Urinary and Serum Concentrations of Diclofenac after Topical Application to Horses*

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INTRODUCTION
Veterinarians commonly administer NSAIDs to horses for the treatment of a wide variety of inflammatory conditions. Systemic administration was the standard of care in the past, even if the inflammation was limited to a small anatomic area. The side effects of systemic NSAID administration, however, include gastrointestinal ulceration, renal toxicity, and injection-site reactions.\textsuperscript{1} In contrast, topical NSAID administration is associated with fewer side effects, primarily because the total dose administered is far below the toxic threshold while therapeutic drug concentrations are still

CLINICAL RELEVANCE
The liposomal cream formulation of the NSAID diclofenac, which is approved by the FDA for use in horses, has been shown to be an effective, safe, and convenient way to treat localized areas of inflammation in horses. The results of this study revealed urinary and serum concentrations of diclofenac after topical administration of 1\% liposomal diclofenac cream for 10 days at the labeled dose and at 2\times and 4\times the labeled dose. These results demonstrate the slow absorption and elimination of 1\% liposomal diclofenac cream and may be useful when estimating the withdrawal time needed before a competition to prevent an inadvertent positive drug test.

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achieved locally. Topical NSAID administration in humans is efficacious for the treatment of rheumatoid arthritis, osteoarthritis, and other inflammatory conditions and has an improved safety profile compared with orally administered agents.2,3

The phenylacetic acid NSAID diclofenac sodium is a nonselective cyclooxygenase and lipoxygenase inhibitor that is commonly used in human medicine (Voltaren, Novartis Pharmaceuticals).4 The safety profile of topical diclofenac has been clearly established in humans; its efficacy is equivalent or superior to that of other commonly used NSAIDs, while the risk for gastrointestinal irritation and renal toxicity is relatively low.5,6

Although there is not a diclofenac product approved for oral or parenteral administration in horses, the FDA recently approved a 1% diclofenac liposomal cream formulation (Surpass [1% diclofenac sodium] Topical Anti-Inflammatory Cream, IDEXX Pharmaceuticals) for topical administration to horses for control of joint pain and inflammation associated with osteoarthritis. The results of a clinical field trial indicate that the product is safe, easy to use, and effective in reducing lameness caused by degenerative joint disease.7 Therefore, this NSAID cream provides a new therapeutic modality for veterinarians to use in the treatment of a variety of musculoskeletal injuries and inflammatory conditions.

Little is known regarding systemic absorption and elimination of this 1% diclofenac liposomal cream when applied according to the label recommendations or at extra-label dosages. The elimination pattern of this agent is particularly relevant to veterinarians treating racehorses or performance horses that are routinely subjected to drug-testing programs. Although the FDA-approved label indicates that diclofenac cream should be applied to only one joint twice daily, it is possible that equine practitioners will occasionally treat multiple sites on a single horse. Therefore, the objectives of this study were to determine the concentrations of diclofenac in serum and urine samples collect-

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**Topical NSAID administration is associated with fewer side effects than systemic administration.**

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**MATERIALS AND METHODS**

**Subjects and Treatments**

Six Thoroughbred geldings, 5 to 11 years of age and weighing 520 to 587 kg, were used in this study, which was approved by the University of Florida Institutional Animal Care and Use Committee. The horses were healthy on physical examination at the start of the study. They were exercised on a high-speed treadmill three times a week and maintained on pasture with free access to water and mixed hay. The same six horses were used for the three consecutive treatments, which consisted of the application of 7.2 g of 1% diclofenac cream to one, two, or all four fetlocks twice daily for 10 days. Because of the prolonged treatment period, it was assumed that systemic residual drug concentrations after one treatment would have no significant effect on the ultimate urine and serum concentrations produced by the following treatment. Therefore, a minimum withdrawal period of 3 days between the final application of one treatment...
The elimination pattern of topical diclofenac is particularly relevant to veterinarians treating horses routinely subjected to drug-testing programs.

Analytical Procedure for Determination of Diclofenac and 4-Hydroxydiclofenac in Test Samples—Analytical Standards

Sodium diclofenac and 4-hydroxydiclofenac (diclofenac metabolite) were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved and diluted in high-performance liquid chromatography grade methanol (99.9%) to working solution concentrations of 2 and 20 µg/ml. Meclofenamic acid (internal standard) was obtained from Parke-Davis (Ann Arbor, MI) and likewise dissolved and diluted to a working standard concentration of 2 µg/ml.

Sample Preparation Urine Samples

Three milliliters of each thawed urine sample was pipetted into centrifuge tubes containing 30 µl of the 2 µg/ml internal standard meclofenamic acid working solution and vortex-mixed for 30 seconds. Samples were incubated with 2 ml of 1.0 M acetate buffer (pH 5) and 1 ml of fresh β-glucuronidase solution for 2 hours in a 60°C water bath. The hydrolyzed samples underwent solid-phase extraction on octyl-benzyl sulfonic acid columns (United Chemical Technologies, Bristol, PA) that had been preconditioned by successive washings of methanol, water, and 0.1 M phosphate buffer (pH 6). Loaded columns were rinsed with water, 1.0 M acetic acid, and hexane and allowed to dry under moderate vacuum.

Analytes of interest were eluted with 3 ml of ethyl acetate–hexane solution (50–50) and evaporated to dryness under nitrogen in a 60°C water bath. The residue was reconstituted in 250 µl of methanol and transferred to chromatography vials. All test, calibration, and positive control samples were extracted and analyzed in duplicate. Drug-free urine was fortified to concentrations of 4, 8, 12, 16, 20, 50, 100, 400, and 800 ng/ml (calibration samples) and 5, 14, 30, and 700 ng/ml (positive control samples).

The limit of quantitation (LOQ) of the urine assay was 4.0 to 8.0 ng/ml. The LOQ was determined to be the positive control sample with the lowest concentration for which theoretic and experimental concentration values differed by less than ±20% and for which duplicate preparations resulted in reported

collected as previously described. Serum and urine samples were stored frozen at −20°C until analyzed by liquid chromatography–mass spectrometry (LC-MS).

protocol and the first application of the next was determined to be adequate for the purposes of this study.

For the first treatment protocol, one front fetlock of each horse was treated with 7.2 g of the product (i.e., 1× the label dose) twice daily for 10 days, and urine and blood samples were collected before treatment and 0.25, 8, 12, 24, 48, and 72 hours after the last treatment was applied. For the second treatment protocol, two fetlocks of each horse were treated with 7.2 g of product (i.e., 2× the label dose) twice daily for 10 days, and urine and blood samples were collected as previously described. For the third protocol, all four fetlocks of each horse were treated with 7.2 g of product (i.e., 4× the label dose) twice daily for 10 days, and urine and blood samples were
concentrations with less than ±20% variation. In addition, nonquantitating diclofenac and 4-hydroxydiclofenac ions demonstrated signal:noise ratios of greater than 3:1. Calibration and positive control samples were prepared and analyzed concurrently with the analysis of the administration samples and were considered acceptable if all positive controls demonstrated both accuracy and precision consistent with the ±20% criteria.

Serum Samples
One-milliliter aliquots of each thawed serum sample were pipetted into centrifuge tubes; 2 µl of phosphate buffer (pH 2), 5 ml of dichloromethane, and 2 µl of 2 ng/µl meclofenamic acid solution were added to each tube. The tubes were capped, mixed end-over-end for 10 minutes, and centrifuged at 3,100 rpm for 10 minutes. The organic layer was transferred to conical tubes and evaporated to dryness under nitrogen at 60°C; the resulting residue was constituted in 50 µl of methanol. The tubes were vortex-mixed for 30 seconds, and the contents were transferred to chromatography vials. All test, calibration, and positive control samples were extracted and analyzed in duplicate. Drug-free serum was fortified to concentrations of 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 10, 12, 16, 20, 30, and 50 ng/ml (calibration samples) and 0.2, 0.4, 0.8, 1.0, 4.0, 12, and 30 ng/ml (positive control samples). The LOQ of the serum assay was 0.2 to 0.8 ng/ml.

Sample Analysis
LC-MS data were obtained on a MicroMass Quattro Micro (Cary, NC) in tandem with a Waters 2695 Separations Module (Waters Corporation, Milford, MA) run in positive electro-spray ionization mode with MassLynx v3.5 acquisition software (MicroMass). Chromatographic separation was accomplished on an Atlantis dC18, 3µm, 2.1 × 150 mm column (Waters Corporation) with 0.1% (v/v) formic acid in Milli-Q (Millipore, Billerica, MA) water (component A) and 0.1% (v/v) formic acid in acetonitrile (component B). Flow rate was 0.25 ml/min, and column temperature was held at 35°C. The gradient consisted of an increase from 30% component B to 70% component B over a 3-minute period followed by a 6-minute increase to 90% component B.

Serum samples were analyzed for diclofenac only, whereas urine samples were analyzed for both diclofenac and 4-hydroxydiclofenac. Analytes of interest and respective retention times were 4-hydroxydiclofenac at 7.4 minutes and diclofenac at 8.8 minutes, with internal standard meclofenamic acid detected at 9.7 minutes. For compound identification and quantification purposes, a single transition of the ion at mass:charge ratio 296 to 278 (m/z 296>278) was selected for meclofenamic acid. Two transitions were utilized for 4-hydroxydiclofenac: m/z 312>266 for quantification and m/z 312>231 for compound verification purposes. Three transitions were utilized for diclofenac analysis: m/z 296>215 for quantitation and m/z 296>250 and m/z 296>278 for compound identity verification purposes. The peak area ratios for diclofenac and 4-hydroxydiclofenac from each test sample, calibrator, and positive control urine samples were calculated by dividing the area of the ions at the highest urine concentrations of diclofenac occurred 6 hours after the final application of the cream.
m/z 215 and 266, respectively, at the retention times of diclofenac and 4-hydroxydiclofenac by the area of the ions at m/z 278 at the retention time of meclofenamic acid. The resulting peak area ratios versus the concentrations of the corresponding calibrators were plotted, and the calibration line was determined by linear nonweighted regression. A correlation coefficient of at least 0.999 was deemed acceptable. Concentrations of diclofenac and 4-hydroxydiclofenac in test and control samples were determined from the slope and intercept of the corresponding regression equation.

RESULTS
After 10 days of topical administration of 1% diclofenac liposomal cream at 1×, 2×, and 4× the recommended label dose, diclofenac and 4-hydroxydiclofenac were present in all urine samples collected from 0.25 to 72 hours after the final application (Tables 1 and 2). As shown in Figures 1 and 2, the highest urine concentrations of diclofenac and 4-hydroxydiclofenac occurred 6 hours after the final application of the cream at both 1× and 4× the label dose. For example, following application of 1× the label dose twice daily for 10 days, the highest urine concentrations occurred 6 hours after the last dose was applied, with mean peak concentrations of diclofenac and 4-hydroxydiclofenac of 256.2 ± 92.9 and 119.6 ± 49.7 ng/ml, respectively (mean ± SD). After appli-

| TABLE 1. Urine Diclofenac Concentration (ng/ml; Mean ± SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days |
|---|---|---|---|
| Time after Dosing (hr) | 1× Label Dose | 2× Label Dose | 4× Label Dose |
| 0.25 | 103.6 ± 119.4 | 247.5 ± 283.5 | 411.3 ± 236.7 |
| 6 | 256.2 ± 96.8 | 283.6 ± 153.3 | 502.1 ± 192.0 |
| 12 | 99.5 ± 50.4 | 153.4 ± 99.2 | 301.6 ± 173.7 |
| 24 | 56.5 ± 33.0 | 97.0 ± 73.5 | 179.6 ± 75.1 |
| 48 | 23.6 ± 9.1 | 76.3 ± 31.8 | 157.9 ± 81.4 |
| 72 | 12.2 ± 10.0 | 64.1 ± 38.7 | 121.2 ± 35.3 |

| TABLE 2. Urine 4-Hydroxydiclofenac Concentration (ng/ml; Mean ± SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days |
|---|---|---|---|
| Time after Dosing (hr) | 1× Label Dose | 2× Label Dose | 4× Label Dose |
| 0.25 | 72.9 ± 34.24 | 178.5 ± 84.1 | 263.6 ± 154.7 |
| 6 | 119.3 ± 49.7 | 173.5 ± 51.7 | 273.4 ± 195.07 |
| 12 | 79.5 ± 36.8 | 161.5 ± 28.7 | 259.3 ± 64.1 |
| 24 | 43.1 ± 22.8 | 84.6 ± 59.4 | 166.9 ± 13.1 |
| 48 | 20.5 ± 8.1 | 46.5 ± 14.5 | 111.7 ± 41.1 |
| 72 | 14.1 ± 5.8 | 53.1 ± 20.1 | 102.3 ± 30.0 |
cation of 2× the label dose, the highest mean concentration of diclofenac also occurred in the samples collected 6 hours after the final application, but 4-hydroxydiclofenac concentrations were higher in the samples collected 0.25 hours after the final application (Figures 1 and 2). After administration of 1×, 2×, and 4× the label dose twice daily for 10 days, the decline in diclofenac and 4-hydroxydiclofenac concentrations occurred gradually over the next 3 days, with both the parent compound and the metabolite present in concentrations above the LOQ of the assay (4.0 to 8.0 ng/ml) 72 hours after the last application of each dosage regimen.

After 10 days of topical administration of 1% diclofenac liposomal cream at 1×, 2×, and 4× the recommended label dose, diclofenac was present in all serum samples collected from 0.25 to 48 hours after the final application (Figure 3 and Table 3). Similar to the results of the urine analysis, peak diclofenac concentrations were usually detected in the samples collected 6 hours after the final application. Mean concentrations of diclofenac in serum samples collected 0.25 and 6 hours after the final application of the 2× label dose, however, were essentially the same at 3.8 ± 1.7 and 3.8 ± 1.2 ng/ml, respectively. After 6 hours, serum
concentrations of diclofenac declined slowly and remained above the LOQ of the assay (0.2 to 0.8 ng/ml) for 48 hours after all dosing regimens and for 72 hours after application of 2× and 4× the recommended label dose.

**DISCUSSION**

The diclofenac liposomal formulation used in this study is unique in equine medicine. Liposomes are microscopic vesicles composed of membrane-like lipid layers surrounding an inner compartment. The layers are composed of phospholipids, which are lipophilic at one end and hydrophilic at the other. Liposomal preparations are often used to transport hydrophilic compounds across membranes. The compounds are dissolved in the aqueous phase of the liposome, which is located between the lipid layers and in the central core. Although effective, the amount of a hydrophilic drug that can be delivered by this method is generally limited. However, because diclofenac is highly lipophilic, a relatively large amount of the drug will also dissolve in the lipid portion of the liposome. This maximizes the amount of drug that can be transported across cell membranes and delivered to the site of inflammation when the preparation is applied topically.

The term *locally enhanced topical delivery* (LETD) has been used to describe this local ac-

![Figure 3. Serum diclofenac concentration (mean ± SD) versus time from six horses after the final topical administration of three different doses of diclofenac liposomal cream twice daily for 10 days.](image)

**TABLE 3. Serum Diclofenac Concentration (ng/ml; Mean ± SD) versus Time after the Final Topical Administration of Three Different Doses of Diclofenac Liposomal Cream Twice Daily for 10 Days**

<table>
<thead>
<tr>
<th>Time after Dosing (hr)</th>
<th>1× Label Dose</th>
<th>2× Label Dose</th>
<th>4× Label Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>1.6 ± 0.9</td>
<td>3.8 ± 2.1</td>
<td>5.3 ± 1.6</td>
</tr>
<tr>
<td>6</td>
<td>2.6 ± 0.8</td>
<td>3.8 ± 1.0</td>
<td>9.1 ± 2.2</td>
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<tr>
<td>12</td>
<td>1.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>4.5 ± 1.0</td>
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<tr>
<td>24</td>
<td>0.8 ± 0.4</td>
<td>1.5 ± 0.5</td>
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<tr>
<td>48</td>
<td>0.4 ± 0.0</td>
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<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>72</td>
<td>&lt;LOQ</td>
<td>1.2 ± 0.4</td>
<td>2.4 ± 1.1</td>
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</table>
cumulation of drug into a target tissue.\textsuperscript{9} LETD was demonstrated in a recent study of seven horses with subcutaneously implanted tissue cages, which revealed that the highest mean concentration of diclofenac (76.2 ± 29 ng/ml) was detectable in the carrageenan-induced inflammatory transudate within 6 to 18 hours after topical application of diclofenac liposomal cream.\textsuperscript{10} In addition, this transudate had lower prostaglandin E\textsubscript{2} concentrations than the transudate from placebo-treated tissue cages.

Compared with other conventional creams and ointments, liposomal formulations provide better penetration and sustained release of therapeutic agents.\textsuperscript{11} The results of this study are consistent with these previous reports. For example, the highest concentrations of diclofenac in urine and serum were generally detected 6 hours after administration. In addition, urine and serum concentrations of diclofenac declined slowly over 2 to 3 days after the last application. These findings indicate that the liposomal formulation of diclofenac is slowly absorbed following topical application, which is consistent with the prolonged effect observed in the tissue cage inflammatory study.\textsuperscript{10}

Systemic administration of NSAIDs can be associated with side effects that include gastrointestinal ulceration and renal toxicity. The results of this study indicate that topical application of diclofenac in a liposomal formulation is unlikely to result in serum concentrations that would be associated with systemic toxicity. For example, even when applied at 4× the label dose twice daily for 10 days, serum concentration of diclofenac did not exceed 15 ng/ml in any of the horses. Although the pharmacokinetics of diclofenac in horses following systemic administration has not been determined, oral administration of recommended therapeutic doses in children (1 to 2 mg/kg) resulted in peak serum concentration of 2.4 ± 1.3 µg/ml.\textsuperscript{12} This would be consistent with concentrations of therapeutic doses of NSAIDs in horses, which are typically in the µg/ml range.\textsuperscript{13–15} Thus, serum concentrations associated with systemically active doses of NSAIDs are 100× or more than those occurring after topical application of diclofenac liposomal cream to horses.

As an NSAID, diclofenac is regulated in most medication control programs under which performance horses compete. As such, the results of this study may be useful in assisting veterinarians in determining adequate withdrawal times to avoid positive postcompetition tests. For example, the United States Equestrian Federation (USEF) allows diclofenac to be present in serum collected from horses competing under their Therapeutic Substance Rule as long as the concentration does not exceed 5 ng/ml.\textsuperscript{16} To avoid exceeding this maximum per-

\textbf{Urine and serum concentrations of diclofenac declined slowly over 2 to 3 days after the last application.}
Because urine concentrations of diclofenac were determined for only 72 hours after administration in this study and because the limits of detection for the testing methods used by these organizations are not known, it is difficult to predict withdrawal time for these rules. A minimum of 1 week may be sufficient, but veterinarians should consult with regulatory authorities of the USEF and FEI for more specific recommendations.

In a similar manner, many different analytical methods are used in the United States to detect NSAIDs such as diclofenac in urine samples collected from horses after racing. Therefore, it is not possible to recommend a blanket withdrawal time for horses racing in different jurisdictions. Nevertheless, some generalities can be made. If the jurisdiction uses a thin layer chromatography–based method to screen for diclofenac, at least 24 hours or the mandatory withdrawal period for that jurisdiction would be recommended when one site is being treated with diclofenac cream. If two or four sites on a horse are being treated, the withdrawal time should be increased to a minimum of 48 or 72 hours, respectively. These withdrawal time estimates are based on typical limits of detection for NSAIDs using thin layer chromatography, which range from 200 to 500 ng/ml. If the jurisdiction is testing for diclofenac using an instrument-based method, such as the LC-MS method described in this study, or an immunoassay, a minimum withdrawal time of 96 hours or 1 week, respectively, should be followed because of the increased sensitivity of these methods compared with thin layer chromatography.

**CONCLUSION**

If applied correctly, diclofenac liposomal suspension is a safe and effective method to treat focal sites of inflammation in horses. Because of the large variation in drug-testing methodologies used by laboratories carrying out testing for show and racing authorities, withdrawal guidelines vary from a minimum of 24 hours to at least 1 week. Veterinarians, horse owners, and trainers should consult with the regulatory authorities and racing laboratories in their jurisdiction for additional guidance.

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**REFERENCES**


**The USEF allows diclofenac to be present in serum as long as the concentration does not exceed 5 ng/ml.**


