Preliminary quantitative analysis of paracetamol, metacetamol, and orthocetamol in equine plasma from Japan using liquid chromatography-electrospray ionisation-tandem mass spectrometry

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ABSTRACT

Paracetamol—also known as acetaminophen—is widely used as nonprescription analgesics and antipyretic medications. Previous studies have reported that paracetamol is ubiquitously found in human and equine urine, and its structural isomers metacetamol and orthocetamol are found in equine urine. However, the sources and routes of paracetamol exposure currently remain unclear and its concentration in Japanese horses has not been investigated. Therefore, we determined the plasma concentrations of paracetamol, metacetamol, and orthocetamol in postrace samples collected for six months from 155 racehorses of the Japan Racing Association. We also analysed the feeds commonly fed to racehorses in Japan to confirm whether they are a source of paracetamol.

After the plasma was spiked with aliquots of paracetamol-d_4 as an internal standard, enzyme-hydrolysed and unhydrolysed analytes were removed using solid-phase extraction. The feeds were spiked with the internal standard after being homogenised using a blender and underwent liquid–liquid extraction followed by solid-phase extraction. The extracts were analysed using liquid chromatography-electrospray ionisation-tandem mass spectrometry.

The hydrolysed and unhydrolysed paracetamol plasma concentrations ranged from approximately 0.5–40 ng/mL. The paracetamol–conjugate ratio was approximately 1 to 0.5. Similar results were obtained for orthocetamol concentrations. In contrast, metacetamol was mainly detected following hydrolysis at up to 2 ng/mL. Furthermore, timothy grass and concentrated feeds were determined to contain approximately 0.5–3 ng/g of paracetamol and 50–200 ng/g of orthocetamol. However, if those feeds had been the horses' main paracetamol source, the plasma paracetamol concentrations should have been much lower than the detected levels based on their daily feed rations.

This preliminary study revealed that paracetamol, measured in ng/mL, was detected in every equine plasma sample analysed in Japan. An additional study with a large number of samples, including urine, will be required to obtain more detailed information about equine paracetamol exposure.

KEYWORDS

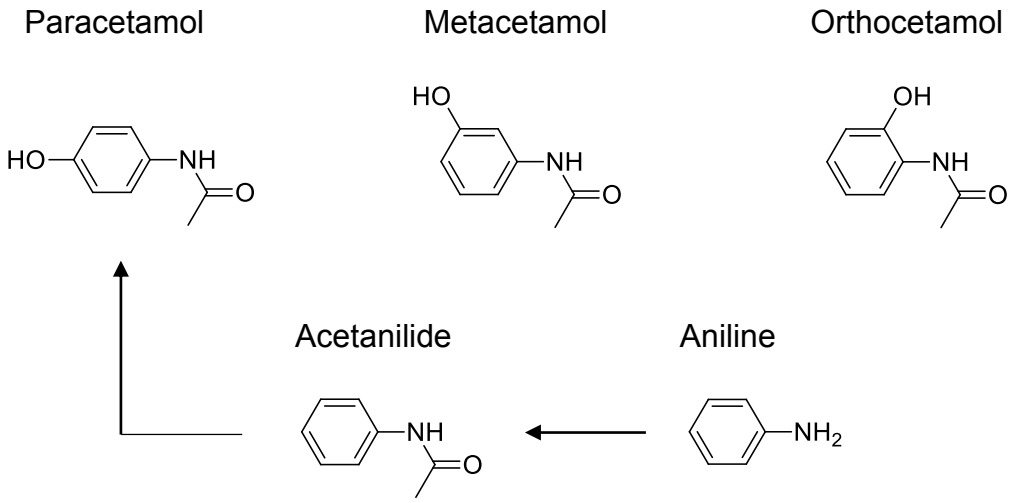
Paracetamol; Metacetamol; Orthocetamol; Plasma; Horse

INTRODUCTION

Paracetamol—also known as acetaminophen—is widely used as nonprescription analgesics and antipyretic medications. Although it is readily available as an over-the-counter drug, the therapeutic use of paracetamol for racehorses in Japan is not common because it is associated with liver toxicity (Cook *et al.*, 2015). However, paracetamol and its structural isomers metacetamol (classified as an analgesic) and orthocetamol are often observed in plasma and urine specimens collected from horses in Japan following a race (Fig. 1). de Kock *et al.,* (2008) reported that these compounds were detected in racehorse urine in low to sub microgram per millilitre concentrations in Southern Africa. They also mentioned that orthocetamol was found in much of the feed and grass available to racehorses, whereas metacetamol, as well as paracetamol and its metabolic counterpart acetanilide, were not detected in feed.

In a human study, Modick *et al.,* (2014) reported that paracetamol could be detected in all analysed urinary samples at a median (range) concentration of 61.7 (0.65–2,274,296) ng/mL taken from 2098 adult and control populations in their institute; information about the use of paracetamol for these subjects was unknown. According to that report, aniline is metabolised into paracetamol via acetanilide (Fig. 1) and the occupational exposure to aniline

Figure 1: The structure of paracetamol and related compounds.



is a possible source of the internal body burdens from paracetamol. Another possibility for horses' exposure to paracetamol is river water. Although Santos *et al.,* (2013) reported that the concentrations of paracetamol and its glucuronide, which is inactive, in a river were up to 0.25 and 3.57 ng/mL in the north part of Portugal, racehorses are generally well controlled and only drink tap water, which should not contain paracetamol.

Consequently, the sources and routes of paracetamol exposure among horses currently remain unclear. In addition, its concentration in Japanese horses has not been investigated. Therefore, we determined the plasma concentrations of paracetamol, metacetamol, and orthocetamol in postrace samples randomly collected for six months from 155 racehorses of the Japan Racing Association (JRA). We also analysed the feeds commonly fed to racehorses in Japan to confirm whether they are a source of paracetamol.

MATERIALS AND METHODS

Materials

Acetanilide, paracetamol, formic acid, LC/MS-grade acetonitrile, and LC/MS-grade methanol were acquired from Wako Pure Chemical Industries (Osaka, Japan). Metacetamol, orthocetamol, and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Paracetamol-d_4 (100 µg/mL in methanol), to be used as the internal standard (IS), was purchased from Cerilliant Co. (Round Rock, TX, USA). The 1 mL and 3 mL OASIS HLB cartridges were purchased from Waters (Milford, MA, USA). β-Glucuronidase (from *Pomacea canaliculata*, 22,000 Fishman units/mL) was supplied by Nippon Bio-Test Laboratories, Inc. (Saitama, Japan). High-purity water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). All other reagents used in this study were of analytical grade.

Sample Collection

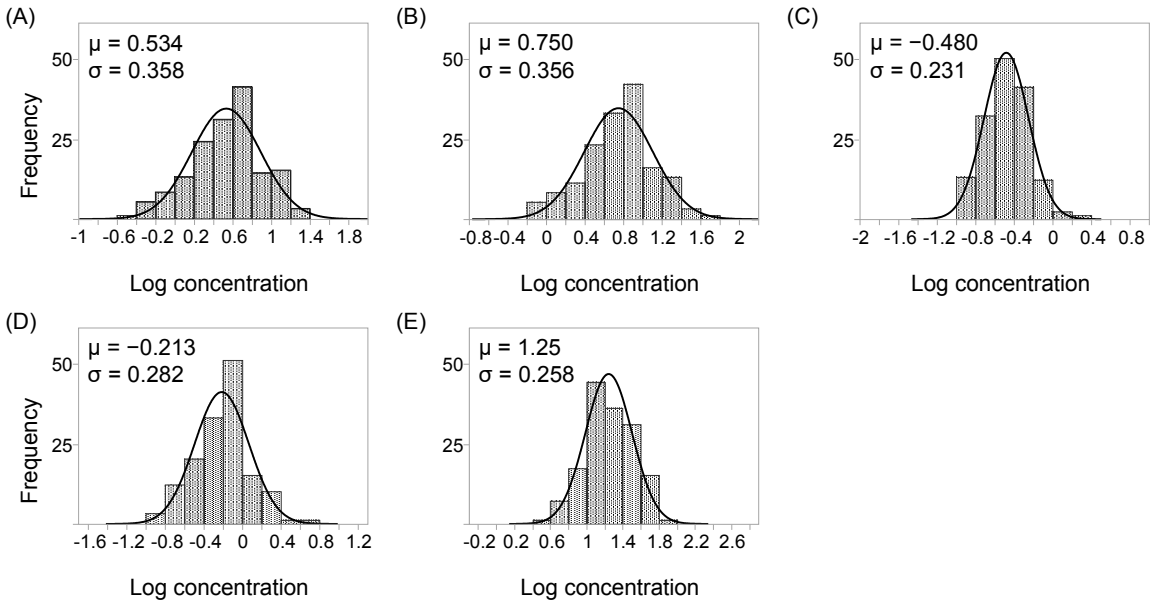
Plasma samples for this study were drawn from postrace samples (n = 155) of the JRA that had been randomly collected for routine drug screening between October 2016 and April 2017. Samples were stored at −40 °C prior to our analysis for approximately two months.

Instruments and Conditions

A Shimadzu Nexera series liquid chromatograph consisting of binary pumps, an online degasser, an autosampler, and a column oven (Shimadzu, Kyoto, Japan) was linked to a linear ion trap quadrupole mass spectrometer equipped with a turbo ionspray interface (QTRAP 4500, SCIEX, Framingham, MA, USA). Analytes were separated using an ACQUITY UPLC BEH C_{18} (2.1 mm i.d. × 100 mm, 1.7 µm, Waters) whose temperature was maintained at 40 °C. The mobile phase consisted of solvent A (0.1 vol% formic acid) and solvent B (acetonitrile), with the following gradient elution (percentages indicate the proportion of solvent B): 0–4 min, 3 %; 4–12 min, 3–95 %; 12–16 min, 95 %; and 16–18 min, 3 %. The flow rate was 0.6 mL/min.

Optimized mass spectrometer settings were as follows: curtain gas, 20 psi; gas 1, 40 psi; gas 2, 40 psi; collision-activated dissociation gas, 10 arbitrary units; ionspray voltage, 5500 V; dwell time, 50 ms; and turbo heater temperature, 700°C. For paracetamol and the IS, respectively, the transitions for selected reaction monitoring (SRM) were *m/z* 152.2 to 109.9 and *m/z* 156.0 to 113.7; the declustering potentials were 81 V and 81 V; the collision energies were 21 V and 21 V; and the collision cell exit potentials were 10 V and

Figure 2: The distribution of plasma concentrations of unhydrolysed paracetamol (A), hydrolysed paracetamol (B), hydrolysed metacetamol (C), unhydrolysed orthocetamol (D), and hydrolysed orthocetamol (E) after logarithmic transformation. The solid line represents a normal distribution curve. Postrace samples were collected from Japanese horses for six months (n = 155, 155, 151, 149, and 155 for (A), (B), (C), (D), and (E), respectively).



10 V. Metacetamol and orthocetamol were detected using the same SRM conditions as for paracetamol. All peak integrations and quantitative calculations were performed using Analyst software version 1.6.1. The ratios of the peak area of each analyte to nominal analyte concentrations were fitted by least-squares linear regression using $1/y^2$ weighting factors.

Preparation of Stock and Working Solutions

Standard stock solutions (1.0 mg/mL) were prepared independently in methanol. Standard working solutions containing paracetamol, metacetamol, and orthocetamol at concentrations of 1, 3, 10, 30, 100, 300, 1000, and 3000 ng/mL were prepared by diluting the stock solutions in methanol. The IS working solution (20 ng/mL) was prepared by diluting the stock solution (100 μg/mL) with 1 mol/L acetate buffer at a pH of 5.0.

Plasma Sample Preparation

To make calibration curves in a concentration range of 0.1–300 ng/mL for each analyte, 50 μL of each standard working solution was transferred into a 1.5 mL polypropylene (PP) tube and dried under a nitrogen stream. The residue was reconstituted with 500 μL of the IS working solution; then, 500 μL of PBS was added and vortexed. For plasma samples, 500 μL of the IS working solution and 500 μL of plasma were mixed in a 1.5 mL PP tube. For hydrolysis, 50 μL of β-glucuronidase (22,000 Fishman units/mL) was spiked into each sample and incubated at 60°C for 30 min. Extraction columns (OASIS HLB 1 mL) were conditioned sequentially with methanol (1 mL) and water (1 mL). Each sample was passed through a column and washed with 5 vol% methanol. Analytes and the IS were eluted with 1 mL of methanol. The eluate was then evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 μL of 0.1 vol% formic acid/acetonitrile (9:1) and transferred into centrifuge tubes with polytetrafluoroethylene filters (0.45 μm) and centrifuged (10,000 *g*, 3 min, 4 °C). Filtrates were transferred into autosampler vials and kept in an autosampler at 4 °C until injection.

Method validation

A spike-recovery test was conducted with and without hydrolysis using two kinds of pooled blank plasma that naturally contained different concentrations of those compounds. Each plasma sample was spiked with two concentrations of analytes. Pooled blank samples and spiked samples were analysed in six replicates on three different days to assess the test's precision (coefficient of variance [CV]) and accuracy, which was defined as the measured concentration/(concentration from blank sample + spiked concentration) × 100.

Statistical Analysis

The plasma concentrations of paracetamol, metacetamol, and orthocetamol below the lower limit of quantification (LLOQ) (0.1 ng/mL) were regarded as

Table 1: Concentrations of paracetamol, metacetamol, and orthocetamol in Japanese equine plasma samples that had been collected for six months

Parameter	Paracetamol			Metacetamol			Orthocetamol		
	Hydrolysis		Fold difference	Hydrolysis		Fold difference	Hydrolysis		Fold difference
	(−)	(+)	n = 155	(−)	(+)	n = 3	(−)	(+)	n = 149
Mean (ng/mL)	4.65	7.65	1.66	0.00516	0.374	2.05	0.967	20.5	32.5
SD (ng/mL)	3.78	6.36	0.260	0.0403	0.246	0.363	1.96	12.3	17.5
Median (ng/mL)	3.76	6.27	1.65	0.00	0.323	2.10	0.648	17.6	29.9
Max (ng/mL)	20.7	39.8	2.45	0.426	2.13	2.38	20.8	64.7	95.3
Min (ng/mL)	0.396	0.787	0.977	0.00	0.00	1.66	0.00	1.98	0.993
Number above the LLOQ	155	155	-	3	151	-	149	155	-

n = 155
LLOQ: lower limit of quantification (0.1 ng/mL); SD: standard deviation

zero to calculate the mean, standard deviation, CV, and median. To confirm whether the plasma concentrations had a normal distribution, a Shapiro-Wilk test was conducted after logarithmic transformation using JMP software (SAS, Cary, NC, USA) with a p-value of 0.05.

Feed Sample Preparation

Oat, bran, boiled flaxseed, timothy grass, and four kinds of concentrated feeds were used for this study. Qualitative analyses were conducted to confirm whether the feeds contained acetanilide, paracetamol, metacetamol, and orthocetamol prior to the quantitative measurements. The amount of paracetamol and orthocetamol in a feed was quantified using a standard addition method. In brief, 4 g of feed homogenised using a blender was spiked with aliquots of each concentration of the standard and IS working solutions, and extracted twice with 20 mL of acetonitrile. Each supernatant was transferred into the same separating funnel and washed with 50 mL of saturated saline solution and 50 mL of *n*-hexane. After removing the bottom of the aqueous layer, the middle of the acetonitrile layer (ca. 38 mL) was collected, evaporated to dryness, redissolved in 0.3 mL of methanol, and then diluted with 2.7 mL of water. The reconstituted solution was cleaned up using solid-phase extraction with OASIS HLB (3 mL). The remaining procedures were performed in a similar manner to plasma sample preparation.

Pharmacokinetics Simulation

A pharmacokinetics (PK) simulation was performed using Phoenix WinNonlin software (Certara, Princeton, NJ, USA) under the assumption that horses were fed 5 kg of timothy grass and 200 g of concentrated feed twice a day, which is equivalent to the ingestion of 11.8 µg of paracetamol and 570 µg of orthocetamol. Plasma concentrations of paracetamol and orthocetamol were estimated with a one-compartment model using PK parameters from a previous study of paracetamol administration at an effective dose of 10 mg/kg by i.v. and p.o. (Neirinckx *et al.*, 2010).

RESULTS AND DISCUSSION

Plasma Concentrations

Method validation was conducted prior to plasma sample analysis. Inter- and intra-assay accuracies and precisions were 100 ± 15% and ≤ 15% for all analytes with and without hydrolysis, respectively.

The results of determining the plasma concentrations of paracetamol, metacetamol, and orthocetamol are shown in Table 1. The concentration of paracetamol could be quantified from all samples above the LLOQ of 0.1 ng/mL. The median and (range) plasma concentrations of unhydrolysed and hydrolysed paracetamol were 3.76 and (0.396–20.7) ng/mL, and 6.27 and (0.787–39.8) ng/mL, respectively. The average ratio of free to conjugated paracetamol was 1 to 0.66. For metacetamol, almost all the plasma samples had a concentration below the LLOQ without hydrolysis. The metacetamol concentration after hydrolysis was between 0 and 2.13 ng/mL (median 0.323 ng/mL). Those results indicated that metacetamol detected in plasma mainly existed as the conjugated form. For orthocetamol, all samples, except for six unhydrolysed plasma samples, could be quantified. The median and (range) concentrations of unhydrolysed and hydrolysed orthocetamol were 0.648 and (0–20.8) ng/mL, and 17.6 and (1.98–64.7) ng/mL, respectively. The mean ratio of free to conjugated orthocetamol was approximately 1 to 30.

Toutain *et al.,* (2002) proposed that the effective plasma concentration (EPC) and irrelevant plasma concentration (IPC) can be calculated using the following equations:

Equation 1: EPC = standard dose (per dosing interval)/ plasma clearance (per dosing interval)

Equation 2: IPC = EPC/safety factor (e.g., 500)

According to those equations, the EPC and IPC for paracetamol were 12 µg/mL and 24 ng/mL, respectively. The EPC and IPC were calculated using a standard dose of 10 mg/kg, a plasma clearance of 0.21 L/h·kg (Neirinckx *et al.*, 2010), and an effective period of about 4 h for human (Oscier *et al.*, 2009). It is unlikely that the measured free paracetamol concentration was potent

enough to cause antipyretic effects because all the plasma samples were below the IPC of 24 ng/mL and conjugated paracetamol has little effect on antipyretic and analgesic actions.

Statistical Analysis

The plasma concentrations of these compounds fit the normal distribution curve after logarithmic transformation ($p < 0.05$), except for the unhydrolysed metacetamol concentration, because almost all the samples had concentrations below the LLOQ. Those histograms are shown in Fig. 2. The transformed μ (transformed σ) concentrations for unhydrolysed and hydrolysed paracetamol in plasma were 0.534 (0.358) ng/mL and 0.750 (0.356) ng/mL, respectively. The normal values of μ + 3σ were 40.4 ng/mL for the unhydrolysed paracetamol concentration in plasma and 65.8 ng/mL for the hydrolysed concentration. If the preliminary residual limit was set as 100 ng/mL, rounded off to 65.8 ng/mL, for hydrolysed paracetamol in plasma, the risk would be 1 in 4472 for a normal sample to exceed this limit. The transformed μ, transformed σ, and the normal value of μ + 3σ were −0.480, 0.231, and 1.64 ng/mL for hydrolysed metacetamol; −0.213, 0.282, and 4.30 ng/mL for unhydrolysed orthocetamol; and 1.25, 0.258, and 105 ng/mL for hydrolysed orthocetamol, respectively.

Feed Analysis

As a result of qualitative analyses, orthocetamol was detected in all analysed samples, whereas acetanilide and metacetamol were not found in any feeds. Paracetamol was only detected in three kinds of concentrated feeds and timothy grass. Using a standard addition method, the paracetamol and orthocetamol amounts in three kinds of concentrated feeds were 0.5–3 ng/g and 50–200 ng/g, respectively. The amounts detected in timothy grass were approximately 2 ng/g and 100 ng/g, respectively.

PK Simulation

A PK simulation showed that plasma concentrations achieved a steady state of up to 1.5 ng/mL after two cycles of feeding. However, if those feeds had been the horses' main paracetamol source, the plasma paracetamol concentrations should have been much lower than the detected levels based on their daily feed rations. The results of this simulation indicate that there are other sources of paracetamol exposure among equines.

On the other hand, the PK simulation for orthocetamol was consistent with the actual plasma concentration, assuming that orthocetamol has PK properties that are similar to paracetamol.

CONCLUSION

We developed and validated methods for determining paracetamol, metacetamol, and orthocetamol concentrations in equine plasma. Our preliminary study revealed that paracetamol, measured in ng/mL, was detected in every equine plasma sample analysed in Japan. These low plasma concentrations seemed to be irrelevant to the therapeutic effect based on Toutan's equations. The results of feed analyses indicated that we did not find the main source of paracetamol, whereas we determined that the orthocetamol concentrations detected in equine plasma could come from their feed. An additional study with a larger number of samples, including urine, will be required to obtain more detailed information about equine paracetamol exposure.

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AUTHOR'S DECLARATION OF INTERESTS

The author declares there are no conflicts of interest.

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