for maximum residue limit (MRL) calculation (Committee for Veterinary Medicinal Products, 2003).

To the best of the authors' knowledge, only one report is present on the pharmacokinetics of MAA after MT intravenous administration in horses (Klaus *et al.*, 1997). Hence, the aim of the present study was to compare the pharmacokinetic profiles of MAA and AA after intravenous (i.v.) and intramuscular (i.m.) administrations of MT in healthy horses.

MATERIALS AND METHODS

Chemicals and reagents

Pure MAA and AA analytical standard (>99.0% purity) were purchased from Toronto Research Chemicals (Toronto, Canada) and Sigma-Aldrich (St. Louis, MO, USA). The Internal Standard (IS) metoclopramide powder (>99.0% purity) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Horse control plasma samples were collected in untreated healthy horses belonging to the same herd as the six animals selected for the treatments.

Animal treatment and sampling

Six female healthy adult racehorses (Italian trotter breed) aged 9–13 years and weighing 480–590 kg were enrolled in the study, which was performed with approval from the Ethical

Committee for Animal Experimentation of the Kostanay State University. The horses were determined to be clinically healthy on physical examination, serum chemistry and haematological analyses. Animals were evaluated daily (for 1 week) for visible adverse effects by specialized personnel. Horses were acclimatized to the stalls and handlers prior to commencing the study. Animals were deprived of food for 8 h prior to the commencement of the experiment while water was available *ad libitum*. Hay was available *ad libitum* from 2 h after treatment administration.

Animals were randomly allocated to two treatment groups (A = 3 and B = 3) (six slips of paper marked with the numbers 1–6 in a box) according to an open, single-dose, two-treatment and two-period crossover design experiment. Two jugular venous catheters, one in each side (for MT administration and for sample collection, respectively), were placed in each animal 1 day prior to commencement of the study. The group A animals received a single dose of MT (25 mg/kg) by intravenous injection (i.v.) (Biovetalgin, injectable solution 500 mg/mL, BioWet, Drwalew, Poland) while the group B animals received MT at the same dose by intramuscular injection (i.m.), injected in the middle quadrant of the neck muscle (Biovetalgin, injectable solution 500 mg/mL, BioWet, Drwalew, Poland). The dose was selected based on package leaflet recommendations. An interval of 1 week (washout period) was observed to ensure complete metabolism and excretion of MAA and AA. After this period, the groups were rotated and the crossover study completed. By the end of the study, each horse had received MT by both administration routes. The blood (3–5 mL) was collected via previously inserted catheters at assigned times (0, 5, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10 and 24 h). The samples were centrifuged at 1006***g*** within 30 min of collection, and the harvested plasma was frozen immediately and stored at −20 °C. Samples were analysed within 1 week of collection.

HPLC-UV

The analytical method was based on a previously described method (Domínguez-Ramírez *et al.*, 2012) with slight modifications (Giorgi *et al.*, 2015). The HPLC system was an LC Jasco (Jasco Italia, Como, Italy) that consisted of a quaternary gradient system (PU 2089 PLUS; Jasco Italia), in line with an ultraviolet detector (Jasco UV-975; Jasco Italia) set at 254 nm. The chromatographic separation assay was performed with a Luna C18 (2) analytical column (250 × 4.6 mm inner diameter, 5 μ particle size [Phenomenex, Bologna, Italy]) preceded by a security guard column with the same stationary phase (C18(2) [Phenomenex, Bologna, Italy]). The system was maintained at 25 °C. The mobile phase consisted of acetonitrile:ammonium acetate (20 mM) solution, pH 5 (20:80, v/v) at a flow rate of 1 mL/min. The elution of the substances was carried out in isocratic mode.

Sample extraction

The procedure was performed in a 15-mL polypropylene vial. A 0.5-mL aliquot of plasma was added to 100 μL of IS (25 μg/mL). After 30-s vortexing, 0.1 mL of sodium hydroxide (1 N) was added and the sample vortexed again. An aliquot of 4 mL of ethylacetate: methylene chloride (3:7, v/v) was added, then vortexed (30 s), shaken (60 osc/min, 10 min) and centrifuged at 10 956***g*** (rotor radius 5 cm) for 10 min at 10 C. Three millilitres of supernatant was collected in a new 15-mL screw-cap vial. The organic phase was evaporated under a gentle stream of nitrogen (40 °C) and reconstituted with 100 μL of mobile phase. Fifty microlitres of this solution was injected onto the HPLC.

Pharmacokinetic analysis and statistical analysis

The pharmacokinetic calculations were carried out using WINNONLIN v 5.3.1 (Pharsight Corp, Princeton, NJ, USA). The curve fit was performed by a noncompartmental analysis. The pharmacokinetic parameters are presented as mean ± standard deviation.

To make comparisons across treatments, the different parameters were first tested for normal distribution and variance homogeneity. Data were compared with the paired *t*-test or the nonparametric Wilcoxon test, depending on whether the data passed a normality test. In all experiments, differences were considered significant if $P < 0.05$.

RESULTS

The HPLC method was revalidated using control horse plasma. Briefly, MAA and AA were linear in the range of 100–5000 ng/mL. LOD was 30 ng/mL and LOQ was 50 ng/mL. When the metabolite concentrations in the samples exceeded the upper limit of the range, they were re-analysed after appropriate dilution. The intraday and interday precision coefficients of variations were lower than 4.1% and 5.4%, and 6.3% and 6.9% for MAA and AA, respectively. The relative error of accuracy in within-day and between-day assay was lower than 3.9% and 4.3%, and 5.1% and 4.7% for MAA and AA, respectively. No behavioural changes or alterations in health parameters were observed in the i.v. or i.m. groups of animals during or after (up to 7 days) the drug administration.

Plasma concentrations of MAA after i.v. administration of MT were detectable from 5 min to 10 h in all horses of both administration groups. As expected, the C_{max} of MAA was higher in the i.v. group than in i.m. group and this concentration was achieved earlier in the i.v. group (0.083 vs. 1.21 h). One hour after MT injection, the average MAA plasma concentrations of i.v. and i.m. groups became similar. From 1.5 to 8 h, MAA plasma concentrations were higher in the i.m. group than in the i.v. group, although not to a statistically significant extent. The per cent of AUC that was extrapolated to infinity ($AUC_{Extrap\ \%}$) was always <20% in all the subjects. The average pharmacokinetic curves are shown in Fig. 2. The main pharmacokinetic parameters are reported in Table 1. T_{max}, C_{max} ($P < 0.01$) and $AUMC_{0\text{-last}}$ ($P < 0.05$) were statistically different between the groups. Ratio calculated based on the relative $AUC_{0\text{-last}}$ values $AUC_{IM\ MAA}/AUC_{IV\ MAA}$ was 1.06.

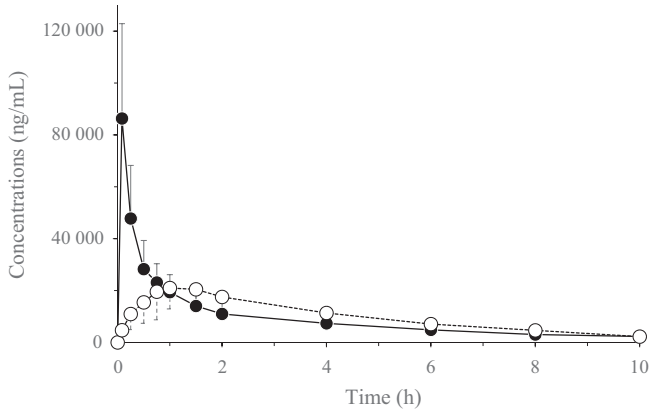

Fig. 2. Mean plasma concentrations of 4-methylaminoantipyrine (MAA) vs. time curves following intravenous (—●—) and intramuscular (–○–) doses of 25 mg/kg metamizole (MT) in healthy horses (n = 6). Bars represent the standard deviations.

Table 1. Main pharmacokinetic parameters of 4-methylaminoantipyrine (MAA) following single intravenous (i.v.) and intramuscular (i.m.) administrations of metamizole (MT) (25 mg/kg) in healthy horses (n = 6)

Parameter	i.v. Mean ± SD	i.m. Mean ± SD
R^2	0.98 ± 0.02	0.97 ± 0.02
λz (1/h)	0.21 ± 0.03	0.24 ± 0.05
$t_{1/2}$ λz (h)	3.34 ± 0.40	3.00 ± 0.56
T_{max} (h)†	0.08 ± 0.00	1.21 ± 0.56
C_{max} (μg/mL)†	86.33 ± 36.55	24.14 ± 8.09
AUC $_{0\text{-last}}$ (h·μg/mL)	94.42 ± 43.60	100.03 ± 22.63
AUC $_{0\text{-}\infty}$ (h·μg/mL)	106.10 ± 50.51	109.56 ± 22.52
Vz/F	1341.81 ± 527.07	1013.74 ± 323.32
Cl/F	286.14 ± 129.53	231.75 ± 51.54
AUMC $_{0\text{-last}}$ (h^2·μg/mL)†	245.27 ± 139.20	345.68 ± 51.55
MRT (h)	3.70 ± 0.93	4.70 ± 0.70

R^2, correlation coefficient; λz, terminal phase rate constant; $t_{1/2}\lambda z$, terminal half-life; T_{max}, time of peak; C_{max}, peak plasma concentration; $AUC_{0\text{-last}}$, area under the plasma concentration–time curve; $AUC_{0\text{-}\infty}$, area under the plasma concentration–time curve extrapolated to infinity; Vz/F, apparent volume of distribution; Cl/F, apparent clearance; $AUMC_{0\text{-}\infty}$, area under the first moment curve from zero to infinity; MRT, mean resident time; SD, standard deviation.
†Statistically different value between the treatment groups.

Plasma concentrations of AA after i.v. administration of MT were detectable from 5 min to 10 h in all horses of both administration groups. These concentrations were lower than those found for MAA. Ratio calculated based on the relative $AUC_{0\text{-last}}$ values $AUC_{IV\ MAA}/AUC_{IV\ AA}$ was 33.7, while the $AUC_{IM\ MAA}/AUC_{IM\ AA}$ ratio was 65.8. The average pharmacokinetic profiles of AA are reported in Fig. 3. AA formation was increased after i.v. compared with i.m. administration of MT, despite the formation rate (slope) being similar between the groups. The main pharmacokinetic parameters are reported in Table 2. $AUC_{0\text{-last}}$ was the only parameter found to be statistically different between the groups ($P < 0.01$). The $AUC_{IM\ AA}/AUC_{IV\ AA}$ ratio was 0.54.

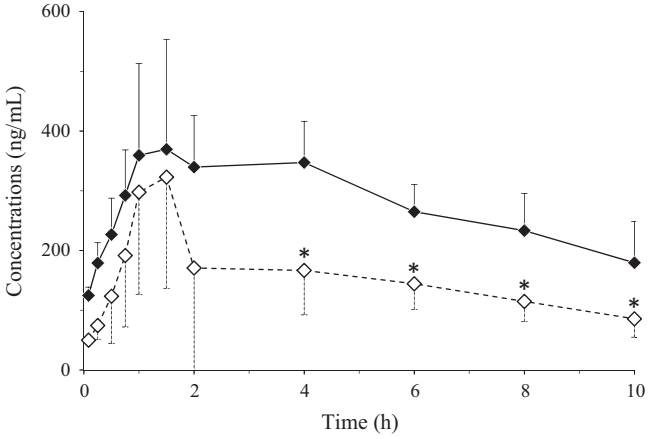

Fig. 3. Mean plasma concentrations of 4-aminoantypyrine (AA) vs. time curves following intravenous (—◆—) and intramuscular (–◇–) doses of 25 mg/kg metamizole (MT) in healthy horses (n = 6). Bars represent the standard deviations. *Statistically different between the groups.

Table 2. Main pharmacokinetic parameters of 4-aminoantypyrine (AA) following single intravenous (i.v.) and intramuscular (i.m.) administrations of metamizole (MT) (25 mg/kg) in healthy horses (n = 6)

Parameter	i.v. Mean ± SD	i.m. Mean ± SD
R^2	0.87 ± 0.21	0.85 ± 0.31
λz (1/h)	0.12 ± 0.05	0.17 ± 0.06
$t_{1/2}$ λz (h)	6.43 ± 2.89	4.27 ± 1.34
T_{max} (h)	2.00 ± 1.04	2.25 ± 1.86
C_{max} (μg/mL)	0.45 ± 0.13	0.36 ± 0.17
$AUC_{0\text{-last}}$ (h·μg/mL)†	2.80 ± 0.53	1.52 ± 0.47
MRT (h)	4.58 ± 0.34	4.44 ± 0.63

R^2, correlation coefficient; λz, terminal phase rate constant; $t_{1/2}\lambda z$, terminal half-life; T_{max}, time of peak; C_{max}, peak plasma concentration; $AUC_{0\text{-last}}$, area under the plasma concentration–time curve; MRT, mean resident time; SD, standard deviation.
†Statistically different value between the treatment groups.

DISCUSSION

Metamizole, a non-narcotic analgesic, has been used to treat pain and fever for almost 90 years in some countries, while in others it is completely unknown or forgotten (Nikolova *et al.*, 2012). MT is known to possess powerful pain-relieving, antipyretic and spasmolytic properties (Levy *et al.*, 1995) and does not have the contraindications or limitations usually observed with opioids or NSAIDs (Avellaneda *et al.*, 2000; Kemal *et al.*, 2007; Baumgartner *et al.*, 2009; Zukowski & Kotfis, 2009; Edwards *et al.*, 2010). MT has been shown to be a safe and important drug for the management of pain, but its use in humans is still controversial. There is plenty of literature attesting to the analgesic efficacy of MT in human beings (Olson *et al.*, 1999; Avellaneda *et al.*, 2000; Kemal *et al.*, 2007; Zukowski & Kotfis, 2009; Edwards *et al.*, 2010; Korkmaz Dilmen *et al.*, 2010).

In veterinary medicine however, the scenario is totally different as the evidence from veterinary studies is not as strong as that from the human literature. There are some data available concerning clinical and side effects of MT in horses (Roelvink *et al.*, 1991), rabbits (Baumgartner *et al.*, 2009), rats (Silva-Moreno *et al.*, 2009) and dogs (Imagawa *et al.*, 2011; Flôr *et al.*, 2013; Teixeira *et al.*, 2013; Zanuzzo *et al.*, 2015) and some concerning the pharmacokinetic profile of its metabolite MAA in horses (Klaus *et al.*, 1997), rats, and dogs (Christ *et al.*, 1973) and sheep (Giorgi *et al.*, 2015).

In the last few years, due to MT attractive pharmacological features, safety profile and low price, there has been a rising interest in its use in the veterinary field. However, previous pharmacokinetic–pharmacodynamic data on MT or active metabolites are insufficient or absent from the literature. For the horse, a single study (Klaus *et al.*, 1997) reporting the pharmacokinetics of MAA after a single i.v. administration of MT is present.

The pharmacokinetic profiles of MAA after i.m. and i.v. administrations of MT were very similar. The significant differences found in C_{max} values were ascribable to the routes of administration of MT. The complete/immediate introduction of MT into the vascular compartment (i.v. injection) may have generated, in the initial minutes, a more rapid metabolic conversion (increasing the C_{max} of MAA) compared with the i.m. injection where an absorption phase is expected.

Despite the abrupt peak of MAA concentration following i.v. administration, no adverse effects were shown in the animals. The absorption phase may also be responsible for the difference in T_{max}. The MAA AUC values (i.v. vs. i.m.) showed that the exposition to the drug over time is similar and the difference in T_{max} is unlikely to be associated with a clinical effect.

Although the i.v. dose of MT was the same, the plasma concentrations of MAA detected in the present study were lower than those in the study of Klaus *et al.* (1997). However, the drug administered in the Klaus *et al.* (1997) study was a combination product (Buscopan compositum, Boehringer-Ingelheim, Ingelheim, Germany) labelled for humans and also containing hyoscine butylbromide. Pharmacokinetic interactions between the two active compounds might have affected the MT metabolism or the MAA kinetics. However, the $AUC_{0-\infty}$ values of MAA are comparable between the studies.

The half-life ($t_{1/2}\lambda z$) reported in this study was shorter than those previously reported in dogs (4–5 h; Liischer, 1993) and horses (4.85 h; Klaus *et al.*, 1997) but similar to that reported in sheep (1.45–3 h; Giorgi *et al.*, 2015). The reason for this discrepancy might be due to a number of factors such as differences in animal species, route of administration, presence of pathophysiological conditions, age of the animals and sensitivity of the analytical method. Volume of distribution is large, a finding in line with MAA being detected in cerebrospinal fluid (Cohen *et al.*, 1998).

Different pharmacokinetic trends have been found for the AA metabolite after i.m. and i.v. administrations of MT. The first part of the curves was similar (AA formation), while the elimination phases, although they had similar slope, showed different AA concentrations. As a result, the $AUC_{0\text{-last}}$ parameter of the i.v. group was double that of the i.m. group. The abrupt peak of MAA concentration following i.v. administration might have saturated the metabolic pathway MAA to AA, metabolizing (oxidizing) a proportion of MAA to FAA. This might explain why the C_{max} values of AA between the groups are not statistically different. Further studies evaluating all the metabolites formed in the horse are necessary to clarify this issue. In humans, the analgesic effect of MT correlates with the concentration of MAA and AA, which differ with regard to their time of onset (MAA >AA) and terminal half-life (MAA: 4–5 h, AA: 5–8 h) (Nikolova *et al.*, 2012). MAA is around 50 times more active than MT as an inhibitor of COX-3 enzyme (Nikolova *et al.*, 2012), while AA is less active than MT. Therefore, both metabolites contribute to the clinically relevant features of rapid onset and long duration of the effect, permitting 6–8 hourly dosing intervals. The half-life of MAA, however, is dose-dependent (Maier, 1999). The other two metabolites, FAA and AAA, are inactive. The metabolites which generate the analgesic action are still unknown in the horse.

According to the drug producer, a 25 mg/kg injection of MT is an effective dose to relieve pain in horses. If we assume that analgesic activity is only attributable to MAA and AA metabolites as in humans, the contribution of AA to the overall therapeutic activity might be negligible due to its negligible plasma concentrations and activity. Hence, it might be presumed that MAA is the main metabolite responsible for the overall analgesic effect. The average plasma concentration of this metabolite (which is likely to produce pain relief in the horse), in other words its minimal effective concentration, can theoretically be calculated as $AUC_{0\text{-last}}/10$ h and approximated to be above 10 μg/mL. Further studies are necessary however, to establish whether the metabolic pattern reported in humans matches that in horses as well as pharmacodynamic studies including a comparison of MT vs MAA, to determine its efficacy in different types of pain.

CONCLUSION

This is the first study reporting the pharmacokinetics of MAA and AA after i.v. and i.m. administrations of MT in horses. The MAA pharmacokinetic profiles were similar between the treatment groups while twice the amount of AA was formed after i.v. administration of MT. Although further studies are needed to understand the metabolic pathway of MT as well as its safety profile, the difference reported in AA concentrations might be clinically negligible in horses.

CONFLICT OF INTEREST STATEMENT

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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