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Original Research

Withdrawal Time of Phenylbutazone Administered to Healthy Horses

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ABSTRACT

Equine medication regulation rules have been established to prevent a horse's performance being altered after the administration of prohibited substances or approved therapeutic medications used for legitimate treatment. Phenylbutazone (PBZ) is a nonsteroidal anti-inflammatory drug widely used in equine medicine and is one of the most frequently identified medications in the anti-doping controls. The aim of this study was to evaluate plasma elimination kinetic of PBZ in healthy horses after both intravenous and oral administration of two commercial products. Phenylbutazone was administered to horses intravenously or orally at a dose rate of 4.4 mg/kg once daily for five consecutive days. Blood (10 mL) samples were collected before the last dose (t_0); and then after 1, 3, 6, 9, 12, and 24 hours; and every 12 hours for 10 days after treatment. Plasma levels of PBZ and its major metabolite oxyphenbutazone were measured by high performance liquid chromatography with ultraviolet detector (limit of detection [LOD] = 0.005 μg/mL). The detection time obtained in this study by both intravenous and oral PBZ administration by using a LOD of 0.005 μg/mL was 84 hours. Using the dose regimen reported here, a withdrawal time of 5 days could be a correct approach to be adopted for the use of PBZ in performance horses.

1. Introduction

Phenylbutazone (PBZ) is a nonsteroidal anti-inflammatory drug (NSAID) widely used in equine medicine for the treatment of conditions associated with pain and inflammation, such as musculoskeletal disorders and soft or hard tissue injuries [1–3]. The drug has analgesic, antipyretic, and anti-inflammatory properties and acts by inhibiting the cyclooxygenase enzyme pathway, thereby preventing the release of inflammatory mediators including prostaglandins, prostacyclin, and thromboxane [1,3]. The drug is available in medicines for both oral (granules and paste) and parenteral administration. Common dosages for oral preparations in horses are between 2.2 and 4.4 mg/kg daily for five consecutive days. Products available for parenteral administration are given by slow intravenous injection at 2.2 to 4.4 mg/kg daily [1].

The use of PBZ and in general of NSAIDs in racehorses can configure the practice of doping when they are intentionally used to conceal pathologic conditions and to allow to race animals that should be excluded. NSAIDs are the most frequently identified molecules in medication regulation tests, and the challenge is to define the limit between therapeutic use and misuse of these drugs is the challenge that equestrian sport organizations are addressing to define protocols for doping control [4,5]. All the equestrian sport organizations, such as Federation Equestre Internationale (FEI), The United States Equestrian Federation (USEF), and Racing Medication Testing Consortium and Association of Racing Commissioners International (RMTC/ARCI), have agreed to establish a general policy that distinguishes between medication control (legitimate drug) and doping control (illegal substances). For prohibited substances,

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where mere drug exposure is enough to incur punishment, the goal is to control any drug exposure using the most powerful analytical methods. This "zero tolerance" rule is not suitable for therapeutic medication control because trace concentrations of therapeutic substances may be detected for a long time after administration despite having no pharmacologic effect on the animal using the current very sensitive analytical detection methods.

The European Horseracing Scientific Liaison Committee (EHSLC) has recommended to European racing laboratories involved in its program to apply a harmonized screening limit (HSL), which appropriately limits the sensitivity of analytical techniques used for medication control [6]. However, as these HSLs are not made public, they are of no practical value for veterinarians or others in the industry who must advise owners or trainers on appropriate withdrawal times (WT) to guarantee that their horses may safely compete after therapeutic treatment.

To help veterinarians propose WT, the EHSLC decided to determine corresponding detection times (DT), for the main veterinary drugs used in horses. A DT, according to the EHSLC definition, is the time at which the urinary (or plasma) concentrations of a drug, in all horses involved in a study, is less than the HSL when controls are performed using routine screening methods. DT proposed by FEI for PBZ in plasma is 168 hours, both after iv and oral drug administration. DTs proposed by Canadian and Australian authorities for PBZ are 96 and 120 hours, respectively. The differences between the three values are very different, and this is due to the different approach adopted to obtain them [7,8]. The FEI DTs are defined on the basis of the extrapolated irrelevant drug concentrations using the model proposed by Toutain and Lassourd [7]. DTs as issued by the EHSLC are not synonymous with WT. A DT is a raw experimental observation, whereas a WT is a recommendation. A WT should be longer than a DT because the WT should take into account the impact of all sources of animal variability (age, sex, breed, training, and racing) and those of the medicinal product actually administered (formulation, route of administration, dosage regimen, and duration of treatment) to avoid a positive test [7].

There is a clear distinction between the European and US perspectives in this field. Plasma threshold levels of 15 and 2 μg/mL of PBZ have been identified by USEF and RMTC/ARCI, respectively [9]. WT of 12 and 24 hours for PBZ before the competition have been also identified by USEF and RMTC/ARCI, respectively [10].

The aim of this study was to evaluate plasma elimination kinetic of PBZ in healthy horses after intravenous and oral administration of two commercial products marketed in Italy to allow more confident estimation of an effective WT.

2. Materials and Methods

2.1. Animals and Experimental Design

The study was conducted according to the Italian law (D.L. 116/1992) and approved by the Ethical Committee for Animal Experimentation of the University of Pisa (0002473/2014).

Six healthy, adult horses (three females and three males; age 8 to 10 years; weighing 450 to 500 kg) were used in this study. Horses were considered clinically healthy on the basis of a physical examination and complete hematologic analyses; they were housed in individual boxes for the duration of the study. Feed ration was identical for all horses and included complete pelleted diet as well as hay. Water was available ad libitum.

Phenylbutazone was administered to horses intravenously (Bute Paste 2 g/10 g; ACME, Reggio Emilia, Italy; Bute solution 200 mg/mL; ACME) or orally (Bute Paste 2 g/10 g; ACME) at a dose rate of 4.4 mg/kg once daily for five consecutive days [1]. Two catheters were placed into both jugular veins, using an aseptic technique. Phenylbutazone solution was administered through the left jugular catheter; samples were collected by the catheter positioned into the right jugular vein. Blood (10 mL) samples were collected before (t_0) and 1, 3, 6, 9, 12, and 24 hours after the last dose (fifth day) and every 12 hours for 10 days thereafter. Blood was collected into lithium–heparin test tubes and centrifuged at 3,000 rpm within 10 minutes from collection. Plasma samples were stored in aliquots at −20°C until analysis.

2.2. Analytical Assay

Phenylbutazone and internal standard (IS) naproxen were purchased from Sigma–Aldrich (Milan, Italy). Oxyphenbutazone (OPBZ) was purchased from Salars (Como, Italy). Solvents high performance liquid chromatography grade were purchased from VWR-PBI International (Milan, Italy). Acetic acid, formic acid, and ammonium formate were purchased from Fluka (Sigma–Aldrich). Stock solutions of 1 mg/mL of NSAIDs were prepared in methanol and stored at −20°C. Working solutions were prepared by diluting stock solutions appropriately with methanol and stored at 4°C.

Molecular imprinted polymer (product code: SupelMIP NSAIDs) was purchased from Supelco (Sigma–Aldrich). Microprocessor pH-meter pH 9219 was purchased from Hanna Instruments (Italy). The vacuum pump used was a Laboport (KNF Neuberger, Trenton). The SPE vacuum manifold was from J.T. Baker (Deventer, the Netherlands).

The chromatographic system consisted of a binary gradient pump SpectraSYSTEM P2000 Thermo Finnigan, with a UV-VIS SpectraSYSTEM 3000 Thermo Finnigan detector, and an autosampler SpectraSYSTEM AS3000 Thermo Finnigan (Waltham, MA). ChromQuest (Thermo Finnigan) software was used for data processing. The column was a SunFire C_{18} (4.6 × 250 mm, 5 μm particle size; Waters, Milford, MA). The detection was done at 254 nm. Separation was done under isocratic conditions at a flow rate of 1.5 mL/min. Mobile phase was acetonitrile–methanol mixture (75%–25%) and acetic acid (0.01 M, 75%–25%, vol/vol). Quantification was performed using internal calibration (IS naproxen). All calculations were performed using peak area ratios of the PBZ and its metabolite OPBZ peaks to the IS peak (peak area ratio). Phenylbutazone, OPBZ, and IS were extracted from equine plasma using a molecularly imprinted polymers solid-phase extraction procedure performed according to