1 **Pharmacokinetics of paracetamol in the Thoroughbred horse following an oral**
2 **multi-dose administration.**

3

4 Pharmacokinetics of paracetamol in the horse

5

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Pharmacokinetics of paracetamol in the Thoroughbred horse following an oral multi-dose administration.

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Abstract

Paracetamol is a widely used, non-opioid analgesic and antipyretic drug. Scientific evidence suggest it is an effective pain treatment in equine medicine. However, there is very little published information about the pharmacokinetics of the drug in the horse. The aim of the research was to determine the pharmacokinetics of paracetamol in equine plasma and urine, to inform treatment of Thoroughbred racehorses. In this

multi-dose study, paracetamol was administered orally at 20 mg/kg to six Thoroughbred horses. Pre- and post-administration urine and plasma samples were collected and analysed using a quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Pharmacokinetic analysis of urine and plasma paracetamol clearance profiles was carried out, which enabled the calculation of possible Screening Limits (SL) that can regulate for a Detection Time of 120 hours. Additionally, an estimation of orthocetamol concentration levels in urine was carried out to investigate any underlying relationship between the para and ortho isomers since both were suspected to contribute to basal levels, possibly due to environmental feed sources.

Keywords: Paracetamol, orthocetamol, pharmacokinetics, screening limit, detection time

1 Introduction

Paracetamol (or acetaminophen, Figure 1a) is a widely used, over the counter, non-opioid analgesic and antipyretic drug. [1, 2, 3] Clinically, paracetamol can be administered orally, rectally or intravenously and can produce analgesia within 40 min, with maximal effect at 1 h (human data). [3] Besides its use as a standalone analgesic, paracetamol is often co-administered with other drugs (such as phenylbutazone), as it aids the overall efficacy of analgesia. Orally administered paracetamol is absorbed mainly from the small intestine and metabolised in the liver. In humans, most of the drug is

1 metabolised through glucuronidation and sulphation. [3] Orthocetamol (or 2-
2 acetamidophenol, see Figure 1b) is a structural isomer of paracetamol. It is believed
3 to have similar analgesic and antipyretic properties to paracetamol and was shown to
4 be subject to the same metabolic reactions. [4,5] Orthocetamol is not currently used as
5 a licensed medication. Previous reports indicate that both compounds are frequently
6 detected in Thoroughbred racehorse urine in South Africa [2] and Japan. [6] This is also
7 supported by routine medication and doping control screening data acquired in the UK
8 (unpublished data).

9 In the United Kingdom, there are no licensed oral (or other) preparations of
10 paracetamol available for horses, only for dogs and pigs. [7] However, preparations
11 licensed for use in humans and other animals can be prescribed under the 'cascade'
12 for pain management in horses. The cascade allows veterinary surgeons to legally
13 prescribe medicines that are not authorised for the relevant clinical case or for the
14 relevant species under treatment when there is no authorised veterinary medicinal
15 product (VMP) available. [8,9] Due to the low cost, fast mode of action and benefits of
16 combined therapy with other drugs [10, 11], paracetamol use has increased in equine
17 medicine. Recently, oral administration of paracetamol at 20 mg/kg to 8 horses was
18 shown to be as effective as flunixin meglumate in a reversible model of equine foot
19 pain.[12]

20 Studies have demonstrated paracetamol as a legitimate candidate for effective pain
21 management in equine medicine. However, as it can have an effect on the nervous
22 system, and other body systems, it is prohibited on race day according to British
23 Horseracing Authority (BHA) Rules of Racing [13] and International Federation of
24 Horseracing Authorities (IFHA) International Agreement. [14] In order to control use of
25 substances that can be legitimately used out of competition the European Horserace

1 Scientific Liaison Committee (EHSLC), an advisory body that aims to harmonise the
2 racing rules in Europe, publishes scientifically derived Detection Times (DTs). DTs are
3 published to inform trainers and their veterinarians, and ensure horses can be
4 effectively and appropriately treated in training but are not subject to the effect of
5 prohibited substances on race day. A Screening Limit (SL) is a concentration at which
6 the drug is considered to no longer have a therapeutic effect in a population of horses,
7 which is typically associated with the relevant DT. [15] The DT may then be used by the
8 veterinary surgeon, to calculate a suitable Withdrawal Time (WT), which includes an
9 appropriate safety margin added to the DT. The determination of the DT for
10 paracetamol is important, given that it is observed at low concentrations in race day
11 samples, and inadvertent environmental exposure cannot be discounted as a possible
12 cause. Similarly to paracetamol, its structural isomer, orthocetamol, appears to be
13 ubiquitous in post-race samples, at levels considerably higher than paracetamol. It is
14 believed that the most likely source of orthocetamol is plants which make up horse
15 feed, whilst the source of paracetamol remains unknown. [2,16]

16

17 The SLs and DTs are deduced from pharmacokinetic data collected experimentally,
18 through a controlled administration study. The data is used to determine the effective
19 plasma concentration (EPC) and irrelevant plasma concentration (IPC), which can
20 help to discriminate between therapeutic and non-therapeutic drug concentrations.
21 Toutain and Lassourd published this approach as a way of establishing cut-off values
22 for drugs which can be present in blood/urine on the day of racing due to ongoing
23 treatment. [17] To date, only one study has determined EPC and IPC values for
24 paracetamol in horse. [16] However, these values were produced based on data

1 published by another research group [18] and some of the parameters were based on
2 human studies.

3

4 The first aim of the research presented here was to carry out a quantitative analysis
5 of plasma and urine samples for paracetamol, following an oral administration of
6 paracetamol at a dose of 20 mg/kg twice per day to six Thoroughbred horses. The
7 second aim was to produce a DT in both matrices to support veterinarians in the
8 legitimate therapeutic use of paracetamol. Additionally, semi-quantitative analysis of
9 orthocetamol in administration urine samples was carried out to establish any potential
10 relationship between the analytes.

11

12 2 Materials and methods

13 2.1 Administration study

14 Six Thoroughbred horses (3 geldings and 3 mares) with a mean ± SD weight of 541.2
15 ± 72.5 kg, aged from 4 – 7 years, fed a normal racehorse diet and housed at the BHA's
16 Centre for Racehorse Studies (Newmarket, UK) were used for this study. The horses
17 were being exercised six days per week with moderate and fast track work. In addition,
18 they were exercised daily on a horse walker. No medications were administered to
19 any horse for at least one month prior to this study. The study was ethically approved,
20 with the horses and personnel involved being licensed under the UK Animals
21 (Scientific Procedures) Act. One blood and two urine control samples were taken from
22 each horse on each of the five days preceding the dosing and immediately before the
23 first dose. Blood was taken via intravenous catheter (Milocath®) placed into the left
24 jugular vein of each horse on the first day of dosing. A dose of 20 mg/kg of paracetamol

1 (500 mg tablets, M & A Pharmachem, Westhoughton, UK) was administered orally in
2 nine twice-daily doses at 9 a.m. and 4 p.m. with only one dose on the fifth and final
3 day (a.m. only). The tablets were dissolved in 30 ml of tap water and administered via
4 a catheter-tipped dosing syringe. The dose was selected based on the available
5 literature. This dose is known to be as effective as flunixin in controlling pain [12] and
6 routinely used by equine veterinarians in the UK. The dosing interval was chosen to
7 reflect a manner in which a twice per day administration would be typically
8 administered. Hay and water were available *ad libitum*.

9 All urine samples were collected as free catch samples into a lined jug, secured at the
10 end of a collection stick. Pre-administration samples were collected twice daily (around
11 7 am and 12 pm) for 7 days prior. All urine voided in each 24 h period following the
12 first and the last paracetamol doses was collected. Additionally, one urine sample was
13 collected shortly before each dose in between doses 3 and 9. Once the administration
14 was completed, samples were collected twice-daily (around 8 am and 4 pm) for six
15 days and once daily (8 am) for five further days up to a total of 14 days after the final
16 dose. Dedicated staff were assigned to monitor the horses (via closed-circuit television
17 (CCTV)) and catch each urine sample during the intense sampling period. Blood
18 samples were collected at 15, 30, 45 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6 h following the first
19 dose and at 1, 3, 5, 7, 9, 13 h after the second dose, then immediately before each
20 consecutive dose up until the final ninth dose. Further samples were collected at 15,
21 30, 45 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 23, 27, 31, 47, 55, 71,
22 79, 95, 103, 119, 127, 143, 151, 167, 175, 191, 215, 239, 263, 335 h after the final
23 dose. Blood samples were collected into lithium heparin tubes and were centrifuged
24 to separate plasma immediately after collection. Samples were collected between

January and August 2019. Urine and plasma samples were stored at -20ºC prior to analysis.

2.2 Chemicals and reagents

Ammonium acetate, potassium dihydrogen orthophosphate, β-glucuronidase from *Helix pomatia* (Type HP-2 at 100,000 units/ml) and pancreatin (8xUSP) were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK). Acetonitrile, ethyl acetate, hexane and methanol were obtained from Fisher Scientific UK Ltd. (Loughborough, UK). Optima grade formic acid was obtained from LGC (Middlesex, UK). Sodium hydroxide solution at 40% (v/v) and HiPerSolv water were obtained from VWR International Ltd. (Lutterworth, UK). Water was purified using a Triple Red Duo Water system (Triple Red Laboratory Technology).

Paracetamol was purchased as powder from Sigma-Aldrich, whilst orthocetamol was purchased as powder from Toronto Research Chemicals (Canada). Deuterium labelled paracetamol-d_4 was purchased as powder from Cayman Chemicals (Cambridge, UK). Stock solutions were prepared at 2 mg/ml (paracetamol and orthocetamol) and 1 mg/ml (paracetamol-d_4) in methanol and stored at -20ºC. Stock solutions of paracetamol and orthocetamol were mixed 50:50 (v/v) to obtain a mixed standard at 1 mg/ml, which was subsequently diluted with methanol to obtain spiking solutions at appropriate concentrations.

2.3 Sample analysis

1 Plasma and urine samples were extracted and analysed using quantitative liquid
2 chromatography-tandem mass spectrometry (LC-MS/MS) methods. Both methods
3 had been previously validated for paracetamol using measures of linearity, intra- and
4 inter-batch precision and accuracy, specificity, selectivity and sensitivity (adhering to
5 unpublished EHSLC quantitative method validation guidelines). The detection of
6 paracetamol was validated in plasma and urine in the ranges of 1 – 20,000 ng/ml and
7 100 – 50,000 ng/ml, respectively. These calibration ranges were shown to be linear
8 with correlation coefficients greater than 0.99 when a weighting factor of $1/x^2$ was
9 applied. The methods for plasma and urine analysis were shown to be accurate and
10 reproducible with low inter-batch variability (detailed in Table 1). This methodology
11 was also used for the semi-quantitative analysis of orthocetamol (an approximation of
12 the concentration); whereby concentrations were still assessed using calibration lines
13 and quality controls (QCs) but without orthocetamol being subject to the wider
14 validation criteria that was applied to paracetamol.

15 According to the literature, paracetamol is excreted in a conjugated form in horses [2].
16 Therefore, a hydrolysis (β-glucuronidase 1,250 units/ml) test was carried out whereby
17 hydrolysed and unhydrolysed urine samples were compared. Both paracetamol and
18 orthocetamol were observed at considerably higher concentrations in the hydrolysed
19 samples, confirming the need for hydrolysis in urine. In plasma, both compounds were
20 analysed unhydrolysed according to unpublished EHSLC guidelines on quantitative
21 method validation in plasma.

22 During plasma analysis, 500 µl of plasma was dispensed and spiked at a concentration
23 of 400 ng/ml of paracetamol-d_4 (internal standard). Following the addition of 1.5 ml of
24 HiPerSolv water and 1 ml of 2 M phosphate buffer pH 6.3, the samples were ready to
25 undergo extraction. Each batch included a calibration curve in duplicate at

1 concentrations of 1 (LLOQ), 10, 100, 500, 1,000, 5,000, 10,000 and 20,000 ng/ml and
2 QC samples in duplicate at concentrations of 10, 5,000 and 15,000 ng/ml. .

3 During urine analysis, 500 µl of urine was dispensed and spiked at a concentration of
4 1,000 ng/ml of paracetamol-d_4. Following the addition of 1.5 ml of HiPerSolv water, 1
5 ml of 2 M phosphate buffer pH 6.3, 100 µl of *Helix pomatia* and 100 µl of pancreatin,
6 samples were incubated at 45ºC overnight. Each batch included a calibration curve in
7 duplicate at concentrations of 100 (LLOQ), 500, 1,000, 5,000, 10,000, 25,000, 40,000
8 and 50,000 ng/ml and QC samples in duplicate at concentrations of 500, 5,000 and
9 25,000 ng/ml. The same extraction protocol was used for both matrices. Solid phase
10 extraction (SPE) was carried out using Varian Nexus (60 mg, 3 ml) cartridges, which
11 were conditioned with 1 ml of methanol followed by 1 ml of water. Incubated urine
12 samples were additionally centrifuged at 1,960 x *g* for 15 min prior to sample loading.
13 Cartridges were washed with 0.5 ml hexane and then dried under full vacuum for 30
14 s, prior to eluting with 2 x 1 ml of 10% methanol in ethyl acetate. Liquid-liquid extraction
15 was performed by adding 1.5 ml of purified water to the SPE eluent. The organic phase
16 was then transferred to a clean tube. Following extraction, both plasma and urine
17 extracts were dried at ambient temperature in a Genevac centrifugal evaporator
18 (Biopharma Process Systems Ltd, UK) and subsequently reconstituted. Plasma
19 samples were reconstituted with 10 µl of methanol and 90 µl aqueous 0.1% formic
20 acid, whilst urine extracts were reconstituted in 100 µl of methanol and 900 µl aqueous
21 0.1% formic acid.

22 Plasma sample analysis was performed on an LC-MS/MS system consisting of a
23 Waters Acquity ultra-high performance liquid chromatographic (UPLC) system
24 interfaced with a Waters Quattro Premier triple quadrupole mass spectrometer
25 (Waters Ltd., Hertfordshire, UK). The mass spectrometer was in positive electrospray

1 ionisation mode at a capillary voltage of 0.9 kV, a source temperature of 120ºC and a
2 desolvation gas temperature of 450ºC. Collision gas was argon at a flow rate of 0.35
3 ml/min. Selected reaction monitoring (SRM) was performed for paracetamol (and
4 orthocetamol) using the precursor ion of *m/z* 151.9. The product ions of *m/z* 109.9 (for
5 quantification), *m/z* 92.8 and *m/z* 64.8 were monitored at a cone voltage of 28 V and
6 collision energy of 16, 22 and 25 eV, respectively. The SRM transition of *m/z* 155.9 to
7 *m/z* 113.9 was used for paracetamol-d_4 (cone voltage of 28 V and collision energy of
8 16 eV).

9 Urine sample analysis was performed on a Waters Acquity UPLC system coupled with
10 a Sciex 4000 triple quadrupole mass spectrometer (Applied Biosystems/Sciex,
11 California, USA). Mass spectrometric analysis was performed using a TurboIon (ESI)
12 source in positive ionisation mode at 5500 V. The source temperature was 550ºC, the
13 curtain gas was 30 units, ion source gases 1 and 2 were operated at 40 and 60 units,
14 respectively and the CAD gas was set to 8. Analysis was carried out in SRM mode
15 and selected transitions, declustering potential (DP), collision energy (CE) and cell exit
16 potential (CXP) are shown in Table 2.

17 Chromatographic separation was achieved on a Waters Acquity HSS T3 (100 mm x
18 2.1 mm, 1.8 µm) reversed-phase UPLC column using 0.1% formic acid in acetonitrile
19 and aqueous 0.1% formic acid as mobile phases. A gradient was operated at 50ºC
20 and at a flow rate of 0.4 ml/min. It was started at 5% organic for 0.5 min, followed by
21 an increase to 45% by 2.5 min and a further increase to 100% by 2.7 min. This was
22 held for 1.5 min before returning to initial conditions of 5% organic at 4.3 min and re-
23 equilibrating for the remainder of the run. The total run time was 5 min.

24

2.4 Pharmacokinetic evaluation

Peak plasma concentrations (C_{max}) with associated times (T_{max}) were determined from evaluation of the data. Oral clearance (CL/F) and half-lives of the elimination phases were estimated using a 2 compartmental model with first order absorption (model 11) with $1/(y_{hat})^2$ weighting within Phoenix WinNonlin 8.0 (Certara, NJ, USA). The average pre-dose paracetamol plasma concentration (background) for each horse was subtracted from their post-dose plasma concentration data prior to model fitting.

The methodology outlined by Toutain and Lassourd [17] was used to estimate the EPC, IPC, irrelevant urine concentration (IUC) and DT. Due to the variability in bioavailability observed within the literature, the EPC for the oral dose of 20 mg/kg BID (total daily dose of 40 mg/kg) was determined using an IV clearance of 3.5 ml/min/kg as determined by Neirinckx *et al.* [18] The IPC creates the basis for the plasma SL, which is the concentration where the drug is no longer pharmacologically significant. The IPC was calculated by dividing the EPC by a safety factor of 500, to ensure there is no significant pharmacological effect for the majority of horses in a population. The IUC was calculated by multiplying the IPC by the steady-state ratio of urine to plasma concentration. DTs were determined from the time post-final dose, where all horses had a concentration lower than the nominal IPC and IUC.

3 Results

3.1 Pharmacokinetic analysis for orally administered paracetamol in plasma

Paracetamol was detected in all plasma samples from six horses (Figure 2), including the pre-administration samples. The concentrations of paracetamol detected in the pre-administration samples ranged from <LLOQ to 7.9 ng/ml. The maximal

1 concentrations (C_{max}) ranged from 15.6 to 22.7 μg/ml and were reached between 0.25
2 and 1 h (T_{max}) after the first dose for horses 1,4 and 6 and between 1.02 and 1.92 h
3 after the second dose for horses 2,3 and 5 (Table 3). Paracetamol remained above
4 the lower limit of quantification (LLOQ) of 1 ng/ml for the entire duration of the study
5 (two weeks after the final dose) for all six horses. Supplementary Figure S1 shows
6 that the paracetamol plasma concentration data over the entire study, adjusted for
7 background paracetamol, gives a good fit to a 2 compartmental model with first order
8 absorption as judged by residual error and correlation between observed and
9 predicted data (r^2 >0.86 for all horses). No significant accumulation was observed
10 based on trough concentrations from repeated administrations. The first half-life ($t_{1/2\alpha}$)
11 is clearly the main elimination phase based on the high contribution to the area under
12 the curve (AUC) and ranged from 3 to 6 h, however, there was an apparent second
13 longer phase starting below 10 ng/ml. The estimated oral clearance (CL/F) from the
14 model ranged from 3.8 to 5.5 mL/min/kg (Table 3).

15

16 For a dose of 20 mg/kg BID (total daily dose of 40 mg/kg) the calculated EPC is 7,937
17 ng/ml based on the literature IV clearance. Using a factor of 500 then gives an IPC of
18 16 ng/ml with a nominal IPC of 20 ng/ml. The latter affords a DT of 120 h or 5 days
19 (Table 4).

20

21 3.2 Pharmacokinetic analysis for orally administered paracetamol in urine

22 Paracetamol was detected in all urine samples from six horses (Figure 3), including
23 the pre-administration samples. The concentrations of paracetamol in the pre-
24 administration samples ranged from <LLOQ to 449.5 ng/ml. The C_{max} ranged from 865

to 2285 μg/ml and was reached between 1.25 and 3.18 h after the second dose for horses 1,2,3 and 5 and 3.50 and 4.83 h after the first and third doses, for horses 6 and 4, respectively. Paracetamol remained above the LLOQ of 100 ng/ml for the entire length of the study (two weeks after the final dose) for five out of six horses. The steady-state urine to plasma concentration ratio was 213 giving a nominal irrelevant urine concentration (IUC) of 4,300 ng/ml and, like plasma, affords a DT of 120 h or 5 days (Table 2).

3.3 Semi-quantitative analysis of orthocetamol in administration study plasma samples

Plasma samples from three of the six horses administered were semi-quantitatively analysed for orthocetamol. The samples were analysed without hydrolysis, therefore orthocetamol peaks were not detected in the majority of the analysed samples. In rare cases where all three of the selected orthocetamol ions were detected, the calculated concentrations remained very low and did not exceed the LLOQ of 1 ng/ml. Based on the results of the first three batches being below the LLOQ, the remaining three administrations were analysed for paracetamol only.

3.4 Semi-quantitative analysis of orthocetamol in administration study urine samples

Orthocetamol was detected in all administration urine samples from six horses (Figure 4), including the pre-administration samples. The lowest concentration of orthocetamol calculated in a pre-dose sample was approximately 4.9 μg/ml, with the majority of the pre-dose samples showing significantly higher concentrations. The C_{max} ranged from 46.5 to 119.4 μg/ml and was reached between 1.67 and 2.25 h after the second and between 3.8 and 4.83 h after the third dose. Orthocetamol was detected throughout

1 the whole sampling period and the lowest concentrations for each animal ranged
2 between 3.3 to 13.4 μg/ml.

3

4 4 Discussion

5 The main aim of this research was to produce pharmacokinetic data for paracetamol
6 in equine plasma and urine. To the best of the authors' knowledge, this is the first
7 paper to present a pharmacokinetic study of paracetamol in urine and to propose an
8 associated DT in both plasma and urine for a 20 mg/kg BID oral regimen, which may
9 be used to advise trainers and their veterinarians on treatment withdrawal prior to
10 racing.

11 To date, there is only one study which investigated the safety and pharmacokinetics
12 of multiple dosing of paracetamol in horse and only plasma data was evaluated. [19]
13 Similarly to the current study, 20 mg/kg of paracetamol was administered twice daily.
14 Rapid absorption and no significant drug accumulation with repeat dosing were noted
15 in both studies. The average C_{max} values were 18.85 versus 15.85 μg/ml [17]). Plasma
16 half-life ($t_{1/2}$) and CL/F were determined to be 3.99 h and 7.9±2.9 ml//min/kg,
17 respectively [19] which is comparable to the herein determined values of $T_{1/2\alpha}$ = 4.22 h
18 (harmonic mean) and CL/F = 4.5±0.7 ml/min/kg. The present study showed an
19 additional second phase with $T_{1/2\beta}$ = 56 h (harmonic mean), however, its contribution
20 to the AUC is minor as it appears 48 hours post last dose at concentrations below 10
21 ng/ml.

22 EPC and IPC of paracetamol in plasma, were previously reported to be 12 μg/ml and
23 24 ng/ml, respectively. [16] This was derived from data published in literature [18] and it
24 should be noted that calculations were based on a 10 mg/kg dose. Additionally, due

1 to a lack of information, the dosing interval recommended in humans was applied.
2 Nonetheless, the EPC and IPC values determined by Ishii *et al.* are only 50% higher
3 than the herein determined values as the total daily doses are 60 and 40 mg/kg,
4 respectively.

5 Only one previous study measured urinary paracetamol concentrations following a
6 single 10 mg/kg dose. [2] The reported C_{max} concentrations were 178 and 307 µg/ml.
7 These concentrations are significantly lower than the urine C_{max} concentrations
8 measured in this study, which ranged from 865 to 2,285 µg/ml. Large variation seen
9 in the C_{max} between different horses can be most likely attributed to the fact that the
10 urine samples were collected at different times for each horse. This may mean that
11 the true C_{max} may be missed in some horses. Five out of six participating horses
12 showed very similar excretion profiles in urine (Figure 3). Considerably higher
13 concentrations are observed for horse 4 and the concentrations do not change
14 between repeat doses as much as they do for the other five horses. Interestingly, the
15 levels of orthocetamol were also significantly higher for horse 4 in comparison with the
16 remaining five horses. The differences in plasma profiles are not as apparent
17 compared to urine; however, the drug does appear to have a more pronounced
18 terminal plasma phase in horse 4 compared to the others.

19 Previously published orthocetamol plasma concentrations, measured in a population
20 of Japanese horses, with a mean of 0.686 ng/ml [16], are in line with the findings of the
21 current study. On the other hand, previously reported urine concentrations, measured
22 in a population of South African racehorses [2], are significantly lower in comparison to
23 the current study, (ranges 0.5 to 7 µg/ml versus 3.3 to 119 µg/ml). As orthocetamol
24 is believed to be present in plants, [2,16] the variations in composition of the horse feed
25 (if any) in different geographical regions could explain the discrepancies between the

urine studies. Interestingly, de Kock *et al.* [2] concluded that the concentrations of orthocetamol remain consistent over time and are not related to paracetamol administration. This is contrary to the findings of the present study, where the urine concentrations of orthocetamol showed a significant increase that coincides with the beginning of paracetamol administration (Figure 4). Furthermore, the T_{max} values for paracetamol and orthocetamol coincide for three out of six horses, with only 1 h difference for the fourth. There is no literature evidence to suggest that paracetamol can be metabolised to orthocetamol and would require the migration of the acetamide group. However, this could potentially be explained by competitive binding between the analytes. Plasma protein binding for paracetamol in the horse is only 50%[18] so it is unlikely that competitive binding between the analytes with plasma proteins will lead to significant increases in orthocetamol concentration. On the other hand, the second longer decay phase observed in plasma and urine is usually indicative of a drug having high affinity but low capacity for a specific tissue in the body. In this case, high concentrations of paracetamol post-dose could displace any orthocetamol bound to this specific tissue, thus releasing it into blood and urine. After the dosing period orthocetamol levels return to similar concentrations to those prior to administration. This theory is supported by orthocetamol having similar physicochemical properties and metabolic fate as its isomer [4,5].

The main aim of this research was to assess the therapeutic levels of paracetamol in equine plasma as well as to propose a DT for paracetamol in equine plasma and urine. The results of this study determined the EPC and IPC to be 8 µg/ml and 16 ng/ml, respectively. Suggested SLs in plasma and urine could be 20 and 4,300 ng/ml, respectively, with a DT of 120 h (5 days). Furthermore, PK simulation indicates that the suggested DT of 120 h is also suitable for an oral paracetamol regimen of 20 mg/kg

given every 12 hours. Any published DTs can be used to aid veterinarians in determining a corresponding WT. However, it is the responsibility of the individual racing authorities to establish their own risk management strategy with regard to DTs and WTs.

Previously published population studies reported a mean background paracetamol concentration of 4.55 ng/ml in plasma [16] and a range of background concentrations from 200 to 3,500 ng/ml in urine, adding that concentrations as high as 5,000 ng/ml were also seen in South Africa. [2] Results from the current study suggest that orthocetamol concentrations tend to exceed background paracetamol concentrations in urine. However, this relationship is reversed following a paracetamol administration. Therefore, the ratio between paracetamol and orthocetamol could be used as an additional tool to support the discrimination of a paracetamol administration from potential environmental exposure.

Data availability statement

Authors elect not to share data.

Conflict of interest:

The authors have no commercial conflict of interests; however, the authors are either employed by, working on behalf of or consulting to a regulatory agency.

Authors' contribution

1 BP, SF and BG contributed to the analytical development method, validation and
2 sample analysis. TM and JHB led the administration study supported by the staff at
3 the BHA CRS who are gratefully acknowledged. The BHA is also thanked for funding
4 the project. PT performed project management, PH, JS and TM performed manuscript
5 editing. SP co-ordinated the data and performed pharmacokinetic analysis. All authors
6 contributed to the writing of the manuscript, and have read and approved the final
7 manuscript.

8

9 Animal Welfare and Ethics Statement

10 The study was ethically approved, with the horses and personnel involved being
11 licensed under the UK Animals (Scientific Procedures) Act.

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30

1 Table 1. Inter-batch precision and accuracy of the methods used for the analysis of

2 paracetamol in equine plasma and urine

	Plasma		Urine	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
QCs	±6.8	±7.4	±7.2	±7.6
Dilution QCs	±8.6	±3.7	±1.2	±12.4

3 *Note:* QC = quality control, dilution QC = a QC diluted and analysed in the same
4 manner as any samples with concentrations above the selected calibration range

5

6

7

8 Table 2. SRM transitions and parameters for the analysis of paracetamol and

9 orthocetamol in urine (SCIEX 4000)

Compound	**Q1 mass (amu, *m/z*)**	**Q3 mass (amu, *m/z*)**	**Dwell (msec)**	**DP**	**CE**	**CXP**
Paracetamol	152.1	110.1	25	71	23	8
		64.9	**25**	**71**	**43**	**4**
		93.0	25	71	33	8
Orthocetamol	152.1	110.0	25	61	40	8
		64.8	25	61	65	4
		91.8	**25**	**61**	**48**	**8**
Paracetamol-d_4	156.2	114.1	25	56	21	6

10 *Note:* Quantitative transitions are highlighted in bold. SRM = selected reaction
11 monitoring, Q = quadrupole, DP = declustering potential, CE = collision energy, CXP
12 = cell exit potential

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1 Table 3. Summary of plasma pharmacokinetic parameters for paracetamol following
2 administration of 20 mg/kg BID for nine doses to six exercised Thoroughbred horses.

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
C_{max} (μg/mL)	22.7	16.0	16.7	15.6	20.7	20.8
T_{max} (h)	1.00[1]	1.15[2]	1.92[2]	0.25[1]	1.02[2]	0.25[1]
Cl/F (mL/min/kg)	4.11	3.82	5.54	4.50	5.07	3.77
$t_{1/2\alpha}$	3.28	3.90	4.12	6.05	4.56	4.25
$t_{1/2\beta}$	15	115	139	79	113	523

3 *Note:* C_{max} = maximal concentration, T_{max} = time the maximal concentration was
4 reached, $t_{1/2\alpha}$ and $t_{1/2\beta}$ = first and second half-lives, Cl/F = oral clearance, [1,2]
5 first/second dose.

6

7 Table 4. Summary of modelled parameters for paracetamol following oral
8 administration of 20 mg/kg BID.

Parameter	**Value**
EPC (ng/ml)	7937
Calculated IPC (ng/ml)	16
Nominal IPC (ng/ml)	20
Plasma DT (h)	120
R_{SS}	213
Nominal IUC (ng/ml)	4300
Urine DT (h)	120

Note: EPC = effective plasma concentration (using IV data from Neirinckx *et al*., 2010), IPC = irrelevant plasma concentration, IUC = irrelevant urine concentration, R_{SS} = steady-state urine to plasma concentration ratio, DT = detection time.

List of figures:

Figure 1. Molecular structure of (a) paracetamol and (b) orthocetamol.

Figure 2. Log plasma paracetamol concentrations versus time in six horses following the administration of nine doses of paracetamol over five days

Figure 3. Log urine paracetamol concentrations versus time in six horses following the administration of nine doses of paracetamol over five days

Figure 4. Urine orthocetamol concentrations versus time in six horses participating in the paracetamol administration study

Supplementary Figure S1

2-compartmental model with first order absorption fit to plasma paracetamol data