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# COMPARATIVE DISPOSITION OF THE NEPHROTOXICANT *N*-(3,5-DICHLOROPHENYL)SUCCINIMIDE AND THE NON-NEPHROTOXICANT *N*-(3,5-DIFLUOROPHENYL)SUCCINIMIDE IN FISCHER 344 RATS

CAROLINE M. HENESEY, GINNY L. KELLNER-WEIBEL, JOAN B. TARLOFF, AND PETER J. HARVISON

*Department of Pharmaceutical Sciences (C.M.H., J.B.T., P.J.H.); and Department of Chemistry and Biochemistry (G.L.K.-W., P.J.H.), University of the Sciences in Philadelphia, Philadelphia, Pennsylvania*

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**ABSTRACT:**

**Disposition of the nephrotoxicant *N*-(3,5-dichlorophenyl)succinimide (NDPS) was compared with that of a nontoxic analog, *N*-(3,5-difluorophenyl)succinimide (DFPS). Male Fischer 344 rats were administered 0.2 or 0.6 mmol/kg [$^{14}$C]NDPS or [$^{14}$C]DFPS (i.p. in corn oil). Plasma concentrations were determined from blood samples obtained through the carotid artery. Urine samples were analyzed for metabolite content by HPLC. Rats were sacrificed at 3 h (DFPS) or 6 h (NDPS) and tissue radiolabel content and covalent binding were determined. [$^{14}$C]NDPS-derived plasma radioactivity levels were 6- to 21-fold higher and peaked later than those from [$^{14}$C]-DFPS. Six hours after dosing, NDPS was 40% eliminated in the urine compared with approximately 90% for DFPS. By 48 h, only 67% of the NDPS dose was eliminated in urine. In contrast, DFPS excretion was virtually complete within 24 h. NDPS underwent oxidative metabolism to a slightly greater extent than DFPS. Distribution of [$^{14}$C]NDPS-derived radioactivity into the kidneys was 3- to 6-fold higher than that into the liver or heart, and was more extensive than with [$^{14}$C]DFPS. NDPS also covalently bound to plasma, renal, and hepatic proteins to a greater extent than DFPS. In summary, NDPS achieves higher tissue and plasma concentrations, covalently binds to a greater extent, and is eliminated more slowly than DFPS. Differences in the lipid solubility of NDPS metabolites and DFPS metabolites may help explain these results. The overall greater tissue exposure of NDPS and its metabolites may contribute to differential toxicity of these analogs.**

*N*-(3,5-Dichlorophenyl)succinimide (NDPS)[1] (Fig. 1) was originally developed for use as an agricultural fungicide (Fujinami et al., 1972). In spite of promising fungicidal activity, subsequent testing in rats demonstrated that this compound induces nephrotoxicity that is selective to the renal proximal tubules (Sugihara et al., 1975; Rankin, 1982; Rankin et al., 1984, 1985). Due to concerns about its toxicity NDPS is not used commercially. However, it has been proposed as a useful model compound for studying chemically-induced kidney damage (Sugihara et al., 1975).

Renal damage after acute exposure to NDPS is characterized by diuresis, proteinuria, glucosuria, increased kidney weights, elevated blood urea nitrogen levels, and decreased accumulation of organic ions by renal cortical slices (Rankin, 1982; Rankin et al., 1984, 1985; Kellner-Weibel et al., 1995). Minor structural changes to NDPS, however, can markedly alter the toxicity of this compound. For example, substituting the chlorines at the 3 and 5 positions of the phenyl ring with fluorines [*N*-(3,5-difluorophenyl)succinimide (DFPS), Fig. 1] reduces toxicity (Yang et al., 1987; Kellner-Weibel et al., 1997). DFPS is nontoxic at a dose 2.5-fold greater than a toxic dose of NDPS (0.4 mmol/kg) and, in fact, is the only 3,5-dihalogenated analog of NDPS that does not exhibit any nephrotoxicity.

Previous studies showed that NDPS undergoes biotransformation by hepatic (Ohkawa et al., 1974; Nyarko and Harvison, 1995), but not renal, cytochromes P-450 (Henesey and Harvison, 1995; Griffin et al., 1996). Furthermore, Rankin et al. (1987a) found that induction or inhibition of microsomal enzymes could markedly alter NDPS-induced renal damage. In addition, several known NDPS metabolites [*N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid (**2-NDHSA**), *N*-(3,5-dichlorophenyl)-3-hydroxysuccinamic acid (**3-NDHSA**), and *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide (**NDHS**)] were more potent nephrotoxicants than the parent compound itself (Rankin et al., 1988, 1989). In contrast, *N*-(3,5-dichlorophenyl) succinamic acid (**NDPSA**) and *N*-(3,5-dichloro-4-hydroxyphenyl) succinamic acid (**NDHPSA**) were less toxic than NDPS (Yang et al., 1985b; Harvison et al., 1992). More recently, we showed that NDPS-induced nephrotoxicity was directly correlated with its conversion to **2-NDHSA**, **3-NDHSA**, and **NDHS** in vitro (Nyarko et al., 1997) and in vivo (Griffin and Harvison, 1998). Thus, it appears that hepatic oxidative

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[1] Abbreviations used are: NDPS, *N*-(3,5-dichlorophenyl)succinimide; DFPS, *N*-(3,5-difluorophenyl)succinimide; **NDPSA**, *N*-(3,5-dichlorophenyl)succinamic acid; **DFPSA**, *N*-(3,5-difluorophenyl)succinamic acid; **2-NDHSA**, *N*-(3,5-dichlorophenyl)-2-hydroxysuccinamic acid; **2-DFHSA**, *N*-(3,5-difluorophenyl)-2-hydroxysuccinamic acid; **3-NDHSA**, *N*-(3,5-dichlorophenyl)-3-hydroxysuccinamic acid; **3-DFHSA**, *N*-(3,5-difluorophenyl)-3-hydroxysuccinamic acid; **NDHS**, *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide; **DFHS**, *N*-(3,5-difluorophenyl)-2-hydroxysuccinimide; **NDHPSA**, *N*-(3,5-dichloro-4-hydroxyphenyl)succinamic acid; **DFHPSA**, *N*-(3,5-difluoro-4-hydroxyphenyl)succinamic acid.

**Send reprint requests to:** Peter J. Harvison, Ph.D., University of the Sciences in Philadelphia, 600 South Forty-third Street, Philadelphia, PA 19104-4495. E-mail: p.harvis@usip.edu

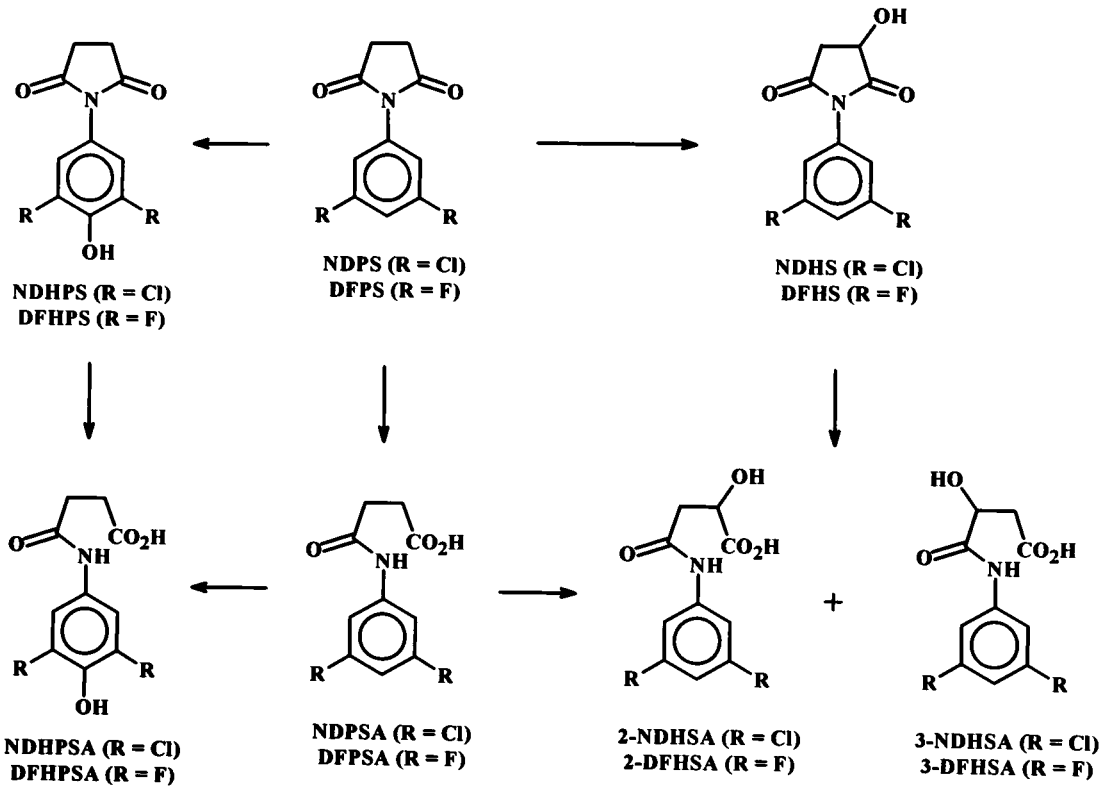


FIG. 1. *Metabolic pathways for NDPS and DFPS.*

biotransformation is critical to the induction of kidney damage by NDPS. Although it is not nephrotoxic (Yang et al., 1987), DFPS undergoes metabolism similar to that of NDPS (Kellner-Weibel et al., 1997). In vivo and in vitro metabolites of DFPS (Fig. 1) included *N*-(3,5-difluorophenyl)succinamic acid (**DFPSA**), *N*-(3,5-difluorophenyl)-2-hydroxysuccinamic acid (**2-DFHSA**), *N*-(3,5-difluorophenyl)-3-hydroxysuccinamic acid (**3-DFHSA**), and *N*-(3,5-difluoro-4-hydroxyphenyl)succinamic acid (**DFHPSA**). In contrast to the results obtained with the NDPS metabolites, we found that **2-DFHSA**, **3-DFHSA**, and *N*-(3,5-difluorophenyl)-2-hydroxysuccinimide (**DFHS**; a structural analog of **NDHS**) were only mild, reversible nephrotoxicants (Kellner-Weibel et al., 1997). Therefore, the difference in the toxicity of these two compounds cannot be attributed solely to a lack of metabolism of DFPS.

The goal of the present work was to directly compare the disposition of NDPS and DFPS in vivo to determine what role (if any) absorption, distribution, metabolism, and excretion play in the differential toxicity exhibited by these two compounds. Our results demonstrated that there are significant differences in the disposition of NDPS and DFPS in the rat. These differences may help account for the fact that NDPS is a nephrotoxicant whereas DFPS is nontoxic.

## Materials and Methods

**Reagents and Syntheses.** All reagents were of highest purity commercially available. [2,3-$^{14}$C]Succinic anhydride (8.4 mCi/mmol) was purchased from Sigma Chemical Co., Inc. (St. Louis, MO). [2,3-$^{14}$C]NDPS and [2,3-$^{14}$C]DFPS were synthesized by reacting [2,3-$^{14}$C]succinic anhydride with 3,5-dichloroaniline or 3,5-difluoroaniline, respectively, according to the method of Fujinami et al. (1972). The radiochemical purity of both compounds (≥99%) was determined by HPLC (see below). Specific activities were 2.15 to 2.26 mCi/mmol for [$^{14}$C]NDPS and 1.55 mCi/mmol for [$^{14}$C]DFPS. NDPS and DFPS metabolites were synthesized as described previously (Shih and Rankin, 1989; Griffin et al., 1996; Kellner-Weibel et al., 1997). Partition coefficient values were estimated using the Pallas software program (CompuDrug, Rochester, NY).

**Animals.** Male Fischer 344 rats (200–270 g, Charles River Laboratories, Wilmington, MA) were used in all experiments. Animals were individually housed in stainless steel hanging cages with a 12-h light/dark cycle at approximately 22°C and 45 to 50% relative humidity. The rats were allowed a 1-week acclimatization period before use in any experiments. Food (laboratory rodent diet #5001, PMI Foods, Inc., St. Louis, MO) and water were freely available unless otherwise noted.

**HPLC Analyses.** All metabolites were separated by reversed phase HPLC on Beckman 5 $\mu$m $C_{18}$ columns (4.2 mm × 25 cm) equipped with Waters $C_{18}$ Guard-Pak cartridges (Waters Corp., Milford, MA). The mobile phase consisted of HPLC grade acetonitrile containing 3% tetrahydrofuran and water containing 2% acetic acid. The assay method used to separate [$^{14}$C]NDPS from its metabolites was described previously (Griffin et al., 1996). [$^{14}$C]DFPS and its metabolites were separated by the method of Kellner-Weibel et al. (1997). Peaks were detected by radioactivity (Raytest Ramona 5LS radiochemical detector) using EcoLume scintillation cocktail (ICN, Costa Mesa, CA). The chromatograms were collected and integrated with the Chromasoft software package (RSM Analytische Instrumente GmbH, Straubenhardt, Germany). [$^{14}$C]NDPS, [$^{14}$C]DFPS, and their metabolites were identified by comparison of their retention times with those of the authentic synthetic standards. The limit of detection for the radiochemical assay is 0.1 to 0.2 nmol for all compounds.

**Absorption Studies.** Rats were placed under general anesthesia using a mixture of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine maleate (0.75 mg/kg). This initial dose was supplemented with ketamine (5 mg/kg) as needed to maintain a surgical plane of anesthesia. The carotid artery was cannulated (PE-10 tubing) to allow blood samples to be withdrawn at timed intervals. Patency was maintained by infusing heparinized saline (100 U/ml; Sigma Chemical Co., St. Louis, MO) at a rate of 18.8 $\mu$l/min using a syringe pump (model 22 syringe infusion pump, Harvard Apparatus, South Natick, MA). Rat body temperature was maintained at 37–42°C throughout the experiment with a heating pad.

To obtain 0- to 2-h samples, rats were dosed with [$^{14}$C]NDPS or [$^{14}$C]DFPS (approximately10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil) between 5 and 10 min after the surgery was completed. Otherwise, animals were dosed first and surgery was performed 1 h before the initial time point. Blood samples (0.5 ml;

no more than 5 samples/rat) were deproteinized with 2 volumes of acetonitrile containing 2% acetic acid and an internal standard (0.133 mM ethyl *N*-(3,5-dichlorophenyl)succinamate or ethyl *N*-(3,5-difluorophenyl)succinamate). The mixture was vortex-mixed and centrifuged (14,000 rpm for 10 min; Eppendorf 5415C centrifuge, Brinkman Instruments, Westbury, NY) to remove the protein. The radiolabel content of an aliquot of supernatant (100 $\mu$l) in EcoLume (10 ml) was determined by liquid scintillation counting (Wallac 1410 Scintillation Counter, Gaithersburg, MD).

**Elimination Studies.** Immediately after dosing with [$^{14}$C]NDPS or [$^{14}$C]-DFPS (approximately10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil), rats were placed in individual metabolism cages (Nalge Co., Rochester, NY) with free access to food and water. Urine was collected at various time points over a 48-h period and the volume was recorded. The urine was then deproteinized and the radiolabel content was determined as described above. NDPS and DFPS metabolite levels in the urine were assayed by HPLC.

**Distribution and Covalent Binding Studies.** Immediately after dosing with [$^{14}$C]NDPS or [$^{14}$C]DFPS (approximately10 $\mu$Ci; 0.2 or 0.6 mmol/kg i.p. in corn oil), the rats were placed in individual metabolism cages with free access to food and water. Urine was collected for 3 or 6 h. Before sacrifice by cervical dislocation, blood samples were obtained by cardiac puncture under general anesthesia (methoxyflurane, Pitman-Moore, Inc., Mundelein, IL). Blood was allowed to clot and then was centrifuged (14,000 rpm for 5 min) to obtain serum. The total blood volume in the animals was estimated using a ratio (13.5 ml of blood/250 g of rat body weight) as described by Davies and Morris (1993). Liver, kidneys, and heart were excised, weighed, and homogenized (Tissumizer, Tekmar Co., Cincinnati, OH) in ice-cold acetonitrile (1–2 vol/g tissue) containing an internal standard (see above). Serum and urine were also deproteinized. All samples were centrifuged (IEC-CENTRA-7R, International Equipment Division, Damon, Inc., Needham Heights, MA) and the radiolabel content of supernatants was determined by liquid scintillation counting as described above.

Protein pellets from liver, kidney, heart, and serum were washed with a series of solvents (0.4 M trichloroacetic acid, 80% methanol in water and 30% ether in ethanol) to remove any unbound radioactivity. The washes were repeated until the supernatants contained less than twice background activity as determined by liquid scintillation counting. Protein pellets were then digested in 1 M NaOH at 70°C for 30 min. Aliquots of the digested protein (100–500 $\mu$l) were counted to determine radiolabel content. The protein concentration was determined using the Bradford method (Bradford, 1976).

**Statistics.** Results are expressed as means ± S.E. from three to five animals. Because a maximum of five blood samples could be obtained per animal, a total of 16 rats (NDPS) or 12 rats (DFPS) was required for the plasma concentration measurements. Each time point in these experiments represents the mean ± S.E. of three to five independent observations. Data were analyzed by the Student's *t* test, a one-way ANOVA, or a two-way ANOVA with compound and tissue as factors. If significance was detected in an ANOVA, a Student-Newman-Keuls test was used to isolate differences between the individual groups. Differences between means were considered statistically significant when $p < .05$.

## Results

Using HPLC with radiochemical detection, the urinary metabolic profiles for NDPS and DFPS in Fischer 344 rats were determined after administration of either compound. Six-hour urinary metabolites of [$^{14}$C]NDPS (0.2 mmol/kg) included **NDPSA**, **2-NDHSA**, **3-NDHSA**, and **NDHPSA** (Table 1). The corresponding difluoro metabolites (**DFPSA**, **2-DFHSA**, **3-DFHSA**, and **DFHPSA**) were found in 6-h urine samples of rats that received [$^{14}$C]DFPS (0.2 mmol/kg). Parent compounds and the hydroxysuccinimide metabolites (**NDHS** and **DFHS**) were not detected in urine. The succinamic acids (**NDPSA** and **DFPSA**) were the major metabolites from each compound, and the amounts of **DFPSA**, **2-DFHSA**, and **3-DFHSA** excreted were significantly greater than the corresponding NDPS metabolites. However, **2-NDHSA** and **3-NDHSA** accounted for a significantly ($p < .05$) larger percentage (47.9 ± 3.0%, data recalculated from Table 1) of the total urinary metabolites than **2-DFHSA** and **3-DFHSA** (40.1 ± 0.6%). The total amount of NDPS metabolites excreted in urine at 6 h was significantly less than the corresponding value for DFPS at 6 h (Table 1).

TABLE 1

*NDPS and DFPS urinary metabolites*

| $^{14}$C-NDPS | | $^{14}$C-DFPS | |
|---|---|---|---|
| Metabolite | Amount excreted | Metabolite | Amount excreted |
| | $\mu mol^a$ | | $\mu mol^a$ |
| **NDPSA** | 8.8 ± 1.8 | **DFPSA** | 28.3 ± 2.1[b] |
| **2-NDHSA** | 4.4 ± 0.4[c] | **2-DFHSA** | 9.5 ± 1.2[b,c] |
| **3-NDHSA** | 4.1 ± 0.3[c] | **3-DFHSA** | 10.3 ± 1.2[b,c] |
| **NDHPSA** | 0.1 ± 0.1[c] | **DFHPSA** | 1.0 ± 0.3[c] |
| Other[d] | 0.6 ± 0.3[c] | Other | ND[e] |
| Total | 17.9 ± 2.1 | Total | 49.1 ± 4.3[b] |

Male Fischer 344 rats were administered $^{14}$C-NDPS or $^{14}$C-DFPS (approximately 10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil) and urine was collected for 6 h. Metabolite content was determined by HPLC.

[a] Values are means ± S.E. of $n = 4$ rats.

[b] Values for DFPS metabolites that are significantly different ($p < .05$) from the corresponding NDPS metabolites.

[c] Values for NDPS or DFPS metabolites that are significantly different ($p < .05$) from **NDPSA** or **DFPSA**, respectively.

[d] Other indicates minor metabolites that were not consistently present in the urine samples.

[e] ND, not detected.

Plasma concentrations were measured for up to 10 h after administration of 0.2 mmol/kg [$^{14}$C]NDPS or [$^{14}$C]DFPS to Fischer 344 rats (Fig. 2). Plasma radioactivity levels were greater (6- to 21-fold difference) for NDPS than DFPS at all time points that were examined. [$^{14}$C]NDPS-derived plasma radioactivity achieved a maximum plateau concentration (186.1 ± 23.6 nmol/ml) that was maintained for approximately 3 to 5 h after dosing. The concentration of radioactivity in plasma (173.4 ± 35.1 nmol/ml) 6 h after the administration of [$^{14}$C]NDPS was not significantly different than that at the 5-h time point, and additional experiments were generally conducted at the later time point (see below). The maximum plasma concentration of [$^{14}$C]DFPS-derived radioactivity (16.0 ± 1.3 nmol/ml) was approximately 10-fold lower than that of NDPS and was achieved in approximately 3 h (Fig. 2).

Urinary excretion of [$^{14}$C]NDPS and [$^{14}$C]DFPS (both administered at a dose of 0.2 mmol/kg) in Fischer 344 rats was determined by measuring the cumulative percentage of radioactivity recovered in urine over 48 h (Fig. 3). The extent of DFPS excretion was significantly greater (ca. 1.5- to 2-fold difference) than NDPS excretion at all of the time points that were evaluated. Six hours after administration, approximately 40% of the total dose of [$^{14}$C]NDPS was excreted in the urine. In contrast, more than 85% of the total [$^{14}$C]DFPS dose was excreted at this time point. Excretion of DFPS in the urine was nearly complete (approximately 95% of the dose) 24 h after administration. By 48 h, we could account for approximately 67% of the [$^{14}$C]NDPS dose in the urine, compared with >97% for [$^{14}$C]DFPS.

We also investigated the distribution of radioactivity into various tissues (liver, kidney, and heart) and fluids (plasma and urine) after administration of [$^{14}$C]NDPS or [$^{14}$C]DFPS (0.2 mmol/kg) to Fischer 344 rats. The extent of distribution was assessed at a time point when the plasma concentration for each compound was maximal (6 h for NDPS and 3 h for DFPS; Fig. 2). At these respective time points we could account for 50 to 60% of the total dose of each compound (Table 2). In both cases, a majority of the radioactivity was recovered in urine. The total amount excreted in urine 6 h after dosing the rats with [$^{14}$C]NDPS was 19.3 ± 1.8 $\mu$mol (data not shown). In contrast,

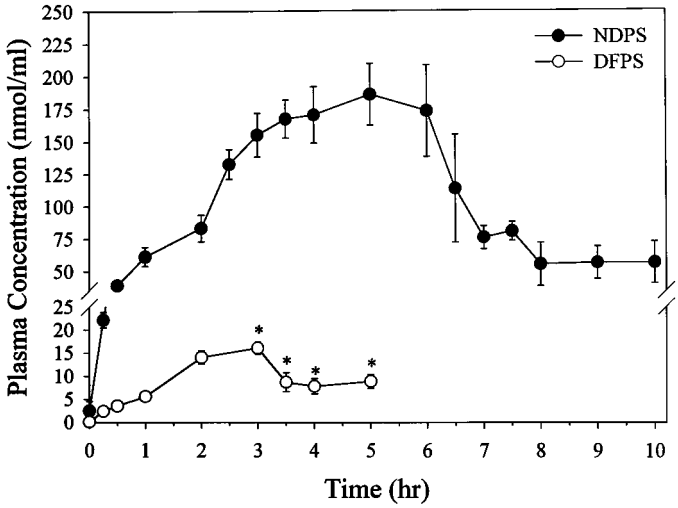


FIG. 2. *Plasma concentration time course for NDPS and DFPS.*

[$^{14}$C]NDPS and [$^{14}$C]DFPS (approximately 10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil) were administered to male Fischer 344 rats. Blood samples were obtained via a cannula inserted in the carotid artery. Radiolabel content of blood samples was determined by liquid scintillation counting. Symbols represent the total amount of radioactivity (i.e., parent compounds plus metabolites) in blood after administration of NDPS or DFPS. Values are means ± S.E. of $N = 3$ to 5 observations (no more than five blood samples were obtained per rat). Error bars that are not shown are contained within the symbols. *DFPS plasma concentration is significantly different ($p < .05$) from the corresponding NDPS value at the same time point.

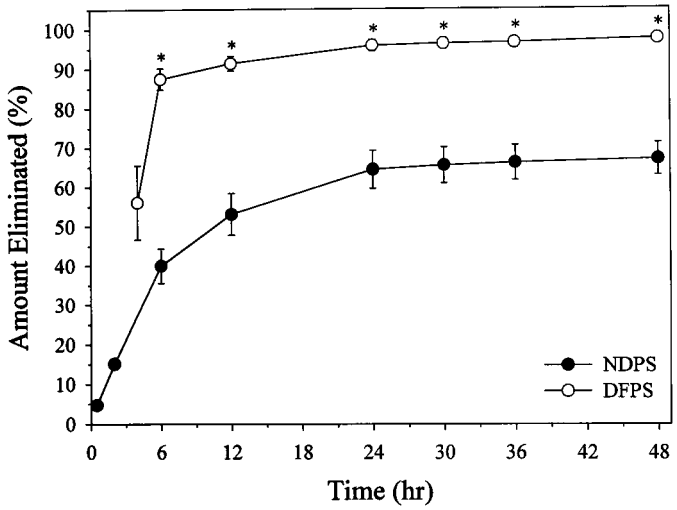


FIG. 3. *Urinary elimination time course for NDPS and DFPS.*

[$^{14}$C]NDPS and [$^{14}$C]DFPS (approximately 10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil) were administered to male Fischer 344 rats. Urine samples were collected over a 48-h period. Radiolabel content of the urine was determined by liquid scintillation counting and is reported as percent of administered dose. Symbols represent the total amount of radioactivity (i.e., parent compounds plus metabolites) in urine after administration of NDPS or DFPS. Values are means ± S.E. of $N = 4$ rats. Error bars that are not shown are contained within the symbols. The results for NDPS at 0.5 h represents the mean of two observations. *percentage of DFPS eliminated is significantly different ($p < .05$) from the corresponding NDPS value at same time point.

a total of 31.2 ± 2.2 $\mu$mol was excreted 3 h after [$^{14}$C]DFPS administration. [$^{14}$C]NDPS-derived radioactivity (expressed as a percentage of total dose) reached higher levels than [$^{14}$C]DFPS-derived radioactivity in all tissues examined, although the difference was significant only in blood. Distribution of radioactivity (expressed in nanomoles per gram tissue weight) into the kidneys was more extensive after administration of NDPS than DFPS (Table 3). In addition,

TABLE 2

*NDPS and DFPS distribution*

| Tissue | Amount of radioactivity (% administered dose)[a] | |
|---|---|---|
| | $^{14}$C-NDPS | $^{14}$C-DFPS |
| Kidney | 0.8 ± 0.2 | 0.14 ± 0.02 |
| Liver | 1.2 ± 0.3 | 0.4 ± 0.1 |
| Heart | 0.05 ± 0.02 | 0.013 ± 0.003 |
| Blood | 4.9 ± 1.5 | 0.8 ± 0.1[b] |
| Urine | 42.7 ± 1.3 | 59.2 ± 4.2[b] |
| Total | 49.6 ± 1.5 | 60.6 ± 4.4 |

Male Fischer 344 rats were administered $^{14}$C-NDPS or $^{14}$C-DFPS (approximately 10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil). Samples were taken at 6 h for NDPS and 3 h for DFPS. Radiolabel content was determined by liquid scintillation counting.

[a] Values are means ± S.E. of $n = 4$ rats.

[b] Values that are significantly different ($p < .05$) from the corresponding NDPS value in the same tissue.

TABLE 3

*NDPS and DFPS tissue concentrations*

| Tissue | Concentration[a] | |
|---|---|---|
| | $^{14}$C-NDPS | $^{14}$C-DFPS |
| | *nmol/g tissue* | |
| Kidney | 198.8 ± 45.2 | 38.4 ± 3.9[b] |
| Liver | 59.4 ± 15.8[c] | 20.7 ± 3.3 |
| Heart | 35.1 ± 15.1[c] | 5.9 ± 1.9 |

Male Fischer 344 rats were administered $^{14}$C-NDPS or $^{14}$C-DFPS (approximately 10 $\mu$Ci; 0.2 mmol/kg i.p. in corn oil). Samples were taken at 6 h for NDPS and 3 h for DFPS. Radiolabel content was determined by liquid scintillation counting.

[a] Values are means ± S.E. of $N = 4$ rats.

[b] Values that are significantly different ($p < .05$) from the corresponding NDPS value in the same tissue.

[c] Values that are significantly different ($p < .05$) from the corresponding values in the kidney for the same compound.

NDPS-derived radioactivity was significantly higher in the kidneys than in the other tissues. Although distribution of DFPS-derived radioactivity into the kidneys was greater than into the liver or heart the differences were not statistically significant. The concentration of [$^{14}$C]NDPS-derived radioactivity in serum (169.0 ± 54.7 nmol/ml) was significantly greater ($p < .05$) than [$^{14}$C]DFPS-derived radioactivity (28.2 ± 3.3 nmol/ml). These concentrations are comparable to those obtained from the plasma concentration time course experiment (Fig. 2).

Covalent binding to hepatic, renal, cardiac, and plasma proteins was measured 6 h after administration of [$^{14}$C]NDPS and 3 h after dosing the rats with [$^{14}$C]DFPS (Fig. 4). In these experiments the two compounds were administered at doses of 0.2 mmol/kg (Fig. 4A, a non-nephrotoxic dose of NDPS) and 0.6 mmol/kg (Fig. 4B, a nephrotoxic dose of NDPS). [$^{14}$C]NDPS-derived covalent binding was more extensive than [$^{14}$C]DFPS-derived covalent binding in the kidneys, liver, and blood at both doses (Fig. 4, A and B). At a dose of 0.2 mmol/kg NDPS (Fig. 4A), the rank order for covalent binding to proteins was plasma > kidney = liver > heart. In contrast, tissue-specific differences in covalent binding were not observed with 0.2 mmol/kg DFPS (Fig. 4A). Compared with the kidneys, significant differences in the extent of covalent binding to proteins in liver, heart, and blood occurred with 0.6 mmol/kg NDPS; the rank order for binding was blood > kidney > liver > heart (Fig. 4B). This difference was not seen with 0.6 mmol/kg DFPS (Fig. 4B).

To estimate lipophilicities, partition coefficients (log *P* values) for NDPS, DFPS, and their respective metabolites were calculated (Table 4). With the exception of the succinamic acids (**NDPSA** and **DFPSA**), log *P* values for NDPS and its metabolites were 2.5- to 7-fold greater than the log *P* values for the corresponding difluoro compounds. Within each series, partition coefficients for the hydroxylated metab-

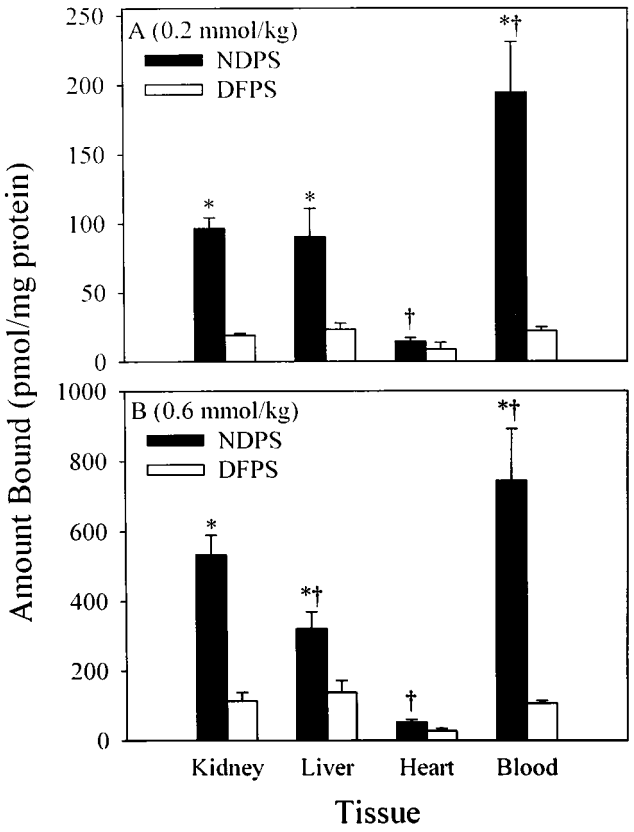


FIG. 4. *Covalent binding of 0.2 mmol/kg (A) or 0.6 mmol/kg (B) NDPS and DFPS to tissue and plasma proteins.*

[$^{14}$C]DFPS or [$^{14}$C]NDPS (approximately 10 $\mu$Ci; 0.2 or 0.6 mmol/kg i.p. in corn oil) were administered to male Fischer 344 rats. Tissue or plasma samples were obtained 6 h after dosing with NDPS or 3 h after dosing with DFPS. Proteins were washed to remove unbound radioactivity and then digested in 1 M NaOH. The amount of covalently bound material was determined by liquid scintillation counting and protein concentration was determined by the Bradford (1976) method. Values are means ± S.E. of $N = 3$ to 4 rats. *covalent binding from NDPS is significantly different ($p < .05$) than that from DFPS for the same dose in the same tissue. †covalent binding is significantly different ($p < .05$) from the kidney for the same compound at the same dose.

TABLE 4

*Calculated partition coefficients for NDPS, DFPS, and their metabolites*

| Compound | Log P[a] | Compound | Log P[a] |
|---|---|---|---|
| NDPS | 1.69 | DFPS | 0.78 |
| **NDPSA** | 2.28 | **DFPSA** | 1.38 |
| **NDHS** | 1.05 | **DFHS** | 0.14 |
| **2-NDHSA** | 1.47 | **2-DFHSA** | 0.57 |
| **3-NDHSA** | 1.47 | **3-DFHSA** | 0.57 |
| **NDHPSA** | 1.90 | **DFHPSA** | 0.99 |

[a] Values were determined using the Pallas software package.

olites (i.e., **NDHS**, **2-NDHSA**, and **3-NDHSA**) were lower than those for the parent compounds.

## Discussion

Hepatic cytochrome P-450-mediated oxidation in the succinimide ring is an important factor in NDPS-induced nephrotoxicity in rats (Rankin et al., 1986, 1987a; Nyarko et al., 1997; Griffin and Harvison, 1998). However, we have recently shown that DFPS (Fig. 1), which is not a nephrotoxicant (Yang et al., 1987), undergoes metabolism similar to that observed with NDPS (Kellner-Weibel et al., 1997). Furthermore, NDPS metabolites that can be derived from succinimide ring hydroxylation (**NDHS**, **2-NDHSA**, and **3-NDHSA**) were potent nephrotoxicants at doses as low as 0.1 mmol/kg (Rankin et al., 1988, 1989). In contrast, the corresponding DFPS metabolites (**DFHS**, **2-DFHSA**, and **3-DFHSA**) were essentially devoid of adverse renal effects at a dose of 1.0 mmol/kg (Kellner-Weibel et al., 1997). Collectively, these results suggest that factors other than biotransformation may also contribute to the difference in nephrotoxicity between NDPS and DFPS. By comparing NDPS and DFPS disposition in parallel experiments, the studies described herein were designed to further assess the roles of absorption, distribution, metabolism, and excretion in NDPS-induced nephrotoxicity. To the best of our knowledge this report represents the first direct comparison between NDPS and a nontoxic analog.

Rat urinary metabolites of NDPS and DFPS included the succinamic acids (**NDPSA** and **DFPSA**), 2- and 3-hydroxysuccinamic acids (**2-/3-NDHSA** and **2-/3-DFHSA**) and *para*-hydroxylated compounds (**NDHPSA** and **DFHPSA**; Fig. 1, Table 1). The parent compounds and the putative hydroxysuccinimide metabolites (**NDHS** and **DFHS**) were not detected in urine. These results are consistent with our earlier findings for both compounds at different time points (Kellner-Weibel et al., 1997; Griffin and Harvison, 1998). Based on prior studies (Nyarko and Harvison, 1995; Griffin et al., 1996), we believe that NDPS undergoes oxidative metabolism to **NDHS**, which then rapidly hydrolyzes to **2-NDHSA** and **3-NDHSA**. A similar hydrolytic reaction involving **DFHS** could account for the presence of **2-DFHSA** and **3-DFHSA** in the urine of rats that received DFPS. NDPS and DFPS both underwent *para*-hydroxylation in the phenyl ring, however, this is probably not relevant to the differential toxicity of the two compounds because it was a very minor route of metabolism. In addition, the *para*-hydroxylated metabolites of NDPS were not nephrotoxic (Harvison et al., 1992). Although the metabolic profiles of the two parent compounds were qualitatively similar, a slightly larger percentage of NDPS metabolism than DFPS metabolism could be attributed to oxidative metabolism (Table 1). Furthermore, previous studies have suggested that succinimide ring hydroxylation is an obligatory step in NDPS metabolic activation (Rankin et al., 1986, 1988). However, this difference probably does not contribute substantially to the contrasting toxic potencies of these compounds, particularly because the oxidative metabolites of DFPS were not nephrotoxicants (Kellner-Weibel et al., 1997).

We found that there were significant differences in absorption and excretion of NDPS and DFPS in rats. The plasma concentration of NDPS increased rapidly during the initial 2 h, then showed a prolonged plateau from about 2 to 6 h. Although DFPS exhibited a similarly shaped plasma concentration curve to that of NDPS, the peak concentration occurred earlier and was approximately one-tenth that achieved after administration of NDPS at the same dose (Fig. 2). NDPS was excreted in urine much more slowly than DFPS (Fig. 3). This observation can be partly explained by the greater lipophilicities of NDPS and its metabolites relative to those of the corresponding difluoro compounds (Table 4). The more lipophilic dichloro compounds should be more readily reabsorbed in the renal proximal tubules, which would decrease their excretion rate in the urine (Vander, 1985). Our calculated log *P* value of 1.69 for NDPS is reasonably close to the experimentally determined value of 1.40 reported by Yang et al. (1985a). These results may also help explain why hydroxylated DFPS metabolites, such as **DFHS**, **2-DFHSA**, and **3-DFHSA**, are not nephrotoxic (Kellner-Weibel et al., 1997). Because these compounds are less lipophilic than the corresponding NDPS metabolites (Table 4) they should be excreted more readily and may not achieve nephrotoxic concentrations in proximal tubular cells. After 48 h we could only account for approximately two-thirds of the

administered dose of NDPS in urine, whereas DFPS excretion was nearly complete within 24 h. The remaining portion of the total NDPS dose may be sequestered in tissues that were not examined. Collectively, the results of the absorption and excretion studies suggest that total systemic exposure to NDPS and its metabolites may be much higher than to DFPS and its metabolites.

Significant differences in the tissue distribution of NDPS and DFPS were also observed (Tables 2 and 3). [$^{14}$C]NDPS-derived radioactivity levels were significantly greater in kidneys and plasma than those that were obtained after administration of [$^{14}$C]DFPS at the same dose. These results imply that tissue exposure from NDPS may be greater than from DFPS and is consistent with the greater lipophilicities of the dichloro compounds (Table 4). Furthermore, the concentration of [$^{14}$C]NDPS-derived radioactivity in the kidneys was significantly greater than that in the liver and heart. This suggests that the kidneys may be able to preferentially accumulate NDPS or its metabolites, which could help explain the selective nephrotoxicity of this compound. In support of this conclusion, Rankin et al. (1987b) previously showed that probenecid, an inhibitor of the renal organic acid transporter, partially protected rats against NDPS nephrotoxicity.

As an index of formation of potential reactive intermediates (Pumford and Halmes, 1997), we measured covalent binding to tissue and plasma proteins after administration of NDPS and DFPS to rats (Fig. 4). In these experiments the compounds were administered at two doses—0.2 mmol/kg (a nontoxic dose of NDPS) and 0.6 mmol/kg (a toxic dose of NDPS; Rankin et al., 1985; Nyarko et al., 1997; Griffin and Harvison, 1998). [$^{14}$C]NDPS-derived radioactivity bound to a greater extent than [$^{14}$C]DFPS-derived radioactivity in all tissues examined except the heart. The smaller amount of covalent binding associated with DFPS could be due to the lower lipophilicities of its metabolites and their reduced distribution into the tissues (Tables 3 and 4). Alternatively, conversion of DFPS to a reactive intermediate could be less extensive than NDPS. Because DFPS and it metabolites are not nephrotoxic (Yang et al., 1987; Kellner-Weibel et al., 1997), the covalent binding observed with DFPS may occur to noncritical intracellular targets or may be below a threshold level required for cytotoxicity.

NDPS covalent binding was also dose- and tissue-dependent. We previously reported that NDPS-induced covalent binding increased with dose, but that there were no differences in binding to hepatic and renal proteins, even at the highest dose (Griffin and Harvison, 1998). However, covalent binding in those experiments was evaluated at a later time point (24 h) when kidney damage was fully expressed and may not represent early events in NDPS-induced nephrotoxicity. In the current investigation, tissue-dependent differences in covalent binding were apparent after a nephrotoxic dose of NDPS. Tissue-selective covalent binding (i.e., plasma > kidney > liver) has been reported for other nephrotoxicants, such as *para*-aminophenol (Crowe et al., 1979) and suprofen (Smith and Liu, 1995). The fact that NDPS-derived covalent binding is higher in kidneys than liver could indicate that a reactive intermediate is either generated by or selectively accumulated in the kidneys. Because NDPS and its oxidative metabolites were not cytotoxicants in isolated renal preparations (Rankin et al., 1988; Aleo et al., 1991; Henesey and Harvison, 1995), it seems unlikely that the kidneys can directly generate the nephrotoxic species. Conceivably, a reactive intermediate of NDPS (or precursor thereof) could be generated in the liver. Migration of a reactive species from the liver to the kidneys through the blood would require that it have a half-life on the order of several seconds (Gillette, 1986).

One possible route for NDPS metabolic activation would involve glucuronidation or sulfation of **NDHS** followed by elimination to form *N*-(3,5-dichlorophenyl)maleimide, which was previously shown to be cytotoxic in renal proximal tubules (Aleo et al., 1991). Alternatively, an acyl glucuronide could be formed from an acidic NDPS metabolite. In fact, acyl glucuronides were implicated in the nephrotoxicity of suprofen (Smith and Liu, 1995) and mefenamic acid (McGurk et al., 1996). In support of these proposed mechanisms, it has been shown that phase II metabolites may be involved in NDPS-induced nephrotoxicity (Rankin et al., 1995, 1996, 1997; Hong et al., 1999). Furthermore, we have obtained preliminary evidence that glucuronide and sulfate conjugates can be formed from NDPS and **NDHS** (Cui and Harvison, 1999).

In conclusion, we have demonstrated that there are significant differences in NDPS and DFPS disposition in rats. These differences may help explain why NDPS is nephrotoxic, whereas its close structural analog DFPS is nontoxic. NDPS-induced renal damage may be due to a combination of hepatic metabolism and increased renal exposure to nephrotoxic metabolite(s).

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