A Unique Metabolite of Nimesulide* †

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Abstract

Nimesulide is a nonsteroidal anti-inflammatory drug recently detected in equine blood and urine samples taken at the race track. The detection of the drug in a blood sample led to the identification of an unknown thin-layer chromatographic (TLC) spot in track urine samples as a metabolite of nimesulide. Characterization of the unknown TLC spot and comparison with the synthesized compound shows that the unknown TLC spot is a previously unreported equine metabolite of nimesulide. The metabolite was identified as resulting from the reduction of the nitro group on nimesulide to an amino group. This reduced nitro metabolite (4-amino-2-phenoxy-methanesulfonanilide) is a major metabolite of nimesulide in the equine.

Introduction

Nimesulide, a sulfonamide, is a nonsteroidal anti-inflammatory drug. Nimesulide belongs to a new type of nonsteroidal anti-inflammatory drugs, the majority of which are carboxylic acids (1). Reviews on the pharmacological properties of nimesulide have been reported (2–6). The major reported metabolites in humans are 4-hydroxynimesulide and N-acylated-hydroxynimesulide (7–10). The N-acylated metabolite involves reduction of the nitro group; however, hydroxylation appears to be the main metabolic conversion. There have not been any studies on nimesulide administered to horses, nor any report of the reduction of nimesulide.

An unknown substance that gave a purple-color reaction with the diazo spray sequence was detected in our laboratory during the routine screening of race track urine samples. This compound was isolated from several urine samples, and its structure was characterized by Fourier-transform infrared detection (FTIR), gas chromatography–mass spectrometry (GC–MS), and nuclear magnetic resonance (1H NMR). The compound, known as “the purple spot”, was not positively identified until nimesulide was found in a blood sample. Nimesulide was close enough in structure to the purple spot to allow us to postulate that it was a urinary metabolite of nimesulide formed by reduction of the nitro group on nimesulide to an amino group.

The nimesulide postadministration urine of a horse was...
found to have the purple spot, and the reduction of nimesulide did indeed produce the same compound as the purple spot. The preparation of the nimesulide reduction product is shown in Figure 1.

We have shown that a major metabolite of nimesulide in the horse is a reduction of the nitro group on nimesulide to an amino group (4-amino-2-phenoxy-methanesulfonanilide).

Experimental

Reagents and chemicals

An analytical standard of nimesulide was purchased from Sigma (St. Louis, MO). Solvents were glass distilled, HPLC grade. All other chemicals were reagent grade. Elemental analysis of 4-amino-2-phenoxy-methanesulfonanilide was obtained from Guelph Chemical Laboratories (Guelph, Ontario, Canada).

Drug administration and sample collection

Nimesulide (2 g) was administered orally to a standard-bred mare by Dr. Mike Weber. Urine samples were collected hourly for 3 h at the Equine Drug Evaluation Centre.

Reduction of nimesulide to form 4-amino-2-phenoxy-methanesulfonanilide

The method used was a modification of the conditions outlined by Pasto and Johnson (11) for reduction of nitro compounds to amines.

Nimesulide (5 g) was dissolved in approximately 100 mL of dichloromethane (DCM), and then 100 mL of 1N HCl was added. Methanol was added by mixing until a single layer was obtained. Iron powder (2 g) was added to this solution, and it was allowed to stand overnight after occasional mixing over 2 h. The volume was reduced under a stream of nitrogen at 40°C. The pH of the mixture was adjusted to 13 with 5M NaOH. The pH adjustment resulted in a thick precipitate, which was removed by centrifugation of the mixture at 3000 rpm for 15 min. The aqueous layer was extracted with DCM and concentrated under nitrogen, which gave a cream-colored solid weighing 0.88 g. The solid was washed with methanol to purify the solid and to remove some of the color. Discoloration occurred after several days at room temperature. Elemental analysis of carbon, hydrogen, and nitrogen gave the following

\[
\begin{array}{|c|}
\hline
\text{Band} & \text{Assignment} \\
\hline
3398 & \text{NH stretch} \\
3330 & \text{NH stretch} \\
3069 & \text{Aromatic CH stretch} \\
1612 & \text{Aromatic CC stretch} \\
1584 & \text{Aromatic CC stretch} \\
1507 & \text{Aromatic CC stretch} \\
1487 & \text{Aromatic CC stretch} \\
1325 & S = O stretch \\
1213 & \text{CN stretch} \\
1153 & S = O stretch \\
\hline
\end{array}
\]

Table 1. FTIR Band Assignments of 4-Amino-2-phenoxy-methanesulfonanilide
percentages: carbon, 55.40 (calculated 56.12); hydrogen, 4.98 (calculated 5.04); and nitrogen, 9.90 (calculated 10.07).

Urine extraction

Urine (100 mL) was adjusted to pH 9.5 with ammonium sulfite buffer at pH 9.5. Approximately 3 g of XAD-2 resin was added, and the sample was mixed for 15 min. The urine was filtered through a column to collect the resin, which was washed with water (15 mL) and eluted with DCM–ethyl acetate 2:1 (7 × 3 mL). The eluant was concentrated under nitrogen and analyzed for nimesulide and 4-amino-2-phenoxy-methanesulfonanilide.

Purification of 4-amino-2-phenoxy-methanesulfonanilide from urine for characterization by IR and $^1$H NMR

The crude urine extract was dissolved in methanol (200 µL) and applied as a streak to a thin-layer chromatography (TLC) plate. The TLC plate was developed with DCM–ethyl acetate (90:10). The band corresponding to the purple diazo compound was scraped off the plate into a 15-mL screw-cap tube, and 500 µL of 1% aqueous ammonium hydroxide was added with ether (7 mL). The mixture was vortex mixed, and the ether layer was filtered through Na$_2$SO$_4$ and concentrated under nitrogen. The residue was streaked onto a TLC plate and developed with CHCl$_3$–MeOH (92:8), and the band corresponding to the purple diazo compound was scraped off into a 15-mL screw-cap tube. Aqueous ammonium hydroxide (500 µL, 1%) and ether (7 mL) were added to the tube. The mixture was vortex mixed, and the ether layer filtered through Na$_2$SO$_4$ and concentrated under nitrogen. The residue was analyzed by GC–MS, FTIR, and $^1$H NMR.

Instrumentation

GC–MS

A Hewlett Packard 5890 GC with a 5970A mass selective detector was used for the analysis of the nimesulide metabolite. The

Table II. $^1$H NMR Assignments of 4-Amino-2-phenoxy-methanesulfonanilide

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Coupling (Hz)</th>
<th>No. Hs</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.93</td>
<td>singlet</td>
<td>3</td>
<td>CH$_3$SO$_2$</td>
</tr>
<tr>
<td>4.05</td>
<td>broad singlet</td>
<td>2</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>6.18</td>
<td>doublet $J = 2.7$ Hz</td>
<td>1</td>
<td>H$_A$</td>
</tr>
<tr>
<td>6.32</td>
<td>singlet</td>
<td>1</td>
<td>NH</td>
</tr>
<tr>
<td>6.44</td>
<td>doublet of doublets $J = 8.6, 2.4$ Hz</td>
<td>1</td>
<td>H$_B$</td>
</tr>
<tr>
<td>6.98</td>
<td>doublet $J = 7.8$ Hz</td>
<td>2</td>
<td>H$_C$</td>
</tr>
<tr>
<td>7.16</td>
<td>triplet $J = 7.4$ Hz</td>
<td>1</td>
<td>H$_D$</td>
</tr>
<tr>
<td>7.3–7.4</td>
<td>multiplet</td>
<td>3</td>
<td>H$_D, H_E$</td>
</tr>
</tbody>
</table>
column used was a DB XLB-14, 30 m × 0.32-mm internal diameter with 0.25-μm film thickness. The temperature program was 70°C to 300°C at 20°C/min with a 5 min hold. The injector was maintained at 250°C, and the detector was at 310°C.

**FTIR**

4-Amino-2-phenoxy-methanesulfonanilide was analyzed as a film between KBr plates on a Perkin Elmer 16 PC FTIR. The following bands were measured: 3398, 3330, 3069, 2818, 1612, 1584, 1487, 1325, 1213, and 1153 cm⁻¹.

**1H NMR**

4-Amino-2-phenoxy-methanesulfonanilide was dissolved in CDCl₃ and analyzed on a Bruker AM300. The following Hs were measured: 2.93 (singlet, 3H); 4.05 (broad singlet, 2H); 6.18 (doublet, 1H, J = 2.7 Hz); 6.32 (singlet, 1H); 6.44 (doublet of doublets, 1H, J = 8.6, 2.4 Hz); 6.98 (doublet, 2H, J = 7.8 Hz); 7.16 (triplet, 1H, J = 7.4 Hz); and 7.4–7.3 (multiplet, 3H).

**Results and Discussion**

Nimesulide postadministration urine extracts showed 4-amino-2-phenoxy-methanesulfonanilide as a major component. Detection of 4-amino-2-phenoxy-methanesulfonanilide was not the result of the degradation of nimesulide or the extraction process itself because spikes of nimesulide extracted and analyzed with the post-administration urines did not show any metabolite being formed.

4-Amino-2-phenoxy-methanesulfonanilide was characterized by GC-MS, FTIR, and 1H NMR.

Mass spectral assignments could be made for ions 278 and 199, which was the base peak. Ion 278 was the molecular ion, and ion 199 was due to the loss of CH₃SO₂. Figure 2 shows the total ion chromatograms and mass spectra of 4-amino-2-phenoxy-methanesulfonanilide isolated from urine and prepared by reduction of nimesulide. The two gas chromatograms and mass spectra were acquired at different times; therefore, the retention times do not match. The urine isolate was analyzed at the start of the search to identify the unknown compound and the synthesized material during the confirmation of the material as a reduction of nimesulide.
The FTIR assignments are shown in Table I. Figure 3 shows the FTIR spectra of 4-amino-2-phenoxy-methanesulfonanilide isolated from urine and prepared by the reduction of nimesulide. The urine extract contained additional bands that were attributed to silica impurities from the TLC scrape of the urine extract, which was the purification process in the isolation of the material.

The $^1$H NMR assignments are given in Table II. The assigned protons are labeled in Figure 4. Figures 5 and 6 show the $^1$H NMR spectra of 4-amino-2-phenoxy-methanesulfonanilide isolated from urine and prepared by the reduction of nimesulide. The urine extract showed impurities related to the TLC scrape purification process, whereas the NMR spectrum of the prepared material had sharper peaks and none of the additional high field peaks found in the material isolated from urine. Off resonance decoupling experiments were performed to aid in the assignment of the aromatic protons. Irradiation at 6.15 ppm resulted in a collapse of the doublet of doublets at 6.44 ppm to a doublet. HA and HB were therefore coupled to each other by approximately 2 Hz. Irradiation at 6.41 ppm resulted in the doublet at 6.18 ppm reducing to a singlet and the multiplet at 7.3 to 7.4 ppm becoming simplified. HB was therefore coupled to a proton in the multiplet between 7.3 and 7.4 ppm in addition to HA. Irradiation at 6.96 or 7.16 ppm resulted in a change in the multiplet at 7.3-7.4 ppm, and, conversely, irradiation at 7.36 ppm resulted in the collapse of the doublet of doublets at 6.44 ppm to a doublet, the doublet at 6.98 ppm being reduced to a singlet, and the triplet at 7.16 ppm changing to a singlet.

Conclusion

An unknown substance was detected in equine urine race track samples. This unknown was identified as a metabolite of nimesulide that had not been reported previously. Synthesis of the nimesulide metabolite, as well as analysis of postadministration urine from an oral 2-g administration of nimesulide, has shown that reduced nimesulide is a urinary metabolite in the horse.

Acknowledgments

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Dr. David Muir was very helpful in interpreting the spectra of the unknown compound, which led to the recognition of the unknown as the nimesulide metabolite.

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References


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