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Dapsone-Induced Hematologic Toxicity: Comparison of the Methemoglobin-Forming Ability of Hydroxylamine Metabolites of Dapsone in Rat and Human Blood[1]

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The relative methemoglobin (MetHgb) forming ability of two metabolites of dapsone, dapsone hydroxylamine (DDS-NOH) and monoacetyldapsone hydroxylamine (MADDS-NOH), were compared in rat and human whole blood. Concentration-response curves for the two metabolites were generated *in vitro* in whole blood. Data were fit to both the E_{max} and Sigmoid E_{max} models. The E_{max} values for MetHgb formation in rat blood for MADDS-NOH and DDS-NOH fitted to the E_{max} model were 83 (8) and 84 (2)%, while the EC_{50} values were 1087 (283) and 828 (104) μM, respectively (mean ± SD). Neither these values nor those generated for the Sigmoid E_{max} model differed significantly between the two metabolites. Similarly, the E_{max} values in human blood for MADDS-NOH and DDS-NOH fitted to the E_{max} model were 79 (5) and 80 (2)%, while the EC_{50} values were 90 (17) and 95 (19) μM, respectively. These values also did not differ between the two metabolites using either pharmacodynamic model. MetHgb was produced at the same rate, reached similar peak concentrations, and exhibited the same rate of decline with both metabolites. The area under the MetHgb content versus time curve did not differ between the two metabolites. These data demonstrate that MADDS-NOH and DDS-NOH are equipotent and equally efficacious in their MetHgb-forming ability. Investigation of the disposition of these metabolites is necessary to assess their relative role in dapsone-induced toxicity *in vivo*.

Dapsone (DDS), widely used in the treatment of leprosy and dermatitis herpetiformis, is currently being investigated for the treatment and prevention of *Pneumocystis carinii* pneumonia in patients with the Acquired Immunodeficiency Syndrome. The use of DDS is associated with numerous side effects, including methemoglobinemia, hemolytic anemia, agranulocytosis, the sulfone syndrome, and a morbilliform rash. The most frequent of these adverse effects are the hematologic toxicities.

Early evidence indicated that DDS-induced methemoglobinemia was mediated by metabolites rather than by DDS itself. Hjelm and DeVerdier (1965) demonstrated that DDS increased methemoglobin (MetHgb) content in human red blood cells (RBCs) when incubated in the presence of rat liver microsomes and an NADPH-regenerating system, while no change in MetHgb concentration was produced in RBCs incubated with DDS in the absence of an NADPH-regenerating system. Cucinell *et al.* (1972) found a good correlation between the formation of MetHgb and N-oxidation products of DDS in an incubation system of human RBCs, DDS, and rat liver microsomes. These observations led to the conclusion that dapsone hydroxylamine (DDS-NOH) causes the methemoglobinemia. Moreover, Scott and Rasbridge (1973) demonstrated MetHgb formation when DDS-NOH or monoacetyldapsone hydroxylamine (MADDS-NOH) was incubated with human RBCs, an observation confirmed by Israili *et al.* (1973). Subsequently, numerous investigators have substantiated the role of DDS hydroxylamine metabolites in DDS-induced hematologic toxicity (Coleman *et al.*, 1989, 1990a,b,c, 1991; Coleman and Tingle, 1992; Grossman and Jollow, 1988).

While it is clear that both DDS-NOH and MADDS-NOH are capable of inducing hematologic toxicity, the relative role of these two metabolites in DDS-induced toxicity is unclear. The contribution to *in vivo* toxicity will be a function of the relative potency and disposition of these two metabolites. To date, conflicting data regarding the relative potency of DDS-NOH and MADDS-NOH induction of hematologic toxicity have been reported. Scott and Rasbridge

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(1973) examined the relative potency of these two arylhydroxylamines in normal and glucose-6-phosphate dehydrogenase (G6PD) deficient human RBCs *in vitro*. They found that MADDS-NOH formed approximately 50% more MetHgb than did DDS-NOH in RBCs from normal subjects and marginally greater MetHgb in RBCs from patients with G6PD deficiency. Moreover, DDS-NOH produced more hemolysis in G6PD deficient RBCs than did MADDS-NOH (9.4 vs 5.2%, respectively). In contrast to these results, Israili *et al.* (1973) observed essentially equal MetHgb forming ability by DDS-NOH and MADDS-NOH in isolated human RBCs, while Grossman and Jollow (1988) found the two metabolites to be approximately equipotent as hemolytic agents in the rat. The study by Scott and Rasbridge (1973), as well as that from Israili *et al.* (1973) is unconvincing due to the use of a single concentration to compare the two metabolites. The objective of the present study was to compare the potency and efficacy of DDS-NOH and MADDS-NOH in producing MetHgb in rat and human whole blood *in vitro*.

METHODS

Chemicals. Platinum oxide and triethylphosphite were procured from Aldrich Chemical Company (Milwaukee, WI). Absolute ethanol was obtained from AAPER Alcohol and Chemical Company and 4-acetamidophenyl-4′-nitrophenyl sulfone from Parish Chemicals (Ovem, UT). Ammonium chloride, chloroform, and ammonium hydroxide were obtained from Fisher Scientific (Chicago, IL). Acetonitrile, methanol, sodium phosphate, and potassium phosphate were purchased from Curtin Matheson Scientific (Novi, MI). Remaining chemicals were obtained from Sigma Chemical Company (St. Louis, MO). All chemicals were used as received unless otherwise noted.

Subjects. Male Sprague-Dawley rats weighing 175–200 g were purchased from Charles River Laboratories (Danover, MA). Animals were kept in a 12-hr light–dark, temperature- and humidity-controlled facility with free access to food and water and were acclimated for at least 1 week prior to the experiment. Animals used as blood donors were anesthetized with ether and exsanguanated via cardiac puncture. Blood was collected into tubes containing sodium heparin (Vacutainer; Becton-Dickinson). All procedures were approved by the Animal Investigation Committee of Wayne State University.

The human volunteers were five male, healthy, drug free individuals between 25 and 35 years of age. Following written informed consent, 5 ml of venous blood was obtained by venipuncture and collected into Vacutainer tubes. The study and written consent form were reviewed and approved by the Human Investigation Committee of Wayne State University.

Synthesis of DDS-NOH. 4-Nitrophenyl-4′-aminophenylsulfone (NADS) was synthesized according to the method of Gabel and Grinberg (1940). 4-Acetamidophenyl-4′-nitrophenylsulfone (3.12 mmol) was refluxed in 50 ml of 18% HCl for 30 min to deacetylate the sulfone. The mixture was cooled and alkalinized with Na_2CO_3 until a yellow precipitate formed. The mixture was then diluted with water and filtered. Recrystalization of this precipitate from 100 vol of 50% ethanol yielded yellow needle-like crystals of NADS.

DDS-NOH was synthesized from NADS according to a modification of the method of Uetrecht *et al.* (1988). Platinum oxide (0.044 mmol) was placed in a hydrogenator and 2 ml of absolute ethanol was slowly added. NADS (3.6 mmol), triethylphosphite (30 μl), and absolute ethanol (123 ml) were placed in the bottle. The mixture was hydrogenated at a pressure of 40 psi for 4 hr and then filtered (Millipore 0.45 μ filter). DDS-NOH was obtained by evaporating the filtrate, which was pale yellow in color, on a rotary evaporator. Identification of the product was confirmed by proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra on a General Electric QE-300 spectrometer. All chemical shifts are reported as δ values referenced to tetramethylsilane (TMS) and gave the following proton resonances: (DMSO/TMS) δ 8.98 (s, 1H, NHOH); 8.68 (s, 1H, NHOH); 7.61 (d, 2H, aromatic H-2 and H-6); 7.48 (d, 2H, aromatic H-2′ and H-6′); 6.86 (d, 2H, H-3, and H-5); 6.60 (d, 2H, H-3′, and H-5′); 6.04 (s, 2H, NH_2). The corresponding carbon resonances were also observed: (DMSO/TMS) δ 129.18 (aromatic C-2 and C-6); 123.31 (aromatic C-3 and C-5); 113.27 (aromatic C-2′ and C-6′); 111.91 (aromatic C-3′ and C-5′). The product was further identified by infrared (IR) spectra (KBr pellet) recorded on a Nicolet 5DXB FT-IR spectrophotometer using polystyrene as reference, yielding the following resonances: 3482 and 3422 (benzylic amine); 3302 (NHOH); 1629 and 1596 (aromatic); 1270, 1137, 1104 (SO_2). The purity of the product was determined by the high performance liquid chromatography (HPLC) method described below. It was found to contain 3% dapsone.

Synthesis of MADDS-NOH. MADDS-NOH was synthesized using a modification of the method described by Jackson (1940). An ammonium chloride solution (41.7 mmol) in 25 ml water was added to a solution containing 3.12 mmol of 4-acetamidophenyl-4′-nitrophenyl sulfone in 150 ml of absolute ethanol at 50°C. Zn dust (1.53 g) was then added to the mixture over a period of 3 min with constant stirring at 50°C. The reaction was allowed to proceed for 25 min, and the mixture was then cooled in ice water for 30 min, filtered, and washed with cold 70% ethanol. The filtrate was concentrated to about 60 ml on a rotary evaporator at 40–45°C, and the resulting mixture was filtered and concentrated to about 5 ml. The mixture was refrigerated at 4°C overnight after which the precipitate was collected by filtration and washed with 2 to 3 ml of $CHCL_3$:MeOH:NH_4OH (900:200:3). The resulting solid was dried under vacuum. The remaining filtrate was evaporated, washed with 3 to 4 ml of chloroform, and filtered, and the resulting solid was dried under vacuum. The purity of the combined product (MADDS-NOH) was analyzed by HPLC and was found to contain 3% impurity. The total yield was about 50%. The product identity was confirmed by NMR and IR spectroscopy. All chemical shifts are reported as δ values referenced to TMS and gave the following proton resonances: (DMSO/TMS) δ 10.34 (s, 1H, NHCO); 9.11 (s, 1H, NHOH); 8.75 (s, 1H, NHOH); 7.76 (q, 4H, aromatic H-2, H-3, H-5, and H-6); 7.68 (d, 2H, aromatic H-2′ and H-6′); 6.88 (d, 2H, aromatic H-3′ and H-5′); 2.06 (s, 3H, CH_3). The corresponding carbon resonances were also observed: (DMSO/TMS) δ 169.46 (NHCO); 156.10 (C-1); 143.61 (C-1′); 136.59 (C-4); 130.16 (C-4′); 128.996 (C-3 and C-5); 128.38 (C-2 and C-6); 119.26 (C-2′ and C-6′); 111.867 (C-3′ and C-5′); 24.52 ($CO\underline{C}H_3$). The product was further identified by infrared (IR) spectra (KBr pellet) recorded on a Nicolet 5DXB FT-IR spectrophotometer using polystyrene as reference, yielding the following resonances: 3309 (NHOH); 1675, 1596 (aromatic); 1536 ($NHCOCH_3$); 1144, 1104 (SO_2).

MetHgb assay. An aliquot (50 μl) of blood was hemolyzed by the addition of 3.95 ml of distilled water, followed by addition of 4 ml of phosphate buffer (pH 7.4). MetHgb content in the hemolysate was determined according to the method of Fairbanks and Klee (1987). The reproducibility of the MetHgb assay, measured as the coefficient of variation, was 6.7% ($n = 4$).

HPLC assay. The HPLC assay was that described by Coleman *et al.* (1989). The elution was conducted on a Waters μBondapak C_{18} column (Milford, MA) using a mobile phase of water:acetonitrile:acetic acid:triethylamine (80:20:1:0.05 v/v/v/v) at 1.2 ml/min. Detection was by uv absorbance (254 nm) and peak area was quantitated with an electronic integrator (Hewlett Packard 3396A). Sulfamethazine (SMZ) was used as the internal standard. The retention times of SMZ, DDS-NOH, DDS,

MADDS-NOH, and MADDS were 7.8, 9.8, 12.0, 12.5, and 15 min, respectively.

Stability of hydroxylamine metabolites. The stability of DDS-NOH and MADDS-NOH (10 μM) in phosphate buffer (pH 7.4), with or without ascorbic acid (1 mM), was determined at room temperature. This was essential to determine the requirements for sample preparation and storage. To 10 ml of metabolite solutions, 100 μl of SMZ (6 mM) in phosphate buffer was added as an internal standard. Metabolite solutions were analyzed by direct injection onto the HPLC system at regular intervals over a period of 24 hr.

In vitro MetHgb formation. Metabolite solutions were prepared in methanol at desired final concentrations. A 75-μl aliquot of metabolite solution was then added to a clean, dry culture tube and evaporated under a gentle N_2 stream and heat. Glucose was added to whole blood to create a final concentration of 10 mM. A 75-μl aliquot of this whole blood was added to the test tube containing metabolite and incubated at 37°C in a shaker bath. At the completion of each incubation, a 50-μl aliquot was withdrawn and assayed for MetHgb formation.

Effect of ascorbic acid on DDS-NOH-induced MetHgb. The influence of ascorbic acid on DDS-NOH-induced MetHgb formation was determined at various concentrations of ascorbic acid and DDS-NOH. Aqueous solutions of ascorbic acid (0.1, 1.0, 10 mM) were added to test tubes and the water was evaporated under a gentle N_2 stream and heat. Methanol solutions of DDS-NOH were then added to test tubes and the methanol was evaporated under gentle N_2 stream and heat. Rat whole blood containing glucose (10 mM) was added to the test tubes and incubated for 1 hr at 37°C in a shaker bath. Each incubation was carried out in triplicate. At the completion of the 1-hr incubation, a 50-μl aliquot was withdrawn and assayed for MetHgb as described above. Concentration-response curves were analyzed as described below.

Comparison of MADDS-NOH- and DDS-NOH-induced production of MetHgb in rat and human blood. To assess the relative potency and efficacy of DDS-NOH and MADDS-NOH, MetHgb formation at concentrations ranging from 1 μM to 40 mM was determined for both metabolites in rat and human blood. Each incubation was carried out in quadruplicate for 1 hr as described previously. The mean MetHgb content of quadruplicate incubations at each concentration was used to develop concentration response curves for each subject.

Modeling. The concentration response data from each subject were analyzed by fitting the mean data for replicate samples to both the E_{max} (Eq. (1)) and the Sigmoid E_{max} models (Eq. (2)) using a nonlinear least squares regression program (SIGMAPLOT). The E_0 values used in studies with rat whole blood were the MetHgb content determined in control blood from each rat. For the experiment examining the effect of ascorbic acid on MetHgb, a value of 3% (the average MetHgb content in the rat) was used for E_0 (Coleman *et al.*, 1990b,c, 1991). For the human studies, the average MetHgb content in normal healthy subjects (1%) was used for E_0 in all calculations (Coleman *et al.*, 1991).

$$E = E_0 + [E_{max} * C]/[EC_{50} + C] \quad (1)$$

$$E = E_0 + [E_{max} * C^n]/[EC_{50}^n + C^n] \quad (2)$$

E_{max}, maximum MetHgb response (expressed as % of Hgb present as MetHgb), EC_{50}, concentration which produces an effect which is 50% of E_{max}; E_0, baseline MetHgb content expressed as a percentage of total Hgb; C = molar concentration of metabolite; n = Hill coefficient.

Time-response curves. Differences in the rate of MetHgb formation by the two metabolites were examined by preparing test tubes containing 1 mM DDS-NOH or MADDS-NOH in groups of six. Rat whole blood was incubated for 8 hr with these metabolites under mild shaking at 37°C. Fifty-microliter aliquots were then withdrawn at various time points and assayed for MetHgb as described previously. Area under the curve ($AUC_{0-8\ hr}$) was calculated for each set of time-concentration data using the trapezoidal rule.

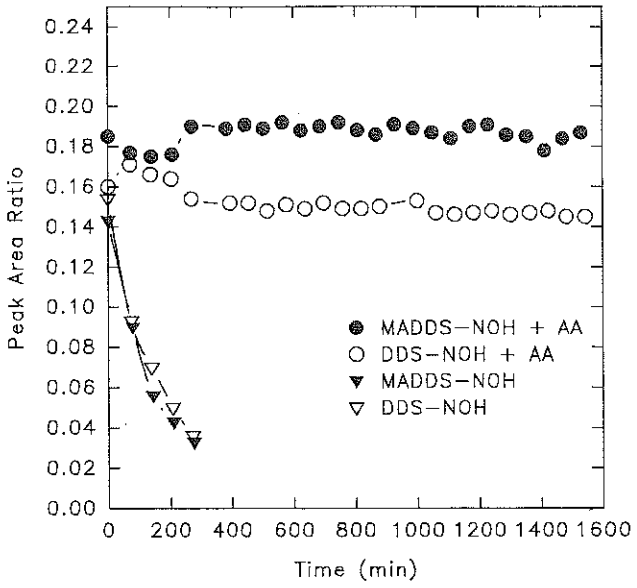

FIG. 1. Stability of DDS-NOH and MADDS-NOH in buffer with and without ascorbic acid. Initial concentrations of hydroxylamines was 10 μM while that of ascorbic acid was 1 mM.

Statistical analysis. All data are presented as mean (±SD) unless otherwise stated. E_{max} and EC_{50} values generated for each metabolite were compared statistically using the paired t test. The differences in the area under the curve ($AUC_{0-8\ hr}$) of MetHgb formation by the hydroxylamine metabolites as a function of time were analyzed using paired t test. A value of $p < 0.05$ was considered significant.

RESULTS

Stability of hydroxylamine metabolites. Figure 1 illustrates the stability of the hydroxylamine metabolites in buffer with and without ascorbic acid. In the absence of ascorbic acid, DDS-NOH and MADDS-NOH degrade, both compounds exhibiting a half-life of 120 min. Ascorbic acid prevents the degradation of these metabolites. While the data in Fig. 1 indicate stabilization for up to 1600 min, repeat studies indicate variable stability after 8 hr (data not shown). At concentrations higher than 1 mM, ascorbic acid interferes with the HPLC assay for the hydroxylamine metabolites; therefore the influence of higher concentrations of ascorbic acid on the stability of DDS-NOH and MADDS-NOH was not examined. Moreover, no extraneous peaks appeared on the HPLC chromatograph of samples with substantial degradation.

Effect of ascorbic acid on DDS-NOH-induced MetHgb. Figure 2 illustrates the effect of ascorbic acid on DDS-NOH-induced MetHgb formation. The mean E_{max} values generated using the E_{max} model are shown in Table 1. The E_{max} value in the presence of 10 mM ascorbic acid was signif-

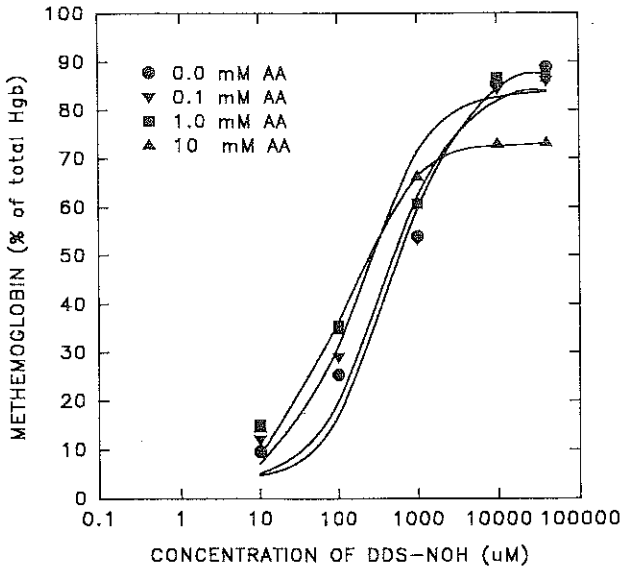

FIG. 2. Concentration-response curve for methemoglobin formation induced by DDS-NOH in rat whole blood in the presence and absence of various concentrations of ascorbic acid.

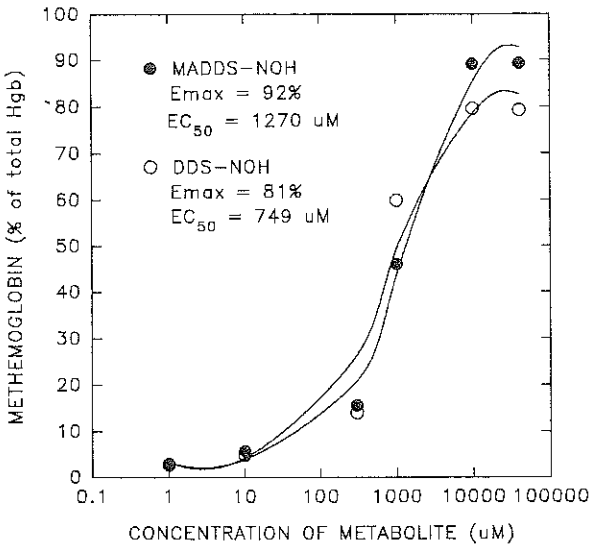

FIG. 3. Representative concentration-response curve for DDS-NOH and MADDS-NOH in rat whole blood fitted to the E_{max} model. Solid lines represent the computer generated fit.

icantly lower than in incubations with lower concentrations of ascorbic acid. This observation suggests that ascorbic acid reduces the efficacy of DDS-NOH to cause MetHgb formation. If efficacy is reduced, the concentration which produces 50% of the E_{max} will also be reduced, since E_{max} itself has been reduced. However, this reduction will occur as a function of reduced efficacy and not potency. Therefore, it is not valid to assess potency using EC_{50} when efficacy is changed. In this situation, it is more appropriate to compare potency by assessing the concentration necessary to achieve a given magnitude of effect in both control and treatment conditions. Therefore, we chose to determine the $EC_{42.9}$ values, the concentration resulting in 42.9% MetHgb (the value that equals 50% of E_{max} in the absence of ascorbic acid), to compare potencies. The $EC_{42.9}$ in the presence of 10 mM ascorbic acid was statistically different from the control. This observation suggests that at high concentrations, ascorbic acid enhances the potency of DDS-NOH.

TABLE 1
Effect of Ascorbic Acid on DDS-NOH-Induced Methemoglobin Formation *in Vitro*

Ascorbic acid (mM)	E_{max} (%)[a]	$E_{42.9}$ (μM)[a]
0.0	86 (5)	458 (162)
0.1	82 (4)	373 (107)
1.0	81 (2)	188 (58)
10	70 (2)*	148 (28)*

[a] Data presented as means (SD) of triplicate incubations.
*, $p < 0.05$ compared with values obtained at 0.0 mM ascorbic acid.

Comparison of MADDS-NOH and DDS-NOH production of MetHgb in rat blood. The concentration-response curves for DDS-NOH- and MADDS-NOH-induced formation of MetHgb in rat blood were fitted to both the E_{max} (Fig. 3) and the Sigmoid E_{max} (Fig. 4) models. The pharmacodynamic parameters for the two metabolites generated using the E_{max} or Sigmoid E_{max} models are shown in Table 2. Both models generated similar E_{max} values, but the EC_{50} values generated from Sigmoid E_{max} were slightly less. The EC_{50} and the E_{max} values did not differ significantly between metabolites using either pharmacodynamic model.

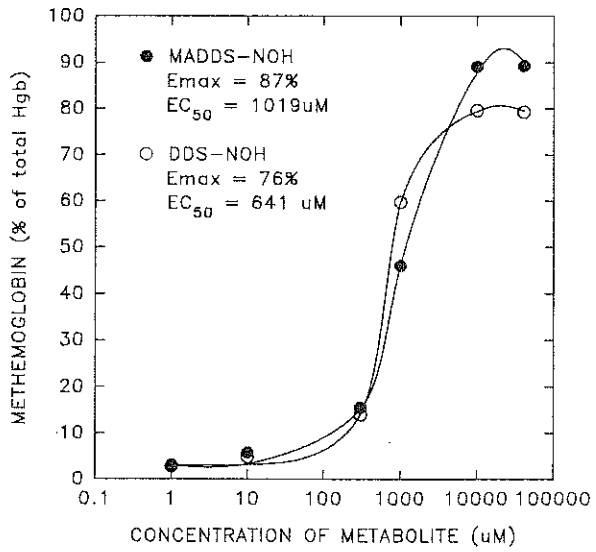

FIG. 4. Representative concentration-response curve for DDS-NOH and MADDS-NOH in rat whole blood fitted to the Sigmoid E_{max} model. Solid lines represent the computer-generated fit.

TABLE 2
Results of Pharmacodynamic Modeling of Methemoglobin Formation in Rat and Human Whole Blood

Source	Model	E_{max} (%)[a]		EC_{50} (μM)	
		MADDS-NOH	DDS-NOH	MADDS-NOH	DDS-NOH
Rat	E_{max}	83 (8)	84 (2)	1087 (283)	828 (104)
	Sigmoid E_{max}	78 (7)	79 (2)	845 (178)	681 (60)
Human	E_{max}	79 (5)	80 (2)	90 (17)	95 (19)
	Sigmoid E_{max}	78 (5)	81 (4)	88 (17)	98 (14)

[a] Data presented as means (SD).

Comparison of MADDS-NOH and DDS-NOH production of MetHgb in human blood. Representative concentration-response curves for the two metabolites, together with the model generated curves, in blood donated from human subjects is shown in Figs. 5 and 6. Data from each human subject were also fitted to both the E_{max} (Fig. 5) and the Sigmoid E_{max} models (Fig. 6). Unlike the rat species, both models generated similar E_{max} and EC_{50} values. The EC_{50} and the E_{max} values determined for DDS-NOH and MADDS-NOH did not differ (Table 2).

Time-response in rat blood. Figure 7 illustrates the time course for MetHgb formation induced by DDS-NOH and MADDS-NOH in rat whole blood *in vitro*. Peak MetHgb content for both metabolites occurred at approximately 100 min after initiation of the incubation. Neither the rate nor the magnitude of MetHgb formation differed significantly between the two metabolites. $AUC_{0-8\ hr}$ measured for each curve are shown in Table 3. The $AUC_{0-8\ hr}$ values determined for DDS-NOH and MADDS-NOH did not differ significantly.

DISCUSSION

Hydroxylamine metabolites have been proposed as mediators for a wide variety of toxicities associated with arylamine xenobiotics (Uetrecht, 1992). Methemoglobin formation represents a convenient pharmacodynamic response to develop models for comparison of hydroxylamine potency and efficacy *in vitro*. Recent studies have reported determinants of methemoglobin formation as mediated by DDS-NOH (Coleman *et al.*, 1989, 1990a,b,c; Coleman and Tingle, 1992; Grossman and Jollow, 1988). However, after DDS administration, two hemotoxic metabolites are formed (DDS-NOH and MADDS-NOH). There is conflicting data in the literature regarding the relative potency of the metabolites in inducing hematologic toxicity (Israili *et al.*, 1973; Scott and Rasbridge, 1973; Grossman and Jollow, 1983). The studies described in the present re-

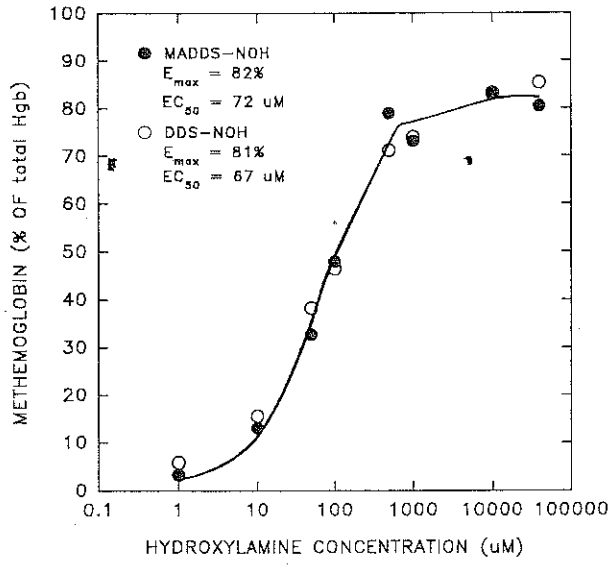

FIG. 5. Representative concentration-response curve for DDS-NOH and MADDS-NOH in human whole blood fitted to the E_{max} model. Solid lines represent the computer-generated fit.

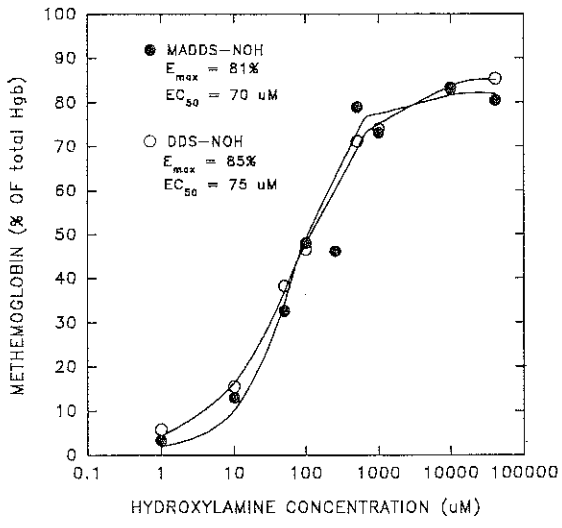

FIG. 6. Representative concentration-response curve for DDS-NOH and MADDS-NOH in human whole blood fitted to the Sigmoid E_{max} model. Solid lines represent the computer-generated fit.

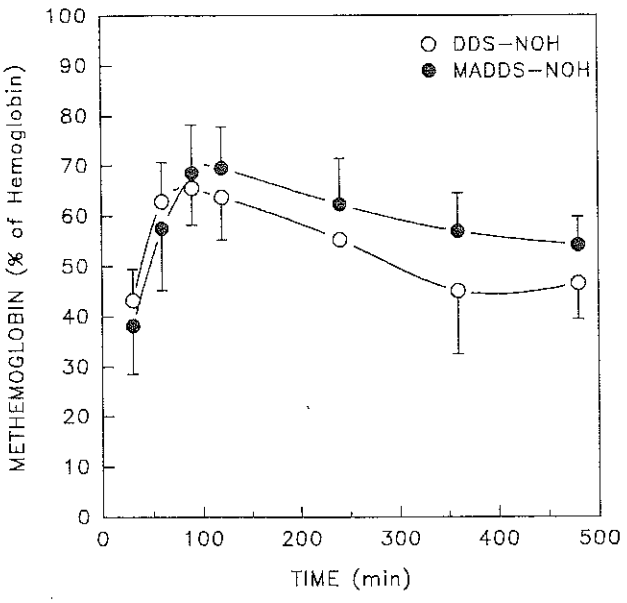

FIG. 7. Time-response relationship for methemoglobin formation rat whole blood in the presence of DDS-NOH and MADDS-NOH. Whole blood was incubated in the presence of 1 mM of the hydroxylamine and 50 μl withdrawn at times indicated.

port were conducted to assess the relative potency and efficacy of these two metabolites in producing MetHgb.

Knowledge about the stability of the hydroxylamine metabolites is important in order to determine incubation conditions and storage requirements for subsequent experiments. Our observation indicates that degradation of these metabolites is quite rapid in phosphate buffer at pH 7.4. However, since both metabolites exhibit a similar degradation rate, the stability of these two metabolites is not a factor which will influence the assessment of their relative potencies. Ascorbic acid at a concentration 100 times that of hydroxylamine metabolites stabilizes these compounds for at least 8 hr.

This stability profile for DDS-NOH is similar to that observed by Grossman and Jollow (1988). These investigators measured DDS-NOH via *in vitro* conversion to the nitroso analog and subsequent HPLC analysis. Thus, their assay measured the sum of the arylhydroxylamine and the nitroso derivative. These investigators observed a half-life of 90 min when DDS-NOH was incubated in a rat RBC suspension or phosphate-buffered saline, which is similar to the 120 min half-life observed in the present study. Hence, oxidation of the arylhydroxylamines to their respective nitroso derivative does not appear to be the cause of the observed instability.

In an earlier study, ascorbic acid was found to reduce xenobiotic induced methemoglobinemia (Bolyai *et al.*, 1972). These investigators found that ascorbic acid consistently reduced the methemoglobinemia induced by sodium nitrite, hydroxylamine, and phenylhydroxylamine. The present study utilized a similar concentration range for ascorbic acid and found that only high concentrations of ascorbic acid lowered the DDS-NOH-induced methemoglobin formation.

Methemoglobin content represents the balance between co-oxidation of the hydroxylamine and hemoglobin and reduction of MetHgb by methemoglobin reductase (Kiese, 1966). The hydroxylamine is co-oxidized to generate a nitroso derivative and methemoglobin. The nitroso derivative is subsequently believed to be reduced by GSH, which in turn is maintained by the hexose monophosphate shunt via NADPH. Methemoglobin is reduced by methemoglobin reductase in the presence of NADH. We have shown that ascorbic acid stabilizes the hydroxylamine metabolites for at least 8 hr in buffer. It is possible that reduction of the nitroso derivative by ascorbic acid or prevention of other chemical reactions of hydroxylamine by ascorbic acid in whole blood increases the hydroxylamine available for oxidizing hemoglobin to methemoglobin. Therefore, less hydroxylamine metabolite is required to cause a given degree of methemoglobinemia in the presence of ascorbic acid than in its absence.

Bolyai *et al.* (1972) also observed that in the presence of high concentrations of ascorbic acid, sodium nitrite-induced methemoglobin levels were reduced at a faster rate than in control. This observation suggests that at high concentrations, ascorbic acid enhances methemoglobin reductase activity, which would result in the observed reduction in efficacy of DDS-NOH. Hence, our observations are consistent with those made by other investigators examining different arylhydroxylamines.

As with other aromatic hydroxylamines, DDS-NOH and MADDS-NOH are believed to undergo redox cycling. To date, there is little published data which support the pres-

TABLE 3
Area under the Time-Response Curve for Methemoglobin Formation in Whole Rat Blood *in Vitro*

	$AUC_{0-8\ hr}$ (%/hr)	
Incubation	MADDS-NOH	DDS-NOH
1	452	457
2	403	423
3	505	369
4	490	450
5	490	402
6	429	374
Mean	462	413
SD	40	37

Note. Whole blood was incubated in the presence of 1 mM of hydroxylamine metabolite and aliquots were withdrawn at various intervals for measurement of MetHgb content. AUC was determined using the linear trapezoidal rule.

ence of such cycling. Coleman and Jacobus (1993) recently demonstrated that from a starting ratio of DDS-NOH to Hgb of 1:16, >30% of the Hgb was oxidized in 5 min. They estimated that one molecule of DDS-NOH may have reacted with up to five molecules of Hgb, an observation which supports redox cycling of the hydroxylamine. Unambiguous demonstration of recycling for DDS-NOH and MADDS-NOH will require analysis of all species in the redox cycling and will probably be best accomplished using purified Hgb. To date, we have been unsuccessful in isolating autooxidation products these hydroxylamines. Assessment of the mechanism of ascorbate reduction of the efficacy of hydroxylamine-induced MetHgb formation will require similar methodology.

The present study is the first in which a quantitative assessment of the relative pharmacodynamics of DDS-NOH and MADDS-NOH has been investigated. Two different pharmacodynamic models, the E_{max} and Sigmoid E_{max}, have been used to analyze the data. These pharmacodynamic models are empirical descriptions of the concentration-response relationship. The E_{max} model is a simpler model based on hyperbolic relationship between concentration and response. The Sigmoid E_{max} model is based on the empirically derived Hill equation and defines the steepness of the concentration-response relationship before saturation. If the steepness can be defined by a hyperbolic relationship, the Sigmoid E_{max} model collapses to E_{max} model. Since the relationship between the models and molecular events involved in methemoglobin formation are neither assumed nor deduced for the fitting of data to the models, we examined both the E_{max} and the Sigmoid E_{max} models. DDS-NOH and MADDS-NOH were found to be equipotent when analyzed by either model. These hydroxylamine metabolites produced a similar maximal methemoglobin response of approximately 80% in both rat and human blood.

Interestingly, the hydroxylamine metabolites were 10-fold more potent in generating methemoglobin in human blood compared to rat blood. We are unaware of similar comparisons with other MetHgb-inducing arylhydroxylamines. This difference may be due to the relative efficiency of regenerating the hydroxylamine amine metabolite from the nitroso form upon oxidation of hemoglobin, the activity of methemoglobin reductase in the two species, or differences in protein binding (which would influence RBC arylhydroxylamine content). Studies with purified hemoglobin from each species may be useful in elucidating the mechanism for this difference. Since the rat is widely used as a model to study the hemotoxicity of xenobiotics, this observation warrants further investigation.

In the above investigations, we chose to examine the extent of MetHgb formation as opposed to the rate of formation. Since DDS and MADDS possess relatively long half-lives, reduction in MetHgb levels produced by their hydroxylamine metabolites will be dependent upon removal of the source of the hydroxylamine metabolites (DDS and MADDS). Hence, for this agent, extent of Hgb formation appears to be the more relevant measurement and was, therefore, the primary focus of our investigations.

The time course of methemoglobin formation by MADDS-NOH and DDS-NOH was first investigated in a limited study by Scott and Rasbridge (1973). They observed a significant difference in the rate of methemoglobin formation between the metabolites in isolated human RBCs, with MADDS-NOH producing methemoglobin more rapidly and to a greater extent. However, the rate of decline after reaching a peak methemoglobin content was also more rapid with MADDS-NOH. In the present study, we observed that both metabolites produced methemoglobin at the same rate, reaching similar peak concentrations, and the rate of decline was also similar with both metabolites. Using the area under the methemoglobin content versus time curve ($AUC_{0-8\ hr}$) as a measure of methemoglobin "exposure," no difference was found between DDS-NOH and MADDS-NOH incubations.

Methodological differences may explain the difference in the observations of the present study and those reported previously. In the investigation by Scott and Rasbridge (1973), the methemoglobin response was determined in isolated human RBCs, whereas in the present study, rat whole blood was used. If MADDS-NOH were more significantly bound to plasma proteins (which may reduce the amount equilibrating into RBCs in whole blood) than DDS-NOH, a potency difference may be masked by our use of whole blood compared with isolated RBCs. Unfortunately, while previous studies indicate that MADDS is more extensively bound than DDS, the extent of protein binding of MADDS-NOH and DDS-NOH is unknown. Thus, the validity of this explanation cannot be assessed with available data. However, we believe that whole blood incubations are more representative of metabolite effect following systemic exposure than those with isolated RBCs.

In summary, we have demonstrated that DDS-NOH and MADDS-NOH are equipotent and equally efficacious in their ability to induce methemoglobin formation in rat and human whole blood *in vitro*. These data indicate that both metabolites may make a significant contribution to DDS-induced toxicity. Studies examining the relative formation and disposition of these metabolites should provide useful additional information in assessing the contribution of these metabolites to DDS-induced toxicity. Furthermore, the observation that DDS-NOH and MADDS-NOH are 10-fold more potent in producing MetHgb in human RBCs than in rat RBCs warrants further investigation.

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