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Selective Effects of Nitric Oxide on the Disposition of Chlorzoxazone and Dextromethorphan in Isolated Perfused Rat Livers

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Running Title: Effects of NO on Chlorzoxazone and Dextromethorphan Disposition

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ABBREVIATIONS: P450, cytochrome P450; NO, nitric oxide; V_{max}, maximum velocity of metabolism; K_m, Michaelis-Menten constant; IPRL, isolated perfused rat liver; SNP, sodium nitroprusside; ISDN, isosorbide dinitrate; CZX, chlorzoxazone; DEM, dextromethorphan; HCZX, 6-hydroxychlorzoxazone; DOR, dextrorphan; MOM, 3-methoxymorphinan; HOM, 3-hydroxymorphinan; ALT, alanine aminotransferase; AST, aspartate aminotransferase; $AUC_{perfusate}$, area under the outlet perfusate-concentration time curve; E, hepatic extraction ratio; C_{in}, inlet concentration; C_{out}, outlet concentration; CL_h, hepatic clearance; CL_{int}, intrinsic clearance; Q, liver perfusion flow rate; $D_{HCZX}^{Perfusate}$, amount of HCZX recovered in the outlet perfusate; CL_{bile}, biliary clearance; D_{HCZX}^{Total}, total amount of HCZX recovered in the outlet perfusate, bile, and liver tissue.

Abstract:

The rapid and direct effects of nitric oxide (NO) donors sodium nitroprusside (SNP) and isosorbide dinitrate (ISDN) on the hepatic and biliary disposition of chlorzoxazone (CZX), a marker of CYP2E1, and dextromethorphan (DEM), a marker of CYP2D1, were studied in a single-pass isolated perfused rat liver model. Livers (n = 30) were perfused with constant concentrations of NO donors (0-120 min) in addition to infusion of CZX or DEM (60-120 min), and periodical outlet and bile samples were collected. Both ISDN and SNP significantly reduced (30% and 60%, respectively) the hepatic extraction ratio of CZX and decreased (50% and 70%, respectively) the recovery of the CYP2E1-mediated metabolite, 6-hydroxychlorzoxazone, in the outlet perfusate and bile. As for DEM, both NO donors increased (up to 3.5-fold) the recovery of the CYP2D1-mediated metabolite dextrorphan (DOR) in the outlet perfusate. However, this was associated with a simultaneous decline (50-75%) in the excretion of the metabolite into the bile, thus resulting in no change in the overall recovery of DOR as a result of NO donor treatment. The decrease in the biliary excretion of DOR was due to NO-induced simultaneous reductions in both the conjugation of DOR and biliary clearance of DOR conjugate. Additionally, both SNP and ISDN significantly reduced the metabolism of DEM to 3-hydroxymorphinan, which is mostly regulated by CYP3A2. These studies in an intact liver model confirm the selectivity of the inhibitory effects of NO donors on P450 enzymes, which was recently reported in microsomal studies, and expand these inhibitory effects to conjugation pathways.

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Cytochrome P450 (P450) enzymes are a major class of heme-containing proteins that participate in the biotransformation of xenobiotics. Studies have shown that the ability of the liver to metabolize drugs is compromised in the presence of infectious diseases or disease states associated with inflammation (Morgan, 1997; Renton, 2001; Renton, 2004; Riddick et al., 2004; Ling and Jamali, 2005). Large quantities of cytokines and nitric oxide (NO) released during inflammation are implicated as the major mediators for the observed down regulation of P450 activities and enzyme levels (Morgan, 1997). The role of cytokines in this down regulation is relatively well established; they are known to inhibit drug metabolism by acting at the level of gene transcription (Ghezzi et al., 1986; Warren et al., 1999). However, the role of NO is still subject to controversy (Sewer and Morgan, 1998), although it has been proposed that NO acts at both transcriptional and post-translational levels (Khatsenko et al., 1993; Wink et al., 1993; Minamiyama et al., 1997).

Very recently (Vuppugalla and Mehvar, 2004a), we reported that the effects of NO on P450 are rapid, concentration-dependent, and enzyme-selective. Additionally, we also showed that the effects of NO are time-dependent, consisting of both reversible and irreversible components (Vuppugalla and Mehvar, 2004b). Further studies (Vuppugalla and Mehvar, 2005) indicated that the inhibitory effects of NO on P450 enzymes are mediated through selective alterations in the V_{max} and/or K_m of various enzymes.

A persistent observation in all of our studies (Vuppugalla and Mehvar, 2004a; Vuppugalla and Mehvar, 2004b; Vuppugalla and Mehvar, 2005) was that although the activities of all the studied enzymes were altered by exposure to NO, CYP2D1 activity was not changed. However, all of these studies were conducted using microsomal preparations obtained from livers exposed to NO donors. Because during the preparation of microsomes, some of the rapidly reversible interactions of NO with heme, such as the formation of nitrosyl-heme complexes, may be lost

(Wink et al., 1993; Gergel et al., 1997; Vuppugalla and Mehvar, 2004b), our observations in microsomal preparation may not be directly extrapolated to intact liver or animals. Therefore, it is not clear whether the lack of effect of NO on CYP2D1 observed in our microsomal preparations (Vuppugalla and Mehvar, 2004a; Vuppugalla and Mehvar, 2004b; Vuppugalla and Mehvar, 2005) is indeed real or due to the experimental model used. Thus, the aim of the present study was to investigate the effects of NO donors on the hepatic disposition of P450 substrates directly in intact isolated perfused rat livers (IPRLs). The effects of sodium nitroprusside (SNP) and isosorbide dinitrate (ISDN) on the hepatic metabolism and biliary excretion of the CYP2E1 marker chlorzoxazone (CZX) and CYP2D1 marker dextromethorphan (DEM) were examined by continuous perfusion of the livers with NO donors for 2 h and infusion of either CZX or DEM during the second hour. We selected CZX and DEM as model drugs because our previous microsomal studies (Vuppugalla and Mehvar, 2004a; Vuppugalla and Mehvar, 2004b; Vuppugalla and Mehvar, 2005) indicated that CYP2E1 and CYP2D1 activities were the most and least affected by NO, respectively.

Materials and Methods

Chemicals and Reagents. CZX, 6-hydroxychlorzoxazone (HCZX), 7-hydroxycoumarin (umbelliferone), DEM, dextrorphan (DOR), 3-methoxymorphinan (MOM), 3-hydroxymorphinan (HOM), 7-hydroxycoumarin glucuronide, SNP, ISDN, uridine 5'-diphospoglucuronic acid triammonium salt (UDPGA), D-saccharic acid 1,4-lactone monohydrate, and β-glucuronidase (Type L-II, from Limpets) were purchased from Sigma-Aldrich (St. Louis, MO). All the other reagents were of analytical grade and obtained from commercial sources.

Animals. Male Sprague-Dawley rats weighing between 250 to 350 g were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Animals were provided with food and water

ad libitum and maintained in a light-controlled room. Texas Tech Health Sciences Center Animal Care and Use Committee approved the study protocol, and all the animals received humane care in compliance with guidelines set by the National Institute of Health (publication no. 85-23, revised 1985, Bethesda, MD).

Isolated Liver Perfusion. Liver isolation and cannulation methods were similar to those reported by us before (Vuppugalla and Mehvar, 2004a). After the cannulation procedure, livers were immediately mounted on a water-jacketed (37ºC), all glass perfusion system (Radnoti Glass Technology Inc., Monrovia, CA) and perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 4.75 mg/l sodium taurocholate and 1.2 g/l glucose. The perfusate was continuously oxygenated with 95% oxygen and 5% carbon dioxide mixture, and the perfusion pressure was monitored using a pressure transducer. Livers were perfused in a single-pass manner at a flow rate of 30 ml/min and were allowed to stabilize for ~10 min before the initiation of the experiments. All the perfusions were conducted for a duration of 2 h. Viability of the livers was examined by bile flow rates, liver enzyme (ALT and AST) levels, inlet pressure, wet liver weight at the end of perfusion, and macroscopic examination of the liver.

Experimental Design and Sample Collection for IPRL Studies. We used SNP and ISDN as NO donors in this study. Because of the unstable nature of SNP in the presence of light (Baaske et al., 1981), all the SNP perfusions were performed under safelight (Delta 1/CPM, Inc. Dallas, TX). The study consisted of 30 isolated livers divided into two groups of CZX (n = 12) and DEM (n = 18). Livers in the CZX and DEM groups were further subdivided into three subgroups (n = 4-6/group). They were perfused for 2 h either without any NO donor (Control) or with a constant concentration (400 μM) of SNP or ISDN, which produces nitrite/nitrate levels of ~15 and 30 μM, respectively, in the outlet perfusate (Vuppugalla and Mehvar, 2004a). Additionally, during the second hour, livers in the CZX or DEM groups were constantly infused

with ~45 μM of CZX (dissolved in a solvent consisting of one part of saline and two parts of 0.1 N NaOH) or ~2.5 μM of DEM (dissolved in saline), respectively. Inlet samples were drawn at 0 (before drug infusion), 15, 25, 40, and 60 min after the start of substrate infusion to assess the stability of each compound as well as the accuracy of infusions. Outlet samples were collected at 5- (0-30) or 10- (30-60) min intervals. Additional samples were also collected from the outlet at the beginning and end of the perfusion for the analysis of liver viability markers (ALT and AST). Bile samples were also collected in preweighed microcentrifuge tubes at 15-min intervals. Bile and perfusate samples and livers, collected at the end of perfusion, were stored at -80ºC for subsequent analysis of CZX, DEM, and/or their metabolites.

Determination of 7-Hydroxycoumarin Glucuronidation in Liver Homogenates. The effects of NO on the in vitro glucuronidation of 7-hydroxycoumarin, a substrate for UDP glucuronosyl transferase (UDPGT), was studied in the homogenates of the Control and ISDN IPRLs (n = 6/group) based on modifications of a previously reported method (Killard et al., 1996). Briefly, IPRLs were first homogenized in 10 mM phosphate buffer (pH 7.4) and the protein contents measured. The incubation mixture (500 μl) contained liver homogenate (1 mg protein/ml), 7-hydroxycoumarin (100 μM), UDPGA (1.25 mM), magnesium chloride (6.25 mM), and saccharic acid 1,4-lactone (6.25 mM) in 10 mM phosphate buffer (pH 7.4). The samples were incubated at 37^{o}C for 20 min when the reaction was stopped by the addition of 500 μl of 0.4 M perchloric acid. After mixing and centrifugation, the supernatants were subjected to HPLC analysis for measurement of 7-hydroxycoumarin and its glucuronide as described below.

Sample Analysis. The concentrations of CZX and HCZX in biological samples were measured both before and after treatment with β-glucuronidase, using a reported HPLC method (Jayyosi et al., 1995). For measurement of total (free plus conjugated) analyte, 150 μl of 0.2 M

sodium acetate buffer (pH 4.75) containing 1000 units of β-glucuronidase was added to 200 µl of perfusate or 10- or 20-times diluted bile or liver homogenates, respectively. The samples were then incubated in a water-bath shaker maintained at 37ºC for 1 h. After incubation, 50 µl of methanol containing 20 µg/ml of umbelliferone (internal standard) and 7 µl of 70% perchloric acid were added, and samples were vortex-mixed and centrifuged at 21,000 g for 3 min. A total of 200 µl of the supernatant was then injected onto the HPLC system. The same procedure, with the exception of β-glucuronidase incubation step, was used for the analysis of unconjugated species.

Dextromethorphan and its metabolites in perfusate, bile, and liver samples were analyzed as follows: To 250 µl of perfusate or diluted bile (500 times) or liver homogenate (20 times), 150 µl of 0.5 M sodium acetate buffer (pH 4.75) containing 500 (perfusate) or 1000 (bile and liver) units of β-glucuronidase was added. The samples were vortex mixed and incubated in a shaking water-bath maintained at 37ºC for 3 h. At the end of incubation, 5 µl of 70% perchloric acid was added, and the samples were vortex-mixed and centrifuged at 9300 g for 5 min. Finally, a 200 µl aliquot of each sample was injected onto the HPLC system. The HPLC method used for the analysis of the drug and metabolites was based on the modifications (Vuppugalla and Mehvar, 2004a) made to a previously reported method (Yu and Haining, 2001). The levels of unconjugated drug and metabolites were also measured using the same procedure, without the β-glucuronidase incubation step.

The concentrations of 7-hydroxycoumarin and its glucuronide in the supernatants of the liver homogenate incubation mixtures were determined using a gradient HPLC analysis reported before (Killard et al., 1996).

Transaminases enzyme levels in the perfusate were measured using commercially available spectrophotometric kits from Sigma-Aldrich (procedure 505).

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Pharmacokinetic Analysis. Except for the direct determination of 7-hydroxycoumarin glucuronide, the concentrations of conjugated species were estimated by subtracting the unconjugated concentrations in the absence of glucuronidase from total concentrations in the presence of glucuronidase. The areas under the outlet perfusate concentration-time curves ($AUC_{perfusate}$) of the drugs and their metabolites were estimated by the linear trapezoidal rule during the substrate infusion. Hepatic extraction ratio (E) was calculated using the following equation:

$$E = \frac{C_{in} - C_{out}}{C_{in}} \quad (1)$$

where C_{in} and C_{out} are the inlet and steady-state outlet concentrations of the drug. For C_{in}, the average concentration of inlet samples taken at 25, 40, and 60 min was used. Similarly, C_{out} was calculated from the last six (CZX) or 3 (DEM) outlet samples. The hepatic (CL_h) and intrinsic (CL_{int}) clearance values were then calculated using the following equations, based on the well-stirred liver model (Pang and Rowland, 1977):

$$CL_h = E \cdot Q \quad (2)$$

$$CL_{\text{int}} = \frac{CL_h}{1 - E} \quad (3)$$

where Q is the perfusion flow rate (30 ml/min). The amount of parent drug or metabolite recovered in the outlet perfusate during the infusion period was estimated by $AUC_{perfusate} \times Q$. Finally, the biliary clearance (CL_{bile}) of glucuronide conjugates of metabolites was estimated by dividing the amount of the conjugate excreted in the bile during the infusion by the respective $AUC_{perfusate}$ of the conjugate.

Statistical Analysis. All the statistical comparisons for IPRL studies were conducted using ANOVA with subsequent Fisher's test. An unpaired, two-tailed t-test was used to test the

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differences in the rate of glucuronidation of 7-hydroxycoumarin in liver homogenates between the Control and ISDN groups. In all cases, a P value of < 0.05 was considered significant. Data are presented as mean ± S.E.M.

Results

Effects of SNP and ISDN on the Viability of IPRLs. Perfusion of the livers with NO donors did not affect the viability of the livers. The average wet liver weights (3.16-3.46% body weight), bile flow rates during the second hour of perfusion (0.40-0.64 ml/h), perfusate AST (8.04-8.22 U/l) and ALT (11.9-13.4 U/l) levels at the end of perfusion, and inlet perfusion pressure with the constant flow rate (30 ml/min) were not significantly different among the Control, SNP, and ISDN groups.

IPRL Studies with Chlorzoxazone (CYP2E1 Marker). The effects of NO donors on the total (free plus conjugated) concentration-time courses of CZX and its metabolite HCZX in the outlet perfusate are presented in Fig. 1. Whereas the concentrations of CZX were the same in the glucuronidase-treated and untreated samples, the metabolite could be detected only in the treated samples (data not shown), indicating that the metabolite almost exclusively exists in its conjugated form. The concentrations of CZX started to rise within 5 min (first sampling point) and reached steady state in ~15 min in all the studied groups (Fig. 1, top). The steady state outlet concentrations (C_{out}) of CZX in the SNP (41.5 ± 1.2 μM) and ISDN (36.1 ± 1.1 μM) groups were significantly ($P < 0.05$) higher than those in the Control group (30.6 ± 0.3 μM). Similarly, the $AUC_{perfusate}$ values for the Control livers were significantly ($P < 0.01$) lower than those for the SNP or ISDN groups (data not shown).

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The outlet concentrations of HCZX formed from CZX were measurable within 5 min of the parent drug infusion and reached steady state in ~20 min in both the SNP and ISDN groups (Fig. 1, bottom). However, the achievement of steady state was delayed (~30 min) for the Control group (Fig. 1, bottom). In contrast to CZX, the steady state concentrations of HCZX in the SNP (1.41± 0.20 μM) and ISDN (1.99 ± 0.09 μM) groups were substantially lower ($P < 0.01$) than those in the Control livers (4.80 ± 0.47 μM). Additionally, the $AUC_{perfusate}$ values for the Control livers were significantly ($P < 0.01$) higher than those for the SNP or ISDN groups (data not shown).

The effects of NO donors on the hepatic disposition parameters of CZX and its metabolite HCZX are presented in Table 1. The *E* value in the Control group indicates that CZX has a low extraction ratio at the input rate used in our studies. Perfusion with SNP or ISDN resulted in 60% or 30% decline, respectively, in *E,* compared with the Control livers ($P < 0.05$). This was due to an NO-induced reduction in the CL_{int} values (Table 1). As the *E*, CL_{int}, and CL_h values indicate (Table 1), the effects of SNP on the hepatic disposition of CZX appears to be more drastic than those of ISDN. In contrast to these kinetic parameters, the binding of CZX to the liver tissue, as reflected in the C_{liver}:C_{out} ratio, remained unaffected by the NO donor treatment (Table 1).

Consistent with the *E* values of CZX, the amount of HCZX recovered in the perfusate ($D_{HCZX}^{Perfusate}$) and the total amount recovered in the bile, liver, and perfusate (D_{HCZX}^{Total}) were considerably lower in the SNP and ISDN groups ($P < 0.01$), compared with the Control values (Table 1). Additionally, both SNP and ISDN treatments resulted in a 90% increase in the liver tissue: outlet perfusate HCZX concentration ratios (Table 1).

IPRL Studies with Dextromethorphan (CYP2D1 Marker). The outlet perfusate concentration-time courses of total (free plus conjugated) DEM, DOR, MOM, and HOM in

Control livers are presented in Fig. 2. Whereas DEM and MOM were present only as unconjugated moieties, the O-demethylated metabolites DOR and HOM were present both in the free and conjugated forms (data not shown); the latter constituted 38-57% and 51-78% of total DOR and HOM, respectively, in the outlet perfusate, with the proportion progressively increasing during the 60 min of perfusion. The steady-state concentration of DEM in the outlet perfusate of control livers (0.0506 ± 0.0110 μM) was very low (Fig. 2), compared with the inlet concentrations (2.4 ± 0.1 μM), reflective of very high *E* of DEM in this group (0.979 ± 0.005). Among the three measured metabolites, the concentrations of DOR were several fold higher than those of the parent drug, whereas the concentrations of MOM and HOM were low and close to those of DEM (Fig. 2). The outlet concentrations of the metabolites, and in particular those of DOR, continued to rise during the 60-min infusion period (Fig. 2).

The effects of SNP or ISDN on the outlet concentration-time courses and perfusate recovery of DEM and its metabolites are presented in Fig. 3 and Table 2, respectively. The concentrations of DEM and most of its metabolites continued to increase with the perfusion time and, therefore, did not reach steady state in the SNP- or ISDN-treated livers (Fig. 3). Except for HOM, the outlet concentrations (Fig. 3) and recovery (Table 2) of the drug and its metabolites in treated livers were higher than those in Control livers, although in some cases statistical significance was not achieved (Table 2). For HOM, whereas SNP caused a substantial increase, ISDN treatment did not cause any significant changes in the outlet concentrations (Fig. 3) or recovery (Table 2). As for the conjugated metabolites, between 35 to 50% of DOR and 55 to 70% of the HOM recovered in the perfusate (Table 2) were in the conjugated form. Furthermore, similar to Control livers, the majority of the total recovery in the perfusate of ISDN- and SNP-treated livers was due to DOR (Table 2). Overall, SNP and ISDN resulted in > 3- and 2-fold

increases ($P < 0.05$), respectively, in the total amount of the drug and metabolite recovered in the outlet perfusate (Table 2).

In addition to the recovery in the outlet perfusate (Fig. 3, Table 2), substantial amounts of the conjugated metabolites (DOR and HOM) were also detected in bile (Fig. 4). In fact, in Control livers, 77% of total (bile plus perfusate) DOR (Fig. 4, top left) and 79% of total HOM (Fig. 4, bottom left) were present in the bile. Both SNP and ISDN substantially ($P < 0.05$) reduced the amounts of conjugated DOR (75% and 50% reductions, respectively) and HOM (88% and 77% reductions, respectively) excreted into the bile (Fig. 4, left panels). This reduction was due to a significant ($P < 0.05$) reduction in the biliary clearance of both DOR and HOM conjugates in the SNP- and ISDN-treated livers (Fig. 4, right panels). The SNP treatment appeared to have a more drastic effect than ISDN on the biliary clearance values of both DOR and HOM (Fig. 4, right panels), although the differences between the two treatments did not reach statistical significance. Nevertheless, unlike the bile or perfusate recoveries, the total amounts of DOR recovered from the bile and perfusate together were similar ($P > 0.05$) in the Control and NO donor-treated groups (Fig. 4, top left). This, however, was not true for HOM recovery, where a substantial decline in the recovery of the metabolite in the bile was also reflected in the total recovery of the metabolite as a result of SNP or ISDN treatment (Fig. 4, bottom left).

The total (parent drug and metabolites) recoveries of the administered dose of DEM in the free or conjugated forms are presented in Fig. 5. Almost all of the recovery was attributed to those in the bile and outlet perfusate; the contribution of the liver to the total recovery of the drug and metabolites was negligible (<2%, data not shown). The total recovery, which ranged from 55% to 64%, was not significantly affected by the NO donors ($P > 0.05$). However, NO donors resulted in a more recovery of the intact (unconjugated) species; 7%, 36%, and 26% of the administered dose of DEM was recovered as intact drug and metabolites in the Control, SNP, and ISDN groups,

respectively. Additionally, in all the groups, ~80% of the recovered material was accounted for by DOR.

Homogenate Studies with 7-Hydroxycoumarin (UDPGT Marker). The rate of formation of 7-hydroxycoumarin glucuronide from 7-hydroxycoumarin in the homogenates of IPRLs in the Control group was 0.300 ± 0.070 nmol/min/mg protein. Perfusion of the livers with ISDN significantly ($P < 0.5$) reduced the glucuronide formation rate to 0.0771 ± 0.0527 nmol/min/mg protein.

Discussion

Although much has been learned about the direct and rapid inhibitory effects of NO on P450 in vitro (Khatsenko et al., 1993; Wink et al., 1993; Minamiyama et al., 1997), the ex vivo or in vivo relevance of this inhibition is not known. Thus, in the present study, we investigated the direct effects of NO donors on the hepatic disposition of CZX (a CYP2E1 substrate) and DEM (a CYP2D1 substrate) using an IPRL model. Our results show that NO alters the disposition of P450 substrates selectively in the intact rat liver. Whereas the metabolism of CZX to HCZX (CYP2E1) was substantially inhibited by both NO donors (Fig. 1), the formation of DOR from DEM (CYP2D1) was not affected (Fig. 4).

The contrasting effects of NO on the hepatic disposition of CZX and DEM, observed in this study, are in agreement with our previous studies (Vuppugalla and Mehvar, 2004b; Vuppugalla and Mehvar, 2004a) conducted in microsomal preparations. Whereas, NO decreased the activities of several P450 enzymes like CYP3A2, 2C11, 2E1, 1A1/2, and 2B1/2 to varying degrees, it did not change the activity of CYP2D1. Moreover, enzyme kinetic studies (Vuppugalla and Mehvar, 2005) in microsomes showed that pretreatment of IPRLs with NO donors decreased

the CL_{int} of the CYP2E1 enzyme by both decreasing V_{max} and increasing K_m for this enzyme. However, unlike CYP2E1, neither the V_{max} nor the K_m value of CYP2D1 was altered by NO donors. The differential effects of NO on the V_{max} values of these enzymes may be due to differences between the two enzymes in the accessibility of heme and/or cysteine thiolate residues to NO (Gergel et al., 1997). Additionally, because NO reacts with thiol groups of amino acid residues in the apoprotein (Minamiyama et al., 1997; Vuppugalla and Mehvar, 2004b), it may affect the binding of substrates to these enzymes, and therefore impact their K_m values, selectively. This is because the degree of involvement of the thiol-containing cysteine residues in the substrate binding may be different for various P450 enzymes (Vuppugalla and Mehvar, 2005). Indeed, it was recently (Paine et al., 2003) shown that the critical amino acids for binding of CYP2D6 to nitrogen-containing ligands are negatively-charged carboxylate-containing amino acids, such as aspartate 301 and glutamate 216. Therefore, a possible binding of NO with the cysteine amino acids of this enzyme is not expected to affect its substrate binding or K_m.

Although HCZX is considered the only major metabolite of CZX (Conney and Burns, 1960; Peter et al., 1990), the metabolism of DEM is more complex (Fig. 6) (Witherow and Houston, 1999). Studies using specific inhibitors of CYP2E1, such as diallyl sulfide (Chen and Yang, 1996), diethyldithiocarbamate (Court et al., 1997), and CYP2E1 antibodies (Jayyosi et al., 1995) have clearly shown that most of the hydroxylation of CZX in rats is catalyzed by CYP2E1. Therefore, CZX appears to be a suitable model for CYP2E1 in rats.

In contrast to CZX, the metabolism of DEM results in multiple metabolites (Chen et al., 1990; Kerry et al., 1993), such as DOR, MOM, and HOM (Fig. 6). Additionally, the O-demethylated metabolites DOR and HOM are subject to subsequent conjugation (Fig. 6) (Kerry et al., 1993). However, among these metabolites, DOR (intact and conjugated) is by far the major metabolite in rats (Fig. 4 and Table 2). This is because the CL_{int} for DEM O-demethylation to

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DOR is >20 fold higher than that of the alternate pathway resulting in N-demethylation of DEM to MOM. Additionally, the sequential metabolism of DOR to HOM has a 100 fold lower CL_{int} than its formation (Fig. 6). Inhibition studies using CYP2D inhibitors such as quinine, dextropropoxyphene, methadone, and propafenone have confirmed that the metabolism of DEM to DOR is mostly via CYP2D (Kerry et al., 1993). Therefore, based on these data, O-demethylation of DEM to DOR appears to be a suitable marker for CYP2D in rat. The presence and absence of the inhibitory effect of NO donors on the metabolism of CZX to HCZX (Fig. 1) and DEM to DOR (Fig. 4, top left), respectively, are in line with our previous observations (Vuppugalla and Mehvar, 2005) showing that perfusion with NO donors resulted in a significant decline in the Cl_{int} of CYP2E1 substrates without any effect on the metabolism of CYP2D1 substrates.

The alternative pathway for DEM metabolism to MOM through N-demethylation is believed to be mostly through CYP3A enzyme in humans (Yu and Haining, 2001), although definitive proofs in rats are lacking. However, the estimated in vitro CL_{int} values (Fig. 6) indicate that the formed MOM is rapidly converted to HOM in Sprague-Dawley rats (Kerry et al., 1993). Consistent with these findings, the total amount (%dose) of MOM found in our Control IPRLs (0.61%, Table 2) was very low, compared with the recovery of HOM or DOR (~9% and 45%, respectively, Fig. 4).

Despite our previous reports (Vuppugalla and Mehvar, 2005) of NO-induced reduction in CYP3A activity, we did not observe a reduction in the MOM levels as a result of SNP or ISDN treatment in our present study (Table 2). Instead, the amounts of HOM were considerably lower in the NO donor-treated groups (Fig. 4, bottom left). Because both in vitro (Kerry et al., 1993) and in vivo (Chen et al., 1990) studies in rats indicate that HOM is mostly formed from MOM, and not from DOR, with the rate limiting step being the formation of MOM (CYP3A), the NO-induced reductions in HOM levels (Fig. 4, bottom left) may be an indication of a decrease in CYP3A

activity. The reasons for the lack of changes in MOM levels as a result of NO donor treatment in our current study are not clear. However, in agreement with our studies, investigations in humans have also shown that the adjusted levels of HOM or HOM plus MOM, and not MOM alone, are significantly correlated with the midazolam (CYP3A marker) clearance (Kawashima et al., 2002).

A novel finding of our study is that both NO donors significantly reduced the formation of the glucuronide conjugates of DEM metabolites (i.e., DOR and HOM) (Fig. 5). Although the effect of NO on P450 enzymes has been investigated relatively extensively, to our knowledge this is the first study to directly demonstrate an inhibitory effect of NO on the glucuronidation pathway. This effect was further confirmed in our liver homogenates using 7-hydroxycoumarin (see Results), which is a known marker of glucuronidation pathway (Bogan and O'Kennedy, 1996; Killard et al., 1996). The NO-induced decline in glucuronide formation (Fig. 5) may be due a reduction in the activity of UDPGT resulted from an interaction of NO with the critical thiol-containing amino acid residues of the enzyme, similar to that reported between the P450 apoprotein and NO (Minamiyama et al., 1997; Vuppugalla and Mehvar, 2004b).

In addition to the decrease in the formation of DOR and HOM glucuronides (Fig. 5), both NO donors, and in particular SNP, significantly reduced the CL_{bile} of these conjugates (Fig. 4). To date, the effects of NO on the transporters responsible for the biliary efflux of drugs and/or metabolites have not been investigated. The limited data available (Song et al., 2002) with regard to transporters within the sinusoidal membrane indicate that pretreatment of hepatocytes with SNP reduces the functional activity of Na+/taurocholate cotransporting polypeptide (NTCP) without any significant effect on the organic cation transporter (OCT). Further studies are warranted to thoroughly investigate the effects of NO on both the sinusoidal and biliary transporters in the liver.

In our present and previous (Vuppugalla and Mehvar, 2004a; Vuppugalla and Mehvar, 2005) studies on this subject, we used SNP and ISDN, two NO donors with different mechanisms,

sites, and modes of NO release (Feelisch, 1998), to assure that the observed effects are indeed due to the generation of NO and not related to nonspecific effects of these drugs. The previous studies (Vuppugalla and Mehvar, 2004a), which utilized the same model used here (single-pass IPRL), indicated that at equal inlet concentrations of ≥ 400 μM, nitrite/nitrate concentrations in the outlet perfusate samples were ≥ two-fold higher for ISDN than that for SNP. In contrast, the amount of nitrite/nitrate found in the bile of SNP-perfused IPRLs was five-fold higher than that after perfusion of the livers with ISDN. The higher outlet concentrations of nitrite/nitrate in ISDN-treated livers was not associated with a more drastic inhibitory effect on the P450 enzymes (Vuppugalla and Mehvar, 2004a), suggesting that total nitrate/nitrite in the outlet perfusate may not be the best marker for the availability of NO in the hepatocytes. Furthermore, previous studies (Feelisch, 1998) have shown that, in contrast to SNP, the metabolism of ISDN produces nitrite/nitrate directly, independent of the formation of NO. Therefore, the perfusate level of nitrite/nitrate after ISDN is likely an overestimation of the liver exposure to NO. Nevertheless, although quantitatively different, the qualitative similarities between SNP and ISDN on their effects on the disposition of CZX and DEM (Tables 1 and 2 and Figures 1-5) suggest that these effects are due to generation of NO.

Conclusion

Our data in an intact liver confirms the P450 enzyme selectivity of the inhibitory effects of NO, previously observed in microsomal preparations. Whereas the metabolism of a CYP2E1 substrate was substantially reduced by NO donors, that of a CYP2D1 marker was not affected. Further studies are needed to determine the relevance of these findings in disease states associated with increased NO release and/or after therapy with NO releasing drugs.

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LEGENDS FOR FIGURES

FIG. 1. The outlet perfusate concentration-time courses of chlorzoxazone (top panel) and 6-hydroxychlorzoxazone (bottom panel). Isolated rat livers were continuously perfused with a buffer free of NO donor (Control) or containing 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of chlorzoxazone (constant infusion of ~45 μM) and its CYP2E1-generated metabolite 6-hydroxychlorzoxazone was studied during the second hour (*n* = 4/group). The symbols and bars represent the average and S.E.M values, respectively.

FIG. 2. The outlet perfusate concentration-time courses of total (free plus conjugated) dextromethorphan (DEM), dextrorphan (DOR), 3-methoxymorphinan (MOM), and 3-hydroxymorphinan (HOM) in Control livers. Isolated rat livers were infused with constant concentrations (~ 2.5 μM) of DEM for 60 min (*n* = 6), and outlet samples were analyzed after incubation with β-glucuronidase. The symbols and bars represent the average and S.E.M values, respectively.

FIG. 3. The outlet perfusate concentration-time courses of total (free plus conjugated) dextromethorphan (top left panel), methoxymorphinan (top right panel), dextrorphan (bottom left panel), and hydroxymorphinan (bottom right panel) in Control (open circles), ISDN (closed circles), and SNP (inverted triangles) groups. Isolated rat livers were continuously perfused with a buffer free of NO donor (Control) or containing 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of dextromethorphan (constant infusion of ~2.5 μM) and its CYP2D1-generated metabolite dextrorphan and two other metabolites was studied during the second hour (*n* = 6/group). The symbols and bars represent the average and S.E.M values, respectively.

FIG. 4. Recovery of total (free plus conjugated) metabolites from outlet perfusate and bile (left panels) and biliary clearance of the conjugated metabolites (right panels) for dextrorphan (top panels) and hydroxymorphinan (bottom panels). Isolated rat livers were continuously perfused with a buffer free of NO donor (Control) or containing 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of the CYP2D- (dextrorphan) and CYP3A- (hydroxymorphinan) generated metabolites of DEM was studied during the second hour by infusing the livers with ~ 2.5 μM of dextromethorphan (n = 6/group). *, and **, significant differences ($P < 0.05$, ANOVA) between treated and Control livers and between SNP and ISDN groups, respectively, in terms of total (perfusate plus bile) metabolite or biliary clearance. Columns and bars represent the average and S.E.M. values, respectively.

FIG. 5. The total (parent drug and metabolites) recoveries of the dose of dextromethorphan in the free or conjugated form. Isolated rat livers were continuously perfused with a buffer free of NO donor (Control) or containing 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of dextromethorphan was studied during the second hour by infusing the livers with ~ 2.5 μM of the substrate (n = 6/group). Columns and bars represent the average and S.E.M. values, respectively.

FIG. 6. Major metabolic pathways of dextromethorphan. The numbers associated with arrows are in vitro intrinsic clearance values (ml/min/mg protein) reported for each reaction in the Sprague-Dawley rat microsomes (Kerry et al., 1993).

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TABLE 1

Effect of NO donors on the hepatic disposition of CZX and HCZX

Isolated rat livers were continuously perfused in the absence (Control) or presence of 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of CZX (constant infusion of ~45 μM) and the amount (D) and concentration (C) of its CYP2E1-generated metabolite HCZX was studied during the second hour (n = 4/group).

	Control	SNP	ISDN
CZX			
E	0.265 ± 0.018	0.0908 ± 0.0170[a]	0.188 ± 0.017[a,b]
CL_{int}, ml/min/g	1.06 ± 0.15	0.366 ± 0.069[a]	0.752 ± 0.049[b]
CL_h, ml/min/g	0.773 ± 0.099	0.330 ± 0.058[a]	0.608 ± 0.027[b]
C_{liver}:C_{out}	9.36 ± 0.32	6.92 ± 0.28	8.53 ± 1.17
HCZX			
$D_{HCZX}^{Perfusate}$, μmol	7.29 ± 0.56	2.36 ± 0.28[a]	3.38 ± 0.13[a]
D_{HCZX}^{Total}, μmol	8.81 ± 0.59	2.71 ± 0.39[a]	4.74 ± 0.25[a,b]
C_{liver}:C_{out}	2.73 ± 0.29	5.20 ± 0.43[a]	5.09 ± 0.34[a]

Values are expressed as mean ± S.E.M.

[a] Significantly different from Control: ANOVA, followed by Fisher's test.

[b] Significantly different from SNP: ANOVA, followed by Fisher's test.

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TABLE 2

Effects of SNP or ISDN on the recovery of dextromethorphan (DEM) and its metabolites in the outlet perfusate

Isolated rat livers were continuously perfused in the absence (Control) or presence of 400 μM of SNP or ISDN for 2 h. The influence of NO donors on the hepatic disposition of DEM (constant infusion of ~2.5 μM) and its CYP2D1-generated metabolite DOR and two other metabolites was studied during the second hour (n = 6/group).

	Control	SNP	ISDN
	Recovery (% dose)		
DEM	1.4 ± 0.3	11 ± 5	14 ± 5[a]
DOR	10 ± 2	35 ± 5[a]	17 ± 3[b]
MEM	0.61 ± 0.20	2.0 ± 0.8	1.4 ± 0.4
HOM	2.0 ± 0.3	5.3 ± 0.9[a]	1.1 ± 0.2[b]
Total	15 ± 2	53 ± 6[a]	34 ± 7[a,b]

Values are expressed as mean ± S.E.M.

[a] Significantly different from Control: ANOVA, followed by Fisher's test.

[b] Significantly different from SNP: ANOVA, followed by Fisher's test.

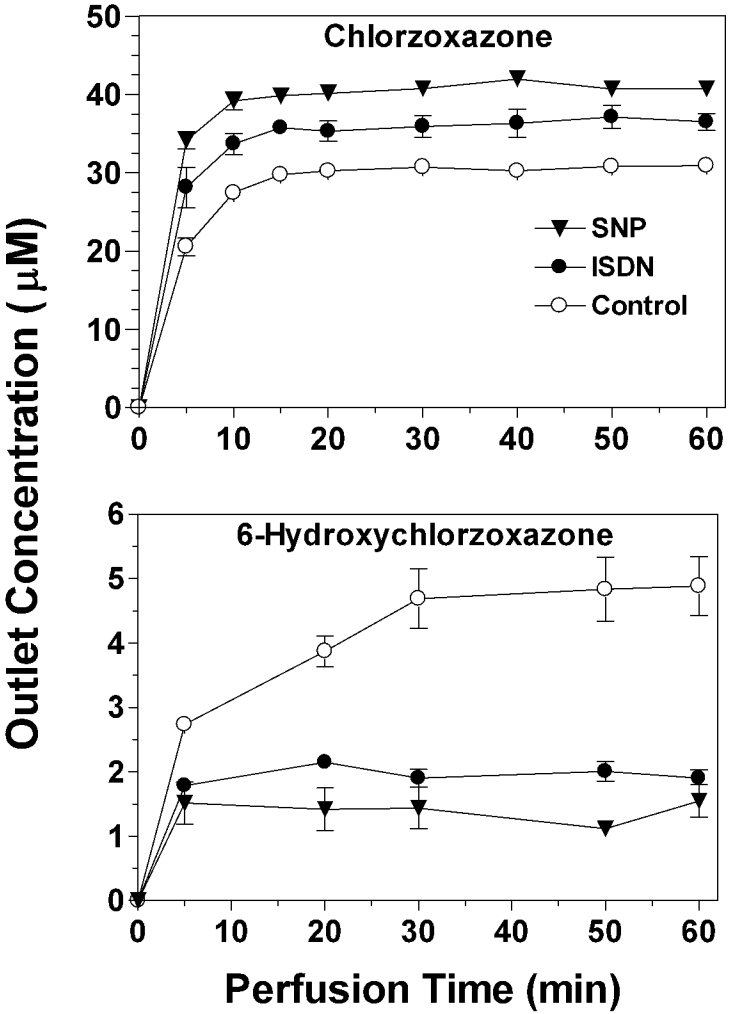
Chlorzoxazone
SNP
ISDN
Control
6-Hydroxychlorzoxazone
Outlet Concentration (μM)
Perfusion Time (min)
0
10
20
30
40
50
60
1
2
3
4
5
6

FIG. 1

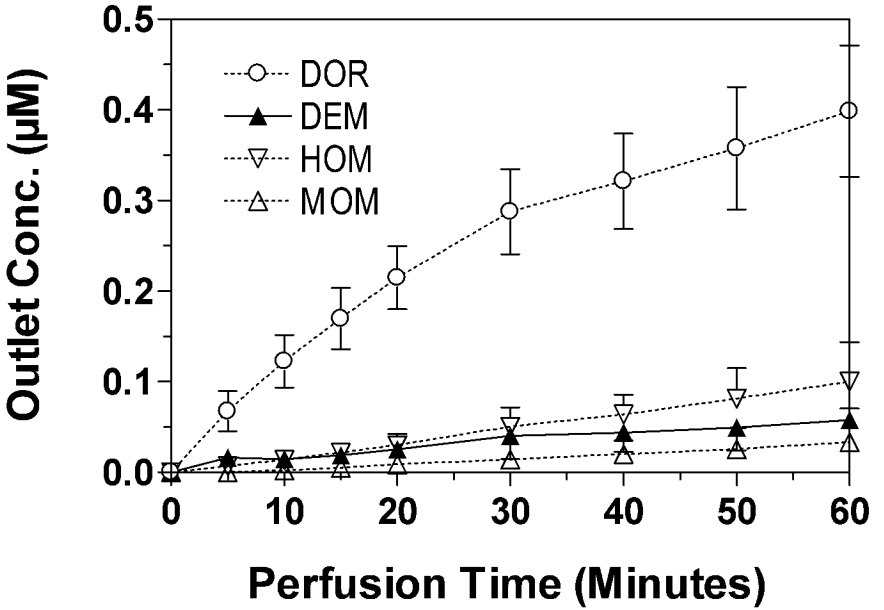
0.5
0.4
0.3
0.2
0.1
0.0
Outlet Conc. (μM)
DOR
DEM
HOM
MOM
0
10
20
30
40
50
60
Perfusion Time (Minutes)

FIG. 2

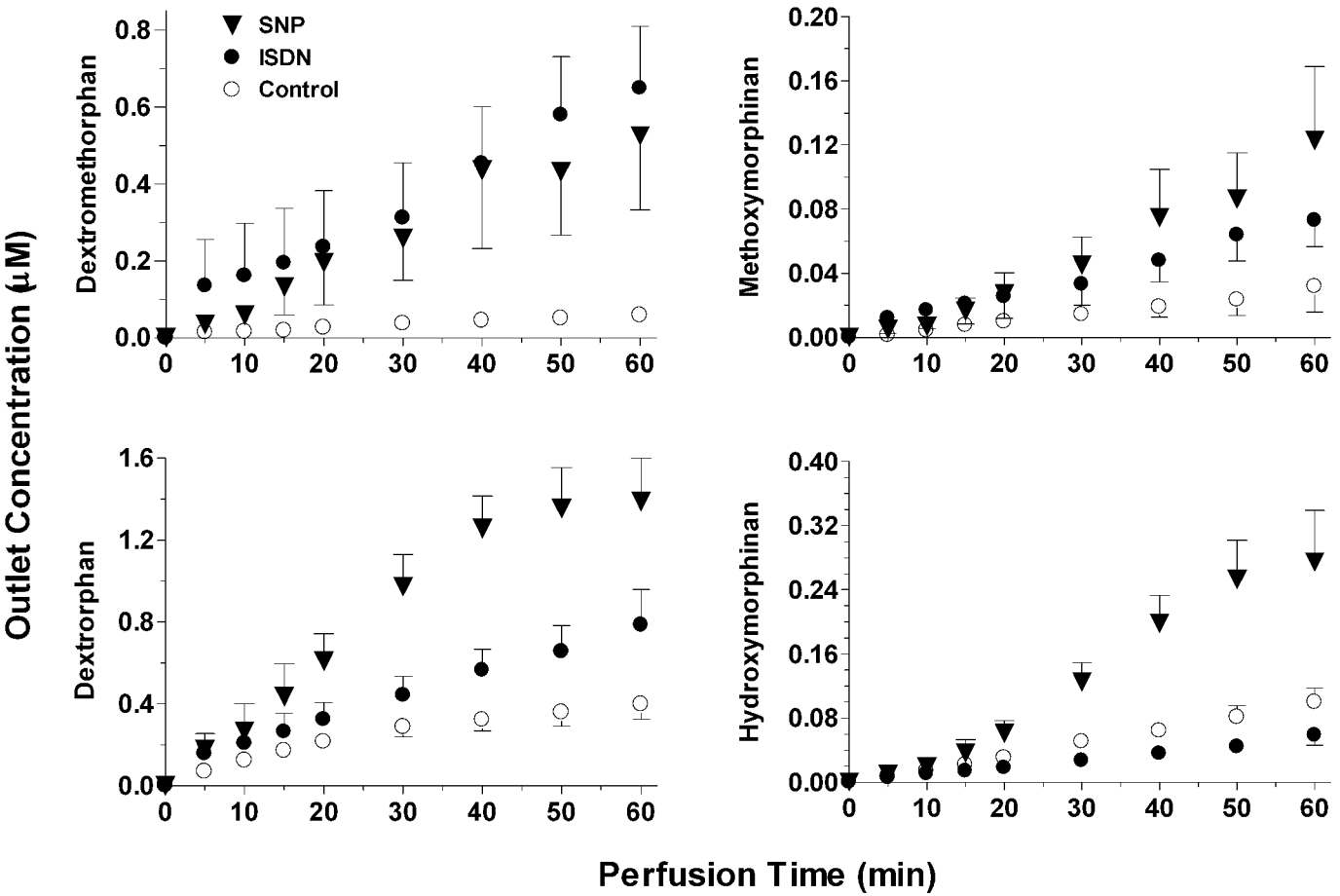
SNP
ISDN
Control
Dextromethorphan
Methoxymorphinan
Dextrorphan
Hydroxymorphinan
Outlet Concentration (μM)
Perfusion Time (min)

FIG. 3

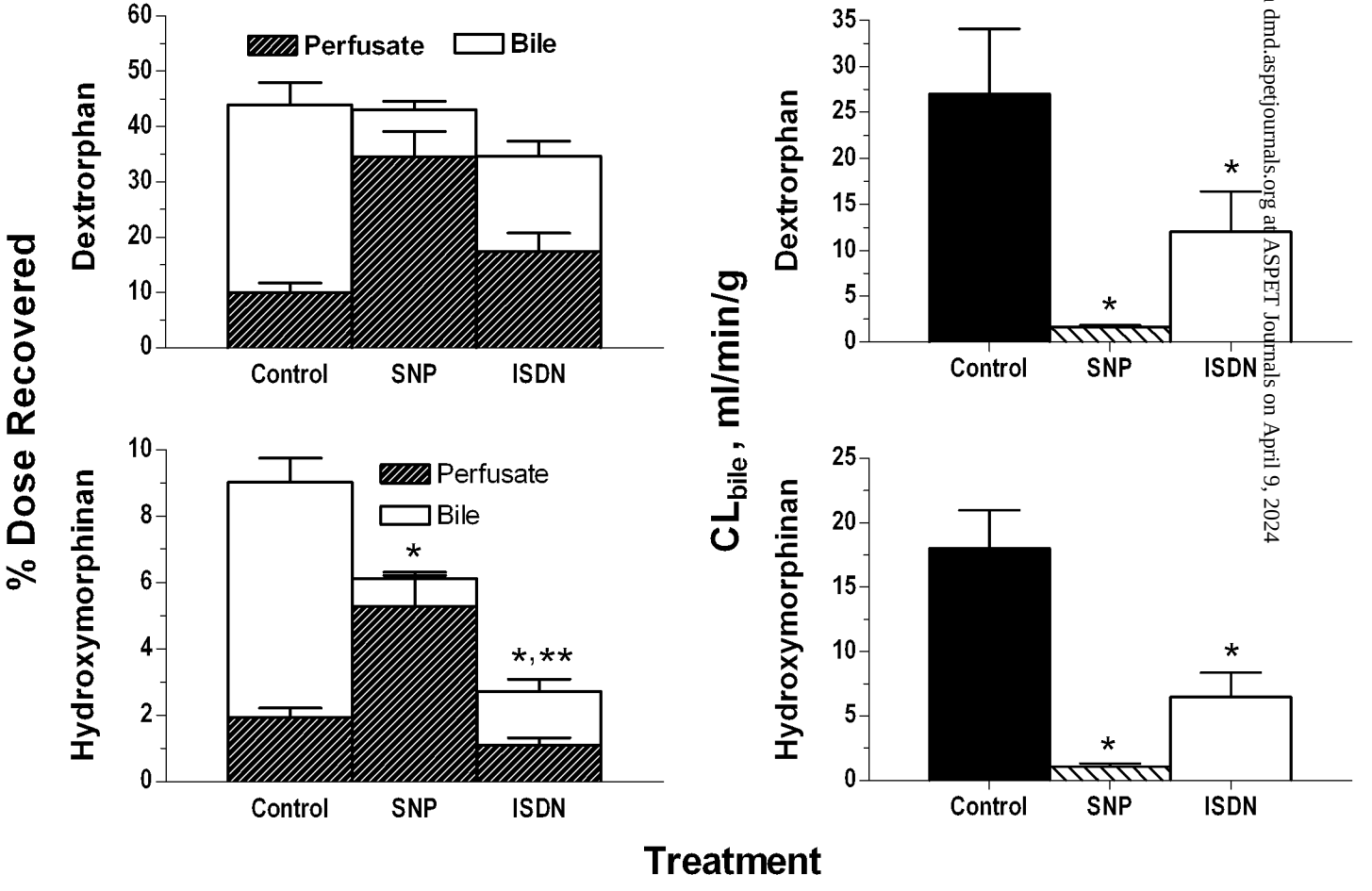

FIG. 4

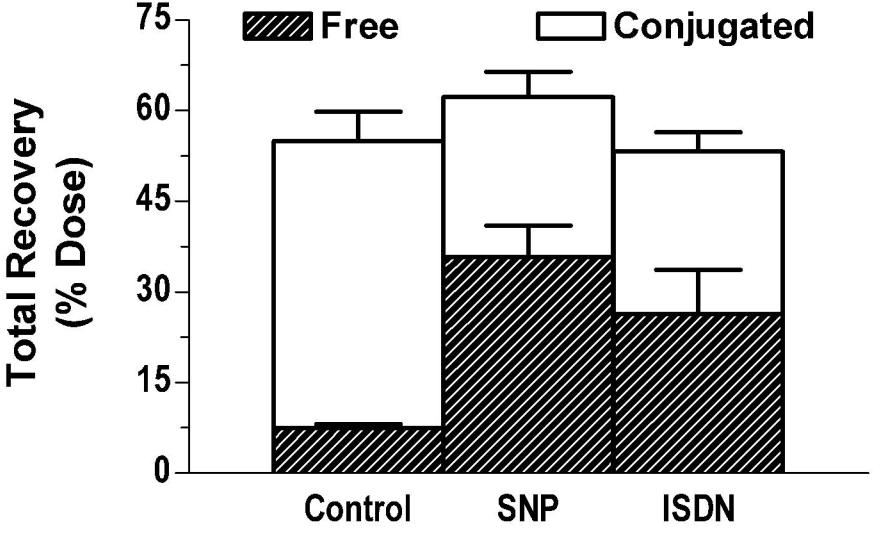
Free
Conjugated
Total Recovery
(% Dose)
75
60
45
30
15
0
Control
SNP
ISDN

FIG. 5

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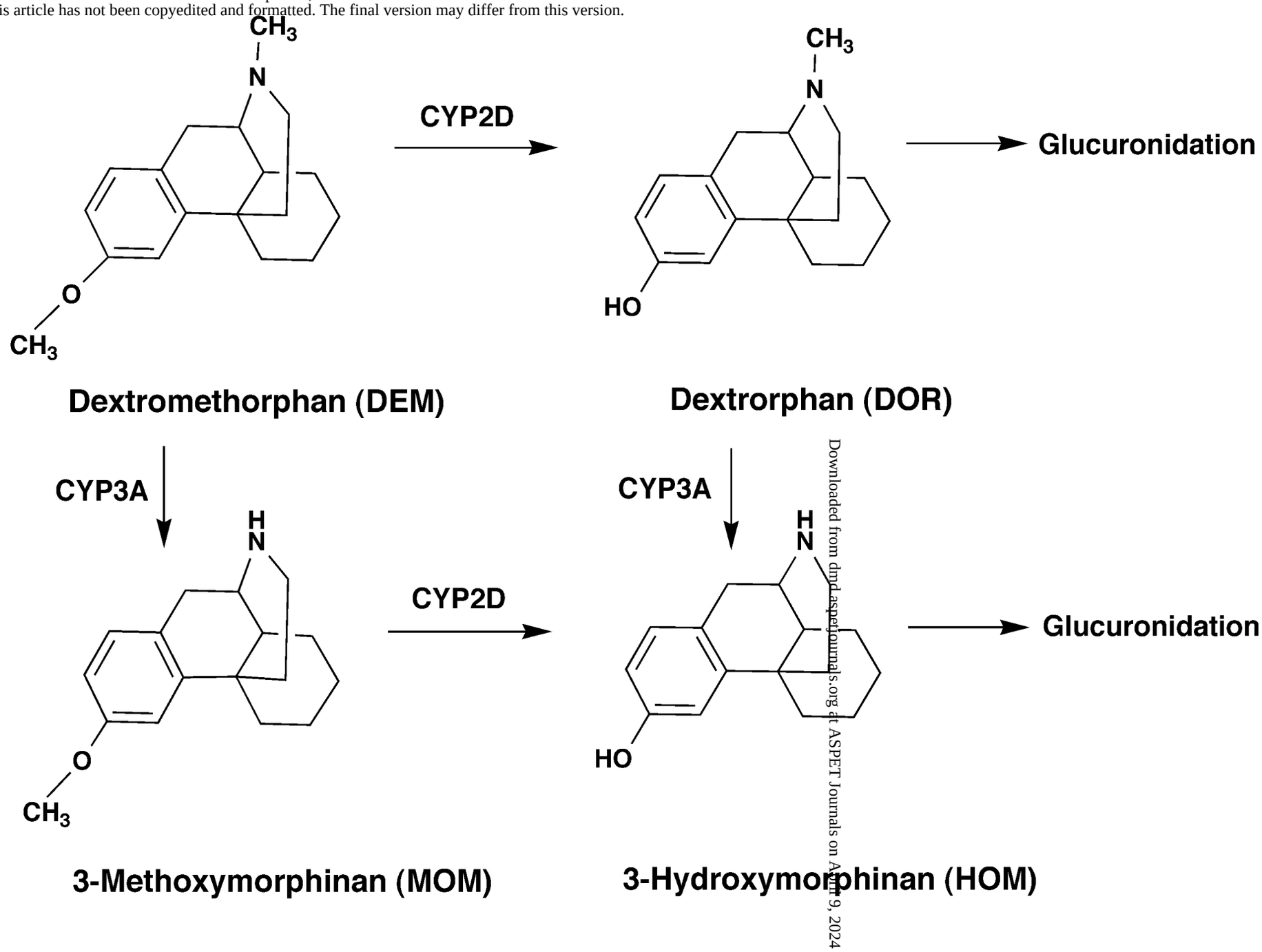

FIG. 6