BIOTRANSFORMATION, EXCRETION KINETICS, AND TISSUE DISTRIBUTION OF AN N-PYRROLO[1,2-C]IMIDAZOLYLPHENYL SULFONAMIDE HERBICIDE IN RATS

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ABSTRACT:
TY029, an N-pyrrolo[1,2-c]imidazolylphenyl sulfonamide herbicide, controls economically important weeds through inhibition of protoporphyrinogen oxygenase. Due to the potential for exposure to this compound in food and animal feed items, a rat metabolism study was required to define the biotransformation of this compound. Animals were exposed to single 50- and 2-mg/kg doses of TY029 [hydantoin-5-14C] by oral gavage. About 90% of the administered dose was excreted within 96 h after oral administration. Excretion plateaued after 48 h, and the cumulative sum of urinary or fecal excretion after 48 h was less than 5% of the orally administered dose. TY029 yielded seven major metabolites. While some metabolites were formed by epimerization around chiral centers, others were generated through hydrolytic bond cleavage and hydroxylations and subsequent oxidation of hydroxyl groups to carboxylic acids. One metabolite, about 6.1% of the dose, was observed only in the urine from low-dose female rats. This metabolite was characterized as a glutamyl conjugate of an extensively oxidized analog of TY029. With the exception of the glutamyl conjugate, the same metabolites were observed in the excreta of all dose groups. However, the relative ratios of the metabolites were different between various dose groups.

TY029 (Fig. 1), an N-pyrrolo[1,2-c]imidazolylphenyl sulfonamide herbicide, is a protoporphyrinogen oxygenase inhibitor that controls economically important weeds in cereals, corn, soybeans, and sorghum. This compound leads to bleaching of the leaves of the target plants and has proven to be very efficacious. The global maximum use rate of this herbicide will be 30 to 40 g of active ingredient/hectare, and the rate of this herbicide will be 30 to 40 g of active ingredient/hectare, g (50 g/ha) in crops such as cotton, soybean, and sorghum. A low-use rate herbicide, is a protoporphyrinogen oxygenase inhibitor that controls economically important weeds in cereals, corn, soybeans, and sorghum. This compound leads to bleaching of the leaves of the target plants and has proven to be very efficacious. The global maximum use rate of this herbicide will be 30 to 40 g of active ingredient/hectare, g (50 g/ha) in crops such as cotton, soybean, and sorghum. A low-use rate herbicide, is a protoporphyrinogen oxygenase inhibitor that controls economically important weeds in cereals, corn, soybeans, and sorghum. This compound leads to bleaching of the leaves of the target plants and has proven to be very efficacious. The global maximum use rate of this herbicide will be 30 to 40 g of active ingredient/hectare, g (50 g/ha) in crops such as cotton, soybean, and sorghum. A low-use rate herbicide.

Materials and Methods

Test Substance. TY029 and its metabolites were synthesized at The DuPont Co. (Newark, DE). Radiolabeled TY029 was synthesized at PerkinElmer Life Science Products (Boston, MA) and supplied as a white powder. The test substance was stable under the conditions of this study. No evidence of physical state or HPLC1 purity profile.

Animals. Male and nulliparous female Crl:CD(SD)IGS BR rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC). At the time of animal dosing, rats were 7 to 12 weeks of age. All animals weighed 173 to 264 g. Upon arrival, rats were maintained under quarantine for at least 6 days. Animal rooms were targeted at a temperature of 23 ± 1°C and a relative humidity of 50 ± 10%. Animal rooms were artificially illuminated (fluorescent light) on a 12-h light/dark cycle. Standard chunk (PMI Nutrition International, Inc., Certified Rodent LabDiet 5002, Richmond, IN) or rodent chow pellets (Rodent Pellet Certified Formula A/I Chow; P.I. Noyes Company, Inc., Lancaster, NH) and tap water were provided ad libitum. Chunk chow was given to rats during the quarantine period and food pellets or chunk chow was given to rats that were on study.

Dose Concentration and Preparation. Oral dosing was chosen as the route of administration to comply with the U.S. Environmental Protection Agency Federal Insecticide, Fungicide, and Rodenticide Act and European Union Commission Directives Guidelines. Dosing solutions were prepared in sterilized glassware by dissolving the test substance in reagent grade water containing 0.5% methyl cellulose and vortexing until completely dissolved (J.C. Maslanka, personal communication). This solution was used for oral dosing via gavage. Unlabeled TY029 was mixed with TY029[hydantoin-5-14C] to achieve the appropriate concentration needed for dosing. Samples of the dosing solutions were analyzed for radioactivity to confirm the purity, concentration, and specific activity. The total dose volume was targeted at ~4 ml/kg (1.0 ml/250-g rat). In the low-dose group, rats received a nominal single oral dose of 2 mg/kg of body weight by gavage. About 30 μCi of radioactivity was delivered to each rat. The low dose was nontoxic (no observable effect level); but high enough to allow for metabolite identification. In the high-dose group, rats received a nominal single oral dose of 50 mg/kg of body weight by gavage. Again, about 30 μCi of radioactivity was delivered to each rat. The high dose was selected to approximate the minimal effects observed on the hematopoietic system and nutritional parameters in the rat developmental and subchronic toxicity studies.

Animal Dosing and Sample Collection. All dosing solutions were analyzed for chemical and radiochemical purity before dosing. For the purposes of...
excreta metabolite identification, a group of eight male and eight female rats was used. Half of each sex group received the high dose and the other half received the low dose of TY029[hydantoin-5-14C] via oral gavage after fasting overnight. Urine and feces were collected (on dry ice) at approximately 0 to 12 and 12 to 24 h after dosing, and each 24-h period thereafter for a total of 96 h, after which the animals were sacrificed. After termination, whole blood (plasma and red blood cells), liver, kidney, heart, lung, thyroid, spleen, brain, fat, carcass, testes, ovaries, pituitary, muscle, adrenals, skin, bone (marrow and mineral), gastrointestinal tract and contents, uterus, and pancreas were collected. These samples were stored frozen (approximately −20°C). Cage wash and feed residue samples were stored at room temperature or refrigerated until processing and analysis.

Measurement of Radioactivity. Radioactivity in urine, plasma, cage washes, and liquid extracts were quantitated by directly assaying aliquots on LSC. For determination of total radioactive residues in solid samples, they were combusted in Packard models 306 and 307 sample oxidizers (Packard Instrument Co., Meriden, CT) for 1 to 1.5 min while trapping the liberated radioactive CO2 and volatiles in CarboSorb E carbon dioxide absorber for liquid scintillation counting. The captured radioactivity was then measured using a Packard Tri-Carb liquid scintillation analyzer, model 2500TR series. Samples were counted by LSC for 10 min. Radioactivity in samples analyzed by HPLC was detected by an on-line Radiomatic Flo-One/Beta Series radioactivity detector. The validity and sensitivity of our on-line detection was corroborated by fraction collection of selected samples over the whole HPLC run and scintillation counting of all fractions on LSC to produce reconstructed chromatograms. For quantification purposes, radioactivity due to each HPLC peak was measured by fraction collection and LSC analysis of the collected fractions. Group data were represented as mean ± S.D. Samples with >10% LSC variability were reanalyzed when possible.

Sample Preparation. The urine, feces, and plasma samples were processed before radioactivity counting and metabolite profiling.

Urine. Urine samples from each collection interval were thawed, and 0.1- to 1.0-ml aliquots were analyzed in triplicate for 14C by LSC. Generally, urine samples collected after 48-h postdose accounted for less than 5% of dose and were excluded from chromatographic analysis. A urine pool was made by combining a defined percentage of the individual samples from one time point within the same dose and sex group. For example, 25% (by weight) of each of the 12- to 24-h urine samples from the four male rats dosed with 50 mg/kg were mixed together. The resulting sample was a pool representative of the 50-mg/kg male urine excreted during the 12- to 24-h time period. In this manner, pooled urine samples were generated across each time point for all animals within the same dose group. Next, a specific percentage of each pooled sample was taken and pooled across all time points. For example, 10% (by weight) of the pooled urine from each of the 0- to 12-, 12- to 24-, and 24- to 48-h pools were combined to yield a 0- to 48-h pool. Approximately 400 µl of each urine sample was centrifuged for 5 min through a 0.45-µm filtration unit in an Eppendorf centrifuge 5414 (Brinkmann Instruments, Westbury, NY) before transfer to an autosampler vial for HPLC analysis.

Feces. Feces from each collection interval were homogenized and aliquots combusted. Feces samples were homogenized by adding water in a 1:1 ratio and vortexing until homogeneous. The CO2 and volatile organics liberated from the combustion were assayed for 14C by LSC to determine total fecal radioactivity for individual rats. Samples were analyzed in triplicate.

For HPLC profiling of the metabolites, fecal homogenates were pooled across animals at a given time point, sex, and dose group to create a pooled fecal homogenate for each collection period in the same manner as described for the urine (above). To generate 0- to 48-h fecal pools for all the animals within the same dose group, a specific percentage of pooled feces from each time point was mixed. For example, 25% (by weight) of the fecal pools from each time period was combined for creating the 0- to 48-h sample. The 0- to 48-h pools were then extracted as described above and analyzed on HPLC. The extraction procedure for the fecal samples is illustrated in Fig. 2. To extract fecal samples, 3 ml of acidic water (1% formic acid) was added to 1 to 2 g of feces in a 50-ml polypropylene copolymer centrifuge tube. After mixing well, the homogenate was extracted with 8 ml of hexane on a wrist-action shaker for 10 min. The mixture was centrifuged at 10,000 rpm for 10 min, and the hexane layers (top) were pooled. Because the hexane extract never contained an appreciable amount of radioactivity (<0.2% of dose), it was discarded. The fecal pellet was then further extracted using 5 ml of a 1:1 mixture of methanol and acidified water (1% formic acid) and centrifuged, as described above, two times. The supernatants were pooled and saved in a separate container. Next, the pellet was extracted using 5 ml of acidified water (1% formic acid) and centrifuged as described above, once. The supernatant was decanted in a separate container and saved. Finally, the pellet was extracted with 5 ml of acidic methanol (0.5% formic acid) and extracted three times as described above. Once again, the supernatant was collected separately and saved. The level of radioactivity in each extract was measured using LSC. All extracts were combined, concentrated using a Speedvac, and filtered using a 0.45-µm centrifugal filter before reanalysis for radioactivity. The pellet was air-dried and combusted to measure the level of unextracted radioactivity.

Plasma. To separate plasma from red blood cells, blood was centrifuged at approximately 4°C for 15 min and 2000g. Plasma was stored at approximately −20°C until needed.

Identification of Metabolites. The primary method of analysis was HPLC.
Structural identification was made in part by cochromatography with authenticated metabolites, and/or purification on HPLC, followed by other techniques described below.

**HPLC method.** The primary HPLC method used a Hewlett-Packard Series 1100 HPLC (Hewlett Packard, Palo Alto, CA), equipped with a diode array detector monitoring at 210 and 254 nm, and an autosampler. For on-line detection of radiolabeled compounds, a Radiomatic Flo-One/Beta Series A500 radiochromatography detector using a 220-μl calcium fluoride solid detector cell was used. The HPLC method used two Zorbax SB-C18 columns (4.6 × 250 mm, 5 μm; Rockland Technologies, Newport, DE) attached in tandem and maintained at 35°C. The flow rate was 1.0 ml/min with a gradient of 9:1 to 0:10 of 0.5% aqueous acetic acid/acetonitrile in 60 min. When synthetic standards were available, this chromatographic method was used to compare retention times with urinary unknowns.

**HPLC/mass spectrometry of urine.** Urine samples were prepared as described above and injected onto the LC/MS for structural determination of metabolites. The system used was a Hewlett-Packard 1100 Series modular LC, equipped with an autosampler, a RAMONA 92 with a 50-μl solid glass, and a Phenomenex Ultragel [ODS (30), 2.1 × 150 mm, 5 μm] column. The gradient was a 9:1, 10 mM acetic acid/methanol to 100% methanol in 55 min. The mass spectra were obtained in the positive electrospray mode using a Quattro II triple quadruple mass spectrometer equipped with a MASSLYNX data system (Micromass Inc., Manchester, UK) operating under sample capillary voltage of 3.5 kV, counter electrode voltage of 30 to 50 V, source temperature of 80°C, scan range of 100 to 700 atomic mass units/3 s, collision energy of 20 eV, and gas cell pressure of approximately, 2.0 × 10^{-3} mBar.

**Nuclear magnetic resonance structural analysis of urine metabolites.** In selected cases, rat urinary metabolites were analyzed using 1H NMR spectroscopy. Each sample was dissolved in D_{2}O (0.05% trifluoroacetic acid) and acquired off-line once the chromatographic run was completed. Each sample was dissolved in D_{2}O (0.05% trifluoroacetic acid) and acquired off-line once the chromatographic run was completed.

**HPLC/infrared of urine.** A representative chromatogram of high-dose male urine is depicted in Fig. 3. As can be observed in Fig. 3, seven radiolabeled compounds were detected in the urine of high-dose males. The spectrum of radiolabeled compound was acquired off-line once the chromatographic run was completed.

**Results.**

One goal of this study was to determine the excretion pattern of the test compound within a given time period (time required for >90% of the dose to be excreted or 7 days). The time necessary for >90% radioactivity to be recovered was determined in a pilot study (data not provided) to be 72 h. The expired air and volatiles did not contain radioactivity in the pilot study, therefore, volatiles were not collected in this study. To ensure sufficient sample collection, excreta were collected up to 96 h after oral dosing. Table 1 summarizes group averages for urinary and fecal excretion of the radioactive dose. The high-dose group excreted >90% of the dose in 96 h while the low-dose group averaged >84% even though both urinary and fecal excretions had plateaued (Table 2). Residue tissues were insignificant (<5% of the applied dose) and the material balance for the high-dose group was >90% while it was in the mid 80s for the low-dose rats (Table 1).

Another objective of this study was to determine the terminal tissue distribution of the radiolabeled test compound and its metabolites 96 h after dosing. As can be observed in Table 3, in all cases less than 0.3% of the dose can be found in the tissues, 96 h after oral administration of TY029.

**Metabolite Profiling and Quantitation as Percentage of Dose.** Any tissue or metabolite accounting for ≥5% of the oral dose requires further studies and/or identification (EPA, 1998). To determine which urine and feces samples required HPLC characterization, the level of radioactivity excreted in each collection period was considered (Table 2). The majority of radioactivity was excreted in the first 48 h and the cumulative sums of radioactivity in the urine or fecal samples collected after 48 h always were <5% of the dose and negligible. Therefore, these latter samples were excluded from further analysis. Pooled samples were analyzed using HPLC/radiochemical detector. A representative chromatogram of high-dose male urine is depicted in Figs. 3.

As can be observed in Fig. 3, seven radiolabeled compounds were detected in the urine of high-dose males. The spectrum of radiolabeled compounds in the urine persisted through all collection periods and

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**Table 1**

Material balance in male and female rats at sacrifice (96 h) after a single oral dose of [14C]TY029

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>S.D.</th>
<th>Mean</th>
<th>S.D.</th>
<th>Mean</th>
<th>S.D.</th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Cage wash</td>
<td>0.93</td>
<td>0.26</td>
<td>3.67</td>
<td>1.70</td>
<td>1.29</td>
<td>0.67</td>
<td>2.22</td>
<td>1.18</td>
</tr>
<tr>
<td>Residual feed</td>
<td>0.66</td>
<td>0.28</td>
<td>2.56</td>
<td>3.39</td>
<td>1.06</td>
<td>0.33</td>
<td>0.73</td>
<td>0.69</td>
</tr>
<tr>
<td>Urine</td>
<td>31.67</td>
<td>14.24</td>
<td>69.21</td>
<td>8.58</td>
<td>37.39</td>
<td>4.62</td>
<td>65.45</td>
<td>6.11</td>
</tr>
<tr>
<td>Feces</td>
<td>57.47</td>
<td>12.24</td>
<td>26.09</td>
<td>4.53</td>
<td>47.15</td>
<td>2.09</td>
<td>16.11</td>
<td>1.22</td>
</tr>
<tr>
<td>Tissues</td>
<td>0.26</td>
<td>0.14</td>
<td>0.06</td>
<td>0.05</td>
<td>0.22</td>
<td>0.10</td>
<td>0.28</td>
<td>0.22</td>
</tr>
<tr>
<td>Total excretion</td>
<td>89.15</td>
<td>2.86</td>
<td>95.30</td>
<td>10.79</td>
<td>84.54</td>
<td>2.92</td>
<td>81.56</td>
<td>7.29</td>
</tr>
<tr>
<td>Material balance</td>
<td>90.65</td>
<td>3.17</td>
<td>101.63</td>
<td>9.66</td>
<td>86.84</td>
<td>2.69</td>
<td>84.80</td>
<td>6.97</td>
</tr>
</tbody>
</table>

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Mean S.D. Mean S.D. Mean S.D. Mean S.D.
additional compounds were not excreted as a function of time. The relative proportion of each metabolite is noted in Table 4.

As noted in the male rats, compounds 1-5 were excreted in the urine of high-dose female rats during all collection periods. Furthermore, mass spectral analysis and spiking the female urine samples with male urine demonstrated that indeed peaks 1-5 were the same in male and female urine (data not shown).

The male and female rats in the low-dose groups exhibited identical urinary excretion patterns to the rats in the high-dose groups (data not provided). The only exception to this was one additional HPLC peak (8, Fig. 3), which represented 6.1% of the dose only in the low-dose female urine (Table 4). Because a synthetic standard for HPLC peak 8 was not available, it was isolated for identification. As will be discussed later, the proposed structure for this metabolite is based only on MS data. Because of sample limitations, the NMR studies, although attempted, were not informative.

Feces samples required more preparation. The details of this work are described for the urine samples. All HPLC peaks found in the fecal extracts of high- and low-dose male and female rats were described in Table 5. The negligible loss of material in the fecal pellets after extraction contained about 3% of the dose and did not require further extraction.

The relative proportion of each metabolite is noted in Table 4. The only exception to this was one additional HPLC peak (8, Fig. 3), which represented 6.1% of the dose only in the low-dose female urine (Table 4). Because a synthetic standard for HPLC peak 8 was not available, it was isolated for identification. As will be discussed later, the proposed structure for this metabolite is based only on MS data. Because of sample limitations, the NMR studies, although attempted, were not informative.

Feces samples required more preparation. The details of this work can be found under Materials and Methods and in Fig. 2. The extraction process was satisfactory because all fecal pellets after extraction contained about 3% of the dose and did not require further studies (Table 5). The negligible loss of material in the fecal pellets was not considered in the calculations of “HPLC peaks as a percentage of dose” (Table 4).

Fecal extracts were subjected to HPLC analysis analogous to that described for the urine samples. All HPLC peaks found in the fecal extracts of high- and low-dose male and female rats were described in the urine analysis, and there were no additional signals. Cochromatography of varying amounts of high-dose male urine, in terms of
radioactivity with the fecal extracts, resulted in coelution of all fecal HPLC peaks with those present in the male urine.

The cumulative excretion of HPLC peaks 1-8 as a percentage of $^{14}$C-dose is presented in Table 4 for each dose group. Although other HPLC signals were observed, based on our calculations, the compounds listed were deemed significant due to their excretion at $\geq 5\%$ of the dose. These compounds were selected for structural elucidation.

Because the high-dose male urine (0–48 h) was inclusive of all significant metabolites, it was used for metabolite identification. Structural elucidation techniques included comparisons of HPLC retention times, NMR, MS, and IR data from authenticated standards (when available) against those of the rat metabolites. When authenticated standards were not available, more detailed NMR, MS, and IR analysis was used to identify metabolites. Once the identities of the metabolites in high-dose male rat urine were established unequivocally, the urine samples from this group were used as authenticated standards, as well.

HPLC/Mass Spectrometric Structural Analysis of Urine Metabolites. All of the metabolites discussed below exhibited a dichloro isotopic mass spectral pattern, consistent with the MS data of the parent molecule. HPLC peaks 1, 2, and 3 (Fig. 3) were studied using mass spectrometry by injecting urine samples onto the LC/MS. HPLC peaks 4, 5, 6, 7, and 8 (Fig. 3) were isolated and studied separately to generate conclusive data using NMR (Fig. 4) and IR.

Compound 1. This HPLC peak was attributed to the parent molecule, TY029 (I) and exhibited the same retention time as the synthetic standard. It showed a m/z 414 (MH$^+$) and produced daughter ions, m/z 68, 88, 116, 132, 185, 236, 283, 299, 300, 386, which were consistent with the synthetic TY029, as well.

Compound 2. This HPLC peak was attributed to the diastereomer of the parent molecule. It exhibited the same retention time as the synthetic standard. It showed a m/z 414 (MH$^+$) and produced daughter ions identical with the synthetic TY029 and its diastereomer (2) standards, as well.

Compound 3. This HPLC peak was attributed to an acidic metabolite (3), which was a hydrolysis product of the parent molecule, and exhibited the same retention time as the synthetic standard. It also showed a m/z 432 (MH$^+$), which was indicative of addition of a molecule of H$_2$O to the parent molecule. It produced daughter ions, m/z 88, 132, 145, 160, 273, 386, which were consistent with the synthetic form of this metabolite.

Compounds 4 and 5. These metabolites exhibited identical mass data and different retention times. The parent ions were m/z 432 (MH$^+$), which indicated an addition of a molecule of H$_2$O to the parent. However, they had substantially different retention times from the acidic metabolite (3) and its diastereomer on the HPLC. Additionally, they yielded pseudomolecular ions with dichloro isotopic patterns at m/z 414 (MH$^+$-H$_2$O, in-source collision-induced dissociation fragmentation), 454 ([M+Na$^+$]) and 464 ([M+MeOH]+H$^+$)). Because of a lack of synthetic standards at the time and inadequacy of MS/MS data in assigning a structure, these compounds were isolated and submitted for NMR and IR analysis.

Compounds 6 and 7. These metabolites exhibited identical mass data and yet different retention times. The parent ions were m/z 446 (MH$^+$). Additionally, they yielded pseudomolecular ions with dichloro isotopic patterns at m/z 428 (MH$^+$-H$_2$O, in-source collision-induced dissociation fragmentation), 468 ([M+Na$^+$]) and 478 ([M+MeOH]+H$^+$)). MS/MS of m/z 428, 446, and 478 all gave a prominent fragment at m/z 102 and weak fragments at m/z 100, 120, 159, 185, and 299. The above data may be explained by either addition of two hydroxyl groups to the parent molecule, opening of the pyrrolidine ring to a carboxylic acid, or oxidizing one of the unsubstituted carbons on the pyrrolidine ring to a ketone in compound 3 (Fig. 5). Because of a lack of synthetic standards and inadequacy of MS/MS data in assigning a structure, these compounds were isolated and studied further by NMR and IR analysis.

Compound 8. This compound was isolated using an HPLC with an on-line radiochemical detector and studied using LC/MS/MS in the negative ion mode. This compound exhibited a m/z 537. It displayed a [M-H]-/[M-H+2]- cluster at m/z 537/539, which indicated that the molecule still contained the $^{14}$C isotope (hydantoin label). Furthermore, this [M-H+2]- exhibited the two Chlorine isotopic pattern. Because peak 8 still contained two Chlorines on one side of the molecule and $^{14}$C on the other, it was concluded that this molecule was not a cleavage product. The LC/MS experiment on peak 8 resulted in m/z 239 as a major fragment. Additionally, the MS/MS experiment on peak 8 resulted in a major fragment at m/z 239 and an ion at m/z 465 (M-73$^+$), which would be consistent with the loss of a glutamate (Fig. 5D). It was, therefore, reasonable to conclude that this metabolite was conjugated to glutamate. The MS cluster at m/z 239/241 ($^{12}$C/$^{14}$C), present as a major fragment, may be explained by cleavage of the urea moiety away from the benzene ring (Fig. 5). The proposed structure has precedence in that its unconjugated analog was reported elsewhere (M. Zhang, personal communication).

Nuclear Magnetic Resonance (NMR) Structural Analysis of Urine Metabolites. Because mass spectrometry data did not provide adequate data on compounds 4-7, they were isolated and studied further using NMR.
Compounds 4 and 5. These metabolites were known to have the same molecular weight (m/z 432) and were thought to be geometric isomers of one another by MS. IR data (below) suggested the presence of intact hydantoin rings. Compound 4 was not present in sufficient amounts for a COSY experiment, but its 1D NMR compared well with the same chemical shift range as the protons on the molecule in question. These signals were resonant in protons c and d). The relative integration was comparable with two metabolites was the complex multiplet between 3.5–3.8 ppm (Fig. 4). The protons labeled c and d in compounds 5 were considered to be diastereomeric.

The COSY data confirmed that the proton geminal to fluorine (g) was coupled to the alpha methylenes (a and b), which were also resonated. According to precedence set by the alcoholic metabolites (4 and 5), and the c and d protons (Fig. 4). COSY data also indicated coupling from g to c and d.

The principal characteristic to note in this pair of metabolites was the complex multiplet between 3.5–3.8 ppm, which is where the ethylene (a and b in Fig. 4). No resonances were present in the region 2.6 ppm shows a complex multiplet consistent with the alpha methylene protons in the two isomers. Consequently, the two samples were considered to be diastereomeric.

Compounds 6 and 7. These metabolites were known to have the same molecular weight (m/z 446) and were suspected to be geometric isomers. As depicted in Fig. 5 (A–C), three tentative structures were considered based on mass spectrometry data. Infrared data (below) indicated that the hydantoin ring was intact, and resonances for the chloromethyl (e) and aromatic protons were the same as those of TY029 in the same solvent system. This ruled out possibility C and left the pyrrolidine ring as the site for metabolism. Inspection of the 1D 1H NMR spectrum showed the proton geminal to the fluorine (g) to be intact as a doublet of doublets (Fig. 4). The region from 2.3 to 2.6 ppm shows a complex multiplet consistent with the alpha methylene (a and b in Fig. 4). No resonances were present in the region from 3–4 ppm, which is where the N-CH2- (c and d) of the parent resonated.

The COSY data confirmed that the proton geminal to fluorine (g) was coupled to the alpha methylenes (a and b), which were also coupled to the hydantoin proton at 4.45 ppm (f). No other couplings were evident. According to precedence set by the alcoholic metabolites (4 and 5), and the above observations, a ring opening at the carbon-nitrogen bond of the pyrrolidine ring was proposed. This ruled out possibility A (Fig. 5) and proved possibility B to be valid. The presence of the carboxylate moiety was supported by the IR data (below), as well. Therefore, the structures for compounds 6 and 7 were reasonably assigned as depicted in Fig. 4.

The principal difference between metabolites 6 and 7 by NMR was in the resonance pattern of the proton geminal to the fluorine. In the former, the resonance was a clear doublet of doublets. In the latter, the multiplet was complex with more fine structure visible. This is due to the difference in dihedral angles between the geminal proton and the methylene protons in the two isomers. Consequently, the two samples were considered to be diastereomeric.

HPLC/Infrared Structural Analysis of Urine Metabolites. Infrared data were acquired from samples recovered from the NMR analysis. The IR structure elucidation work was based on comparisons to the IR spectra of synthetic standards of TY029 and its metabolites. IR spectral analysis of diastereomeric compound 2. Confirmation of the presence of the hydantoin ring was based on the fact that five-membered cyclic imides rings generally have two absorption bands in the carbonyl region. The in-phase C=O band (involves both

### TABLE 4

<table>
<thead>
<tr>
<th>Compound (HPLC Peak No.)</th>
<th>High Dose</th>
<th>Low Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (TY029)</td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td>2 (Diastereomeric isomer)</td>
<td>0.3</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3 (Acidic metabolite)</td>
<td>9.6</td>
<td>7.0</td>
</tr>
<tr>
<td>4 (Alcoholic metabolite)</td>
<td>1.8</td>
<td>12.5</td>
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<tr>
<td>5 (Alcoholic metabolite)</td>
<td>4.8</td>
<td>23.3</td>
</tr>
<tr>
<td>6 (Acidic metabolite)</td>
<td>3.3</td>
<td>4.5</td>
</tr>
<tr>
<td>7 (Acidic metabolite)</td>
<td>8.7</td>
<td>4.6</td>
</tr>
<tr>
<td>8° (Glutamate conjugate)</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (TY029)</td>
<td>32.6</td>
<td>1.2</td>
</tr>
<tr>
<td>2 (Diastereomeric isomer)</td>
<td>4.6</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3 (Acidic metabolite)</td>
<td>17.0</td>
<td>3.8</td>
</tr>
<tr>
<td>4 (Alcoholic metabolite)</td>
<td>4.6</td>
<td>4.9</td>
</tr>
<tr>
<td>5 (Alcoholic metabolite)</td>
<td>10.5</td>
<td>10.7</td>
</tr>
<tr>
<td>6 (Acidic metabolite)</td>
<td>&lt;LOD</td>
<td>1.3</td>
</tr>
<tr>
<td>7 (Acidic metabolite)</td>
<td>&lt;LOD</td>
<td>2.9</td>
</tr>
<tr>
<td>8° (Glutamate conjugate)</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

LOD, level of detection.

* Present at greater than 5% of dose in low-dose female rat urine only.

### TABLE 5

Summary of fecal extraction results for all dose groups

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Pooled Fecal Extraction Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sex</td>
</tr>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
</tbody>
</table>
C=O bonds) in solid phase appeared at 1800–1735 cm\(^{-1}\) and was of medium intensity. The out-of-phase C=O band appeared at 1750–1680 cm\(^{-1}\) and was usually very intense. The solid phase spectrum of TY029 showed absorption bands at 1785 and 1726 cm\(^{-1}\). The ratio between the intensities of the two bands was \(A_{1726}/A_{1785}\) 6.6, which was consistent with a five-membered ring (six-membered ring would have ratio 2).

IR spectral analysis of acidic compound 3. Lack of the hydantoin ring was confirmed by IR analysis. The absorption band at 1660 cm\(^{-1}\), was consistent with a urea type carbonyl. The intense band at 1525 cm\(^{-1}\) confirmed the presence of (CO-NH) in a trans-form (amide II band, C-N-H deformation). The band at 1720 cm\(^{-1}\) was consistent with carboxylic group in the solid form. The bands at 1610 cm\(^{-1}\) and 1408 cm\(^{-1}\) were associated with the antisymmetric and symmetric stretch of the ionized form of the carboxylic acid (CO\(_2\)). The antisymmetric SO\(_2\) most likely overlapped with the 1408 cm\(^{-1}\) band. Carboxylic acids usually show a broad band between 3300–2500 cm\(^{-1}\) as a result of hydrogen bonding, due to O-H stretching, which was observed in the spectrum of compound 3. The band at 3338 cm\(^{-1}\) was assigned to a hydrogen-bonded N-H group from the amide structure.

IR spectral analysis of compounds 4 and 5. The spectra of these isolates were very similar to both TY029 and metabolites 6 and 7. There was a small difference in the position of the bands assigned to the hydantoin structure and a band at 1587 cm\(^{-1}\) instead of 1628 cm\(^{-1}\), and the less intense band at 1386 cm\(^{-1}\), due to the antisymmetric SO\(_2\) vibration, which was known to be very sensitive to hydrogen-bonding. These two compounds yielded a 1628 cm\(^{-1}\) as the antisymmetric stretch of an carboxylic acid ion (CO\(_2\)) and the band at 1440 cm\(^{-1}\) as the symmetric stretch. In fact the spectrum of FCH\(_2\)COO\(^{-}\)Na\(^+\) yielded an absorption band at 1618 cm\(^{-1}\) less intense than in CH\(_3\)COO\(^{-}\)Na\(^+\) at 1576 cm\(^{-1}\) and at 1450 cm\(^{-1}\).

Discussion

The rats in the high- and low-dose groups received a nominal oral dose of 50 and 2 mg/kg TY029, respectively. The total excretion of the low-dose rats was in the low 80s after 96 h of collection (Table 1). However, further collection of excreta did not improve recovery because the excretion of radioactivity had subsided drastically and plateaued before 96 h (Table 2). The cumulative sum of urinary or fecal excretion after 48 h was less than 5% of the orally administered dose. The material balances were between 90–100% in the high-dose groups, and in the mid-80s for the low-dose groups (Table 1). In all
cases, the sum of radioactivity in all tissues was less than 0.3% of the oral dose (Table 3). These data indicated a lack of tissue accumulation.

After a simple clean up, urine samples from all individuals in the same sex and dose group and within each collection period were pooled as described under Materials and Methods. Because all excretions beyond 48 h were negligible, we assumed that the 0–48 h urine pools represented the total urinary excretions within each sex and dose group. Fecal samples required rigorous extractions followed by pooling before metabolite profiling. After extraction, the fecal pellet contained less than 5% of the dose and did not require further studies (Table 5).

Figure 3 depicts a representative urinary HPLC profiles of the high-dose males. Detection capabilities using an on-line radiochemical detector was validated by fraction collection of HPLC runs of urine samples from high-dose male and female groups followed by LSC of each fraction. The reconstructed chromatograms confirmed the ability to detect all significant radiolabeled HPLC peaks on-line.

The 0–48 h chromatogram (Fig. 3, first panel) in the high-dose males was inclusive of all the HPLC signals observed for the urine and feces of all of rats with the exception of one metabolite, which was observed in the urine of low-dose females. This metabolite eluted at around 26 min. The bottom three HPLC panels in Fig. 3 represent different collection periods. While the same profiles were present in HPLC traces of all collection periods, they became less concentrated with time. This is most apparent in peaks 6 and 7, where the concentration effect and the presence of the carboxylic acid moieties have resulted in peak tailing in the 0–12 h collection period.

According to the relative levels of each HPLC peak, the percentage of samples injected onto the HPLC and the amount of urine or feces used, and the percentage of dose present in each HPLC peak was calculated for each dose group (Table 4). According to these data, any compound with a cumulative excretion (urine + feces) of ≥5% of dose in any dose group was selected for identification. HPLC peaks 1, 2, and 3 were identified primarily based on their mass spectral data interpretations as well as comparison of their mass spectral and HPLC retention times to synthetic standards as they became available. Peaks 1 and 2 were determined to be the parent molecule, TY029, and its diastereomer (Fig. 6). Peak 3 had resulted from hydrolytic bond cleavage on the hydantoin ring of the parent molecule and contained a carboxylic acid moiety. The parent molecule was the major urinary excreta in the high-dose female and one of the minor HPLC peaks in the high-dose male rats. Additionally, high-dose female rats seemed to be excreting more of the diastereomeric metabolite (2) than the high-dose male rats. However, in contrast to high-dose female rats, the acidic metabolites (3) seems to be the major urinary metabolite in the high-dose male rats.

Fig. 5. Possible structures proposed for compounds 6, 7, and 8 based on MS data.
A to C, possible structure for metabolites 6 and 7 (m/z 446); D, proposed structures for metabolite 8 (m/z 538).
Mass spectral analysis of compounds 4 and 5 indicated addition of a molecule of water to the parent molecule. However, they exhibited different retention times than the acidic metabolite (3) and its synthetic diastereomer. Therefore, a molecule of water seemed to have been added to a different site than observed in 3. The IR data confirmed the hydantoin ring to be indeed intact in both metabolites. We reached this conclusion because the hydantoin signals observed in the parent molecule, and missing in compound 3, were present in both of these metabolites. The 1H NMR data indicated that the resonances for the chloromethyl and the aromatic protons were identical with those of the parent molecule. Furthermore, two-dimensional 1H-1H COSY data (Fig. 4) supported a contiguous coupling network from the hydantoin proton (f) to the methylene protons (c and d). Additionally, in the parent molecule protons c and d were held in a diastereotopic position yielding 1H NMR resonances at 3.58 and 3.98 ppm. However, in compounds 4 and 5, these protons were only 0.1 ppm apart, indicating a change in their spatial relationship and a lack of ring tension due to its opening. According to the above evidence and precedence in the literature (Tomigahara et al., 1994; and Wu et al., 1999), the cleavage of CH2-N bond in the pyrrolidine ring was proposed. The proposed structures for the diastereomeric metabolites 4 and 5 are shown in Fig. 4 and 6. These metabolites were present in all urine and fecal samples studied. In general, they accounted for more of the excreted radioactivity in males than in females, and in feces than in urine.

It should be noted that the males excreted two additional major metabolites compared with females (6 and 7, Table 4). Metabolites 6 and 7 yielded a m/z of 446 and were suspected to be geometric isomers. Mass spectral data were consistent with any of the three possibilities presented in Fig. 5 A to C. However, IR data indicated an intact hydantoin ring and ruled out possibility C. The 1H NMR data indicated that the resonances for the chloromethyl and the aromatic protons were identical with those of the parent molecule. Inspection of 1H NMR revealed that the methylene protons (a and b), as well as the proton geminal to the fluorine (g), were intact (Fig. 4). Examination of the two-dimensional 1H-1H COSY data, lack of the signals due to the methylene protons alpha to the nitrogen, and plausibility of further oxidation of the carbon bearing the primary alcohol in compounds 4 and 5 into carboxylic acids, favored possibility B in Fig. 5. The presence of carboxylate moieties was supported by IR data.

Compound 8 was only detected in the low-dose female rat urine at appreciable levels (~6% of dose, Table 4). Following a tedious purification process, this metabolite was concentrated and studied further. Mass spectral data, an even number molecular weight (mol. wt. 538), the presence of radioactivity (hydantoin ring) and acidic isotope pattern on the same molecule were indicative of a glutamate conjugate of a highly metabolized TY029 analog (Fig. 5). The features of the proposed structure include an opened pyrrolidine ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring, a hydroxy moiety on one of the pyrrolidine carbons and an acetal (hydrated aldehyde) on the methylene alpha to the fluorine bearing ring.

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A summary of the structures and chemical designations of TY029 and its major metabolites is found in Fig. 6. According to the knowledge of metabolite structures in rats, we proposed the metabolic pathway for TY029 as described in Fig. 7.

In a related study (C.A. Maxwell, E.G. Esrey, and A.M. Brown, personal communication), rat metabolites (2-7) were tested for protoporphyrinogen oxidase inhibition and found to be less potent inhibitors than TY029. The biggest reduction in potency (≥3 orders of magnitude) resulted when one or the other of the rings was opened (i.e., the acid or alcohol metabolites). The diastereomeric metabolite 2 was approximately 50-fold less active than TY029. These observations indicated the importance of intact rings for maximal protoporphyrinogen oxidase-inhibition activity. It also implies that the minimal change in the three-dimensional structure of this hydantoin ring can also significantly reduce activity. Therefore, the proposed metabolic pathway would be consistent with a detoxification process.

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References

