Pharmacokinetic Report

Pharmacokinetics, metabolism and excretion of celecoxib, a selective cyclooxygenase-2 inhibitor, in horses

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Abstract

Celecoxib, a nonsteroidal anti-inflammatory drug is frequently used to treat arthritis in humans with minimal gastrointestinal side effect compared to traditional NSAIDs. The primary aim of this study is to determine the pharmacokinetic profile of celecoxib – a selective cyclooxygenase-2 (COX-2) inhibitor in horses. Six horses were administered a single oral dose of celecoxib at 2 mg/kg (body weight). After oral dosing, the drug reached a maximum concentration (mean ± SD) in blood of 1088 ± 324 ng/mL in 4.58 h. The elimination half-life was 13.60 ± 3.18 h and the area under the curve was 24142 ± 1096 ng.mL/h. The metabolism of celecoxib in horses was via a single oxidative pathway in which the methyl group of celecoxib is oxidised to a hydroxymethyl metabolite and is further oxidised to form a carboxylic acid metabolite. Celecoxib is eliminated mainly through faeces as unchanged drug and as metabolites in urine. Therefore, instructions for a detection time following therapeutic dosing of celecoxib can be set by the racing practitioner and veterinarians to control illegal use in horse racing based on the results of this study.

Keywords: Celecoxib; Elimination; Horses; Metabolism; Pharmacokinetics.

Introduction

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide is the first COX-2 inhibitor to be developed as an analgesic with less toxicity on the gastrointestinal tract (Noble et al., 2000). Despite celecoxib has been clinically proven to be effective as a therapy for many species of animals (Zhang et al., 2000; Paulson et al., 2001; Störmer et al., 2003; Ma et al., 2015) there is limited data reported in horses although being banned as doping agent by horse racing authorities (The International Federation for Equestrian Sports, n.d.). A GC/MS method using TMS derivatization and an LC/MS study have been reported to characterize the metabolites of celecoxib in horse urine (Dirikolu et al., 2001; De Kock et al., 2005). However, the urine samples were collected for a short duration and a clear determination of detection time could not be established in their reports. Further, no detailed study to show data for the drug pharmacokinetics and detection time in horses is available. As such, this study was designated to characterise the plasma pharmacokinetics, metabolism pathways and elimination route of celecoxib given orally to horses, which assess the clinical and animal welfare implications and derive regulatory advice for horse racing industry.

Materials and methods

Animals

Six adult horses (males, male castrates and females) aged 12 to 18 years and weighing approximately 480 kg were used in the study. All animals were tagged, dewormed and housed in air-conditioned stable barns. The horses were given regular walking exercise for 30 minutes twice a day. Regular physical examination was performed on each horse by the local hospital veterinarian working adjacent to this facility. The animals were fed twice a day with hay, alfalfa and grains. They had free access to water. None of the horses had any disease

history in their stable records and they were not treated with NSAIDs or other anti-inflammatory drugs for at least 30 days prior to this administration study. The animals were fasted overnight prior to treatment and food was supplied after 2 hours of the first blood sample collected after drug administration. The study was approved by the Animal Ethics Committee of the Central Veterinary Research Laboratory in Dubai, U.A.E.

Experimental chemicals

Celecoxib and zaleplon were kind donations by Ipca Laboratories Limited, Indore, India, both having an assay purity of ≈ 99.8%. β-glucuronidase from Helix pomatia (type HP2) and NADPH were purchased from Sigma Chemical Co. Ltd.. Hydroxymethyl celecoxib and celecoxib carboxylic acid were obtained from Alsachim (France). All organic solvents were either HPLC grade or LC/MS grade and purchased from Fisher Scientific. Horse liver microsomes were bought from Xeno Tech U.S.A for in vitro studies.

Treatment and sample collection

The study involved administration of a single oral solution dose of celecoxib at 2 mg/kg body weight (bwt) to six horses using a naso-gastric tube. The administration was done in the presence of a clinical veterinary doctor. The drug was mixed with 250 mL of drinking water and poured into the tube using a funnel. The funnel was rinsed with another 100 mL of drinking water to ensure complete delivery of the drug into the horse stomach. Prior to drug administration an 18-guage catheter was placed either into the right or left jugular vein for the collection of blood samples. Blood samples were collected into heparinised tubes at 0, 5, 10, 15, 30, 45 minutes and at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours post medication. The samples were immediately centrifuged, plasma separated and kept frozen at -20°C until use for drug analysis. A custom made diaper was tied to the animal for the

collection of urine and faeces. Urine and faeces samples were collected whenever the animal disposed for seven days after administration and were pooled into morning and evening urine and faeces composites and stored at - 20°C until used for drug analysis.

Plasma sample analysis

Plasma samples were analysed by use of liquid chromatography- mass spectrometry (LC/MS). A previously validated solid phase extraction (SPE) method was carried out using automated extraction modules (RapidTrace, Biotage) and Waters SepPak C18 cartridge was used for the determination of celecoxib. Briefly, the C18 cartridge was sequentially conditioned with 3 mL of methanol and 0.1 M KH2PO4 (pH 6.0). A volume of 1 mL plasma was allowed to pass through the column. Then the column was rinsed sequentially with 1 mL 0.1 M KH2PO4 : methanol (90:10) and 1 mL of 1.0 M acetic acid. Following drying for 2 minutes, the drug and its metabolites were eluted with 4 mL dichloromethane. The organic solvent was dried under a gentle flow of nitrogen on a TurboVap (Biotage) at 40 °C. The residue was reconstituted in 50 µL of mobile phase. In addition to administration samples, quality control samples with the drug spiked at different concentrations (1, 10, 100, 500 and 1000 ng/mL) were also assayed with each set.

A stock standard was prepared by dissolving 1 mg of celecoxib in 100 mL of acetonitrile. The stock standard was further diluted by serial dilution to prepare standard solutions of 1, 100 and 500 ng/mL. Similarly 1 mg of zaleplon was dissolved in 100 mL of methanol. This was further diluted to 1 µg/mL and used as internal standard. Calibration standards ranged from 1 to 1000 ng/mL were prepared by adding appropriate volumes of the standard drug solutions to drug free plasma. Zaleplon was used as the internal standard. A volume of 200 µL (1 µg/mL) of the internal standard was added to the calibration and test sample and extracted as mentioned above.

The LC/MS system consisted of a Surveyor LC pump and auto sampler (Thermo Finnigan) connected to a TSQ Access (Thermo Finnigan) mass spectrometer. Chromatographic separation was performed using a Thermo Hypersil C18 column (100 x 2.1 mm ID., 5 µm particle size) using a mobile phase consisting of acetonitrile and 1% formic acid in water, run in a gradient mode. The mass spectrometer was operated in the negative ion mode performing product ion scans (m/z 380 → 316), (m/z 396 → 302) and (m/z 410 → 366) for celecoxib, hydroxymethyl celecoxib and celecoxib carboxylic acid, respectively. Zaleplon was scanned in the positive ion mode for the transition (m/z 306 → 264).

The present study was able to examine the pharmacokinetics and characterize the metabolic disposition of celecoxib after a single oral dose to horses, applying the proposed highly selective and sensitive LC/MS with a limit of quantitation (LOQ) of 1 ng/mL and a limit of detection (LOD) of 500 pg/mL. No interfering peaks for celecoxib were observed in the chromatograms of blank plasma from the horses used in the study. The intra-day and inter-day assay precision and accuracy for low, medium and high (10, 100, 200 ng/mL) concentration of celecoxib in horse plasma showed a coefficient of variation percentage (CV%) ranging from 5.6 to 9.8 and 4.9 to 8.0, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined for each animal individually utilizing application of the trapezoidal rule for measurements of plasma drug concentration versus time curve to achieve the non-compartmental methodology (Gibaldi & Perrier, 1982; Martinez, 1998; Gabrielsson & Weiner, 2012) using a computerized pharmacokinetic and drug disposition program (Kinetica Version 5.1 SP1, Thermo). For each parameter, the mean and standard deviation (SD) were obtained from the calculation results of six animals (n = 6).

In order to estimate the celecoxib dose for horses from an efficacious dose in human, the following equation was applied (Toutain & Bousquet-Mélou, 2004):

$$Dose_{horse} = \frac{Dose_{human} \times CL_{horse}}{CL_{human}} \quad (1)$$

In addition, determinations of an effective plasma concentration (EPC), irrelevant plasma concentration (IPC), safety factor (SF) and detection time (DT) were based on the following equations (Toutain & Lassourd, 2002):

$$EPC = \frac{standard\ dose\ (per\ dosing\ interval)}{plasma\ clearance\ (per\ dosing\ interval)} \quad (2)$$

$$IPC = \frac{EPC}{SF} \quad (3)$$

$$IPC = C_{max} \times e^{(-\beta \times DT)} \quad (4)$$

Determination of metabolites

Urine samples were pooled as '0' hour (control), 1-12, 12-24 hours and so on up to 192 hours after administration. An aliquot of 10 mL of each urine sample was adjusted to pH 5.2 and approximately 10,000 Fishman units of β-glucuronidase was added and incubated at 37 °C overnight. Another aliquot of 10 mL urine was processed simultaneously without enzyme hydrolysis. Similarly an amount of 10 grams of faeces was weighed and mixed thoroughly with 10 mL of distilled water. The slurry is then centrifuged at 4000 *g* for 15 minutes. A volume of 7 mL of this clear liquid was adjusted to pH 5.2 and enzyme hydrolysed overnight at 37 °C.

The enzyme hydrolysed urine and faeces samples were adjusted to pH 6.0 and again centrifuged for 15 minutes. To 5 mL of each sample 200 µL (1µg/mL) of internal standard was added and subjected to solid phase extraction as described above. The final eluent was dried and reconstituted with 50 µL of the mobile phase for LC/MS analysis.

In vitro metabolism of celecoxib

To characterize the *in vitro* metabolic pathway, celecoxib was incubated with horse liver microsomes. In this study, all the incubations were performed in duplicates in a shaking water bath at 37°C. The experimental protocol is as follows: To 5 µL of celecoxib (1mM) is added 432 µL of 0.1M sodium phosphate buffer (pH 7.4) and 13 µL of the protein (0.5 mg/mL), premixed and allowed to stand at room temperature for 3 minutes. The incubation was commended by the addition of 10 mM NADPH (50 µL) and allowed to react for 30 minutes with constant gentle shaking. After incubation the reaction was quenched by addition of 100 µL of ice cold methanol. The resulting mixture was then extracted with dichloromethane, centrifuged and the organic phase was dried and reconstituted with 50 µL of the mobile phase for LC/MS analysis.

Results

The pharmacokinetic parameters of celecoxib after an oral dose of 2 mg/kg bwt are shown in Table 1. The results show that after oral administration of celecoxib the time to reach peak plasma concentration (t_{max}) was 4.58 ± 1.62 hours and the rate of elimination was calculated to be 0.05 ± 0.01 hr^{-1}, while the terminal half-life (t1/2) was estimated to be 13.60 ± 3.18 hours. The mean ± standard deviation plasma concentration-time profile of celecoxib following oral single dose administration to six horses is graphically presented in Figure 1. Celecoxib was extensively metabolized after oral administration with low levels of the parent drug detected in urine for up to 72 hours. The majority of urinary metabolite consisted of carboxylic acid metabolite which was about 105.53 ng/mL and approximately 3 ng/mL of hydroxymethyl metabolite of celecoxib at 24 hours after oral administration. Moreover, this study shows that the carboxylic acid and hydroxymethyl metabolites of celecoxib could be

detected in urine for 96 and 48 hours respectively. However, the parent drug and carboxylic acid metabolite could be detected in faeces for 120 hours after administration (Figure 2).

Further, the in vitro study using horse liver microsomes evidenced the formation of only two metabolites for the parent drug and no other transformations could be detected using a precursor ion/product ion scan by mass spectrometry (Figure 3).

Discussion

The use of a sensitive and specific analytical technique for the determination of celecoxib in biological samples is of paramount importance in pharmacokinetics study. High performance liquid chromatography with UV or fluorescence detection has been most widely used in the detection of celecoxib (Rose et al., 2000; Störmer et al., 2003; Zarghi et al., 2006). A liquid chromatography-tandem mass spectrometric quantitation of celecoxib in human plasma and rat, employing solid phase extraction was developed by (Bräutigam et al., 2001). Presently mass spectrometry has been predominantly used in the pharmacokinetics and metabolic study of celecoxib (Zhang et al., 2000; Werner et al., 2002; Ma et al., 2015).

The present pharmacokinetic data obtained from horses in this study when compared to humans and other animals is more or less in complete agreement for celecoxib oral administration, irrespective of the species differences (Paulson et al., 2000a ; Paulson et al., 2001; Werner et al., 2002; Itthipanichpong et al., 2005; Park et al., 2012). However, in this study the plotted plasma concentration-time profile shows that there was a rapid increase in celecoxib level in about four hours and then the level decreases gradually reaching detectable levels up to approximately 96 hours following administration (Figure 1). Moreover, the large volume of distribution (Vd/F) and the slow elimination recorded in this study are suggesting extensive distribution of the drug and / or a poor bioavailability in the body of horses.

In the present study, the dosing of 2 mg/kg bwt was based on applying of more or less the same dose as reported for celecoxib urinary metabolism in horse (Dirikolu et al., 2001; De Kock et al., 2005), along with the assumption of equal efficacious plasma concentration between human and horse due to lack of experimental data; and unavailability of celecoxib pharmaceutical preparation in the veterinary market for horses. However, the found plasma clearance in this study (CL/F = 98.48 mL/kg/h) can be of a starting guide to extrapolate a provisional dose from another species assuming that the same overall body exposure (AUC) will produce the same effect in both species (Toutain & Bousquet-Mélou, 2004). Considering the extent of plasma binding is the same between humans and horses with equal bioavailability factor, F, celecoxib oral dose in horses can be extrapolated tentatively by applying equation (1); where the reported human plasma clearance (CL/F) for 3 mg/kg oral administration of celecoxib is 396 mL/kg/h (Brunton et al., 2018), the calculated dose for horses is about 0.75 mg/kg. Consequently, the average plasma concentration that would be achieved in steady-state condition in horses with chronic dosing of 0.75 mg/kg per day is about 317 ng/mL.

Experimental results of this study advise a detection time in horse plasma of approximately 5 days when celecoxib is administered at 2mg/kg bwt based on the achieved analytical LOQ of 1ng/mL. This also was proven by the generic pharmacokinetic/pharmacodynamics approach (Toutain & Lassourd, 2002) based on the determinations of EPC, IPC, SF and the found PK parameters allowing computing of the detection time (DT) by the equations 2, 3 and 4; where the standard dose in this study is 2mg/kg and the found plasma clearance is 98.48mL/kg/h, the calculated EPC is 846.20ng/mL. Applying a default safety factor of 500 (Toutain & Lassourd, 2002) will result in irrelevant plasma concentration of 1.70ng/mL. Where the slope of the terminal phase (β) found in the present study is 0.05 h^{-1} and drug maximum concentration in plasma is 1088 ng/mL, the

computed DT is 5.4 days more or less. Given the above, attention should be given for the change of detection time with a change in celecoxib dose, typically when applying the extrapolated therapeutic dose computed with Eqn (1).

In humans, celecoxib has been found to be extensively metabolized in the liver with < 3% is excreted unchanged and the major route of elimination are faeces and urine (Davies et al., 2000). A reported *in vitro* study for celecoxib metabolism using allelic variant forms of human liver microsomal cytochrome P450 2C9 evidenced the formation of three metabolites, namely: hydroxylated celecoxib, carboxycelecoxib and its corresponding glucuronide (Tang et al., 2001). In addition, it was reported that hydroxylation is the primary pathway of elimination in humans and is similar in several species such as mouse, rat, rabbit, dog and monkey while the carboxylic acid metabolite of celecoxib undergoes further glucuronidation to form carboxylic acid glucuronide and is excreted in urine (Paulson et al., 2000b; Paulson et al., 2001). However, an interesting observation is noticed on the elimination of celecoxib in horses that the parent drug is excreted as the major component compared to a significantly lower level of the carboxylic acid metabolite excreted in faeces (Figure 2). The high concentration of celecoxib in the faeces could be the unabsorbed drug. The two major urinary metabolites of celecoxib in horses were characterized and identified as 4-hydroxycelecoxib and 4-carboxycelecoxib (Dirikolu et al., 2001; De Kock et al., 2005). Moreover, it has been found that there was no appreciable change in the concentration of the carboxylic acid metabolite when using either hydrolysed or unhydrolysed urine suggesting that the drug or its metabolite is excreted without conjugation which is evidenced by the absence of any glucuronic acid conjugate when using a neutral loss screen by LC/MS. Hence, the carboxylic acid metabolite accounts for more than 90% and the parent drug accounts for 1-2% of the elimination of celecoxib in horse urine.

Rules for controlling medication of animals in competitions are established based on the possibility to increase artificially both the physical capability and the presence of a competitive instinct, using drugs. However, an anti-doping policy must not impede the use of legitimate therapeutic medications and most regulatory bodies in the world now distinguish the control of illicit substances (doping control) from the control of therapeutic substances (medication control) (Toutain, 2010). This study indicates that the detection of celecoxib and its carboxy metabolite are key elements for celecoxib doping investigation in horses, due to prolonged elimination profile and considerably high detection concentration in urine and faeces. It is concluded that a detection time of at least 5 days is advised for racing practitioner and veterinarians after administration of oral therapeutic dose of celecoxib at 2 mg/kg (body weight) to control its illegitimate use for horse racing. Attention should be given for computing the detection time upon administration of different oral dose.

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Conflict of interest statement

The authors planned, designed and conducted this study. The authors declare no conflict of interest.

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Table 1

Pharmacokinetic parameters of celecoxib in horses (n = 6) after a single oral solution administration at a dose of 2 mg/kg (body weight). Values are presented as means ± standard deviation (SD).

Pharmacokinetic parameter	Mean ± SD
Drug maximum concentration in plasma (C_{max}) ng/mL	1088 ± 324
Time to reach peak plasma concentration (t_{max}) h	4.58 ± 1.62
Rate of elimination (β) h^{-1}	0.05 ± 0.01
elimination half-life ($t_{1/2\beta}$) h	13.60 ± 3.18
Volume of distribution (Vd/F) mL/kg	1904.13 ± 974.69
Clearance (CL/F) mL/h/kg	98.48 ± 48.16
AUC_{0-t} ng.h/mL	24142 ± 1096

Figure legends

Figure 1: Time course changes (mean ± SD) of plasma concentration of celecoxib following oral administration of a single dose of 2 mg/kg body weight to 6 healthy horses.

Figure 2: Time course changes of faecal excretion of celecoxib and its metabolite COOH-celecoxib.

Figure 3: LC-MS/MS extracted ion chromatogram and mass spectra of (A) celecoxib (RT = 6.65 min), (B) hydroxymethyl celecoxib metabolite (RT = 6.10 min) and (C) celecoxib carboxylic acid metabolite (RT = 6.07) obtained from in vitro metabolism of celecoxib using horse liver mircorsomes.

Figure 1:

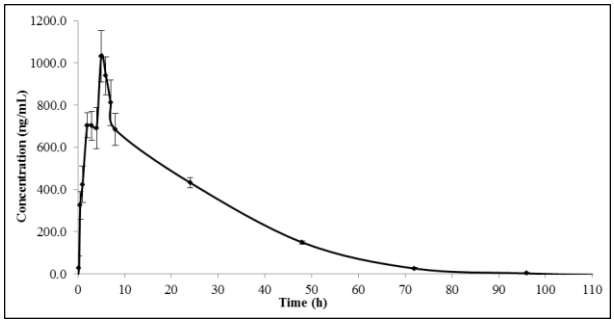

Figure 2:

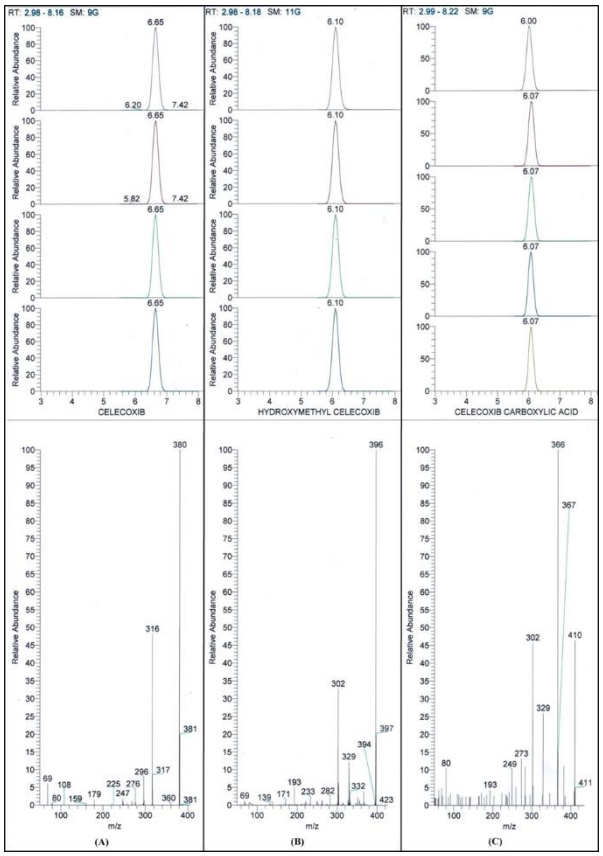

Figure 3: