EFFECT OF SELECTIVE PHASE II ENZYME INDUCERS ON GLUCURONIDATION OF BENOXAPROFEN IN RATS

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ABSTRACT:

The induction of benoxaprofen (BNX) glucuronidation in rats by intragastric administration of three nitrogen heterocycles (quinoline, 2,2'-dipyridyl, or 1,7-phenanthroline at 75 mg/kg daily for 3 days) has been investigated. BNX was administered i.v. at a dose of 20 mg/kg to bile-cannulated rats that had been induced. Blood and bile were collected over 8 h. Liver tissues were also collected at the end of the 8-h study and used to examine conjugation activity of BNX by UDP-glucuronosyl transferases and cytochrome P-450 enzyme activities in vitro. Two methods were used to characterize the true metabolic formation rates of the labile benoxaprofen glucuronide conjugate in vitro, which gave comparable mean values for $K_m$ and $V_{max}$. There appeared to be a trend of increase of the $V_{max}$ of BNX glucuronidation in rat liver microsomes by all three nitrogen heterocycles; however, the induction was only significant with 1,7-phenanthroline. $K_m$ was not noticeably altered by any of the three inducers. No change of measured hepatic microsomal cytochrome P-450 activities in the rat was found. BNX glucuronidation in rats in vivo was increased by all three nitrogen heterocycles with 1,7-phenanthroline more effective than quinoline and 2,2'-dipyridyl. The use of nitrogen heterocycles provides a means to modulate exposure to labile, reactive acyl glucuronides in vivo without apparent changes in oxidative metabolism.

Conjugation with $\beta$-glucuronic acid represents a major route for the elimination and detoxification of drugs and endogenous compounds possessing a carboxylic acid function (Smith and Williams, 1966; Dutton, 1980; Faed, 1984). However, some acyl glucuronides are potentially reactive electrophilic species that have been shown to undergo hydrolysis, intramolecular acyl migration (isomerization), and covalent binding to plasma and/or tissue proteins (Faed, 1984; Spahn-Langguth and Benet, 1992). Covalent attachment of drug to protein in vivo constitutes chemical modification of native proteins and has been suggested as a possible requisite step in the generation of toxic responses to drugs metabolized to acyl glucuronides (Faed, 1984; Van Bremen and Fenselau, 1985; Smith et al., 1986). Moreover, the extent of irreversible tissue binding at a particular time depends on both the stability/reactivity of the reactive acyl glucuronide and the exposure of the organ to the reactive metabolite (King and Dickinson, 1993). Any modulation of the extent of glucuronidation in vivo might, therefore, have a direct impact on the degree of irreversible tissue binding, and consequently, the potential for developing toxicity. Recently, Seitz and Boelsterli (1998) reported an increase of the severity of small intestinal ulcers in rats after 3,4-benzoquinoline treatment, which selectively increased hepatic microsomal diclofenac UDP-glucuronosyl transferase (UGT) activity.

Benoxaprofen (BNX) (Fig. 1) is one of the nine carboxylic acid nonsteroidal anti-inflammatory drugs that were removed from the U.S. and British markets from 1964 to 1993 due to severe toxicity (Dahl and Ward, 1982; Bakke et al., 1984, 1995). It has been postulated that covalent binding of reactive benoxaprofen glucuronide conjugate (BNX-G) to hepatic tissue proteins might be directly related to the few fatal incidents of cholestatic jaundice in elderly patients. Approximately 90% of a single dose of BNX is metabolized in humans to its acyl glucuronide conjugate, of which two-thirds is excreted renally and one-third is eliminated in the feces (Chatfield and Green, 1978). Glucuronidation of BNX is, however, not a major metabolic pathway in rats, and most of the BNX-G formed is reported to undergo biliary excretion (Mohri et al., 1997). Selective modulation of BNX glucuronidation in vivo and in vitro is therefore desired for studies of this reactive metabolite.

The induction of drug-metabolizing enzymes is most often characterized by combined increases in enzymes catalyzing both phase I and phase II reactions. For example, both UGT2 and cytochrome P-450 (CYP) 2B activities can be induced by phenobarbital. Occasionally only phase I reactions are increased, a phenomenon most often identified...
ified with CYP isozymes as the marker. Recently, there has been some interest in a group of nitrogen heterocycles that seems to selectively affect phase II enzymes. Induction studies with this group of compounds have shown that the position of the single heterocyclic nitrogen atom and the addition of ring substituents strongly influence the magnitude and breadth of the induction responses (Le and Franklin, 1997; Vargas et al., 1997). Among them, quinoline (QO), 1,7-phenanthroline (PH), and 2,2'-dipryridyl (DP) demonstrated high selectivity for phase II enzyme induction and, therefore, were selected for this study. Based on the substrates evaluated so far, induction of UGT2B12, UGT2B1, and possibly UGT1A6 was indicated (Le and Franklin, 1997; Vargas et al., 1997). Although the rat UGT isozyme involved in BNX glucuronidation has not yet been reported, from in vitro studies of expressed human isozymes it is known that human UGT2B7 does glucuronidate BNX (Jin et al., 1993).

The objective of this study was to evaluate the effect of oral administration of the three nitrogen heterocycles on the glucuronidation of BNX in vivo in rats. Both the selectivity and the extent of induction of rat UGTs were also assessed in vitro to distinguish the modulation of enzyme activity from the alteration of excretion process. Because of the labile nature of acyl glucuronides under physiological conditions, two approaches suggested by Spahn-Langguth and Benet (1993) for better estimation of true metabolic formation rates in vitro were tested separately for comparison.

Materials and Methods

Rac-BNX was obtained from tablets of Oralflex previously marketed by Eli Lilly (Indianapolis, IL). Anal. Calcd for BNX (C_{16}H_{12}ClNO_3): C, 63.69; H, 4.01; N, 4.64; Cl, 11.75; O, 15.91. Found: C, 63.05; H, 4.31; N, 4.71; Cl, 11.68; O, 16.25. The purity of BNX was confirmed based on analytical HPLC using UV detection at a wavelength of 210 nm. UDP-glucuronic acid, trifluoroacetic acid (TFA), Trizma hydrochloride, Triton X-100, saccharic acid-1,4-lactone (SA), phenylmethylsulfonyl fluoride (PMSF), and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO). QO, PH, and DP (98, 99, and 99% purity, respectively) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade except acetoni-trile and methanol, which were of HPLC grade.

Analytical Methods. The analytical HPLC system consisted of a Bio-Rad (Hercules, CA) Model AS-100 HPLC automatic injector, a Shimadzu RF 535 spectrophotometer detector, a Hewlett-Packard Chemstation (Palo Alto, CA). Cumulative biliary excretion up to 8 h (Ae,bile,0-8h) as well as apparent biliary formation clearance of BNX-G (Cl_{f,bile,app}) [= A_{e,bile,0-8h}(BNX-G)/AUC_{0-8h}(BNX)] for BNX-G were determined. Statistical analyses were performed by general linear model. Differences were considered significant at p values less than 0.05.

Preparation of Rat Liver Microsomes. Hepatic microsomes were prepared separately for each rat from all four experimental groups using a modified method of Wang et al. (1986). Briefly, livers obtained at the end of in vivo studies were homogenized with a Tenbroeck tissue grinder in three volumes of ice-cold 0.25 M sucrose buffer. Homogenate was first centrifuged at 10,000g for 20 min. The resulting supernatant was centrifuged at 100,000g for 60 min. The microsomal pellet was removed, resuspended, and resedimented at 100,000g for 60 min. The resulting microsomal pellet was resuspended in sucrose buffer (protein ca. >30 mg/ml). The microsomal preparations were stored at −80°C. Microsomal protein concentrations were determined according to Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

CYP Enzyme Assays. Erythromycin N-demethylase activity, a marker for CYP3A catalytic activity, was determined by a modified method of Nash (1953). The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.3), 1 to 2 mg of microsomal protein, 0.4 mM erythromycin, and 1 mM NADPH in a final volume of 1.0 ml. The reaction was initiated with NADPH, incubated for 15 min at 37°C, and terminated by the addition of 0.6 ml of 10% trichloroacetic acid. After centrifugation, an aliquot of supernatant (1 ml) was added to 0.5 ml of NASH reagent, and allowed to stand at 50°C for 15 min, then at room temperature for 5 min. The absorbance at 412 nm of each resulting solution was determined. A standard curve was prepared using authentic standards of formaldehyde.

Acetanilide hydroxylase activity, a marker for the activity of CYP1A2, was measured using the HPLC assay method of Liu et al. (1991). The reaction mixture contained 50 mM Tris buffer (pH 7.5), 0.30 mM MgCl_2, 0.2 to 0.5 mg microsomal protein, 0.6 μmol of NADPH, and 1 mg of BSA in a volume of 1 ml. The reaction was initiated by the addition of 25 μl of a 20 mM acetanilide solution. After 20 min of incubation at 37°C, the incubation was terminated and extracted twice with 2 ml of ethyl acetate. Extracts were analyzed by reversed phase HPLC using a 4.6 mm × 25 cm Zorbax Rx C_{18} column and a

![Fig. 1. Metabolism of BNX to BNX-G via uridine diphosphogluconuronyltransferase.](image-url)
mobile phase of 85% KH₂PO₄ buffer (50 mM)/15% acetoniitrite. The flow rate was 1 ml/min, and the eluting hydroxyacetanilide was detected and quantitated by measuring absorbance at 254 nm.

Activity of p-nitrophenol hydroxylase, a marker for the activity of CYP2E1 was measured using the method of Koop (1986). The reaction mixture, maintained at 37°C, contained 0.1 M p-nitrophenol, 2 to 3 mg microsomal protein, and 1 mM ascorbate in a volume of 1 ml. Reactions were initiated by the addition of 10 μl of 100 mM NADPH and stopped after 6 min by addition of 200 μl of ice-cold 1.5 N HClO₄. Aliquots (1 ml) were removed after centrifugation and color was developed by the addition of 100 μl of 10 N NaOH. The absorbance at 510 nm of each resulting solution was determined. A standard curve was prepared using authentic standards of 4-nitrocatechol.

Preparation of Acyl Glucuronide. The principal investigator took a single oral dose of BNX (600 mg, racemic mixture). Urine was then collected over 48 h and immediately adjusted to pH 2 to 4 with phosphoric acid, then refrigerated until extracted. BNX-G was isolated by passing urine at pH 2 through Sep-Pak C18 cartridges. After washing the cartridge, the glucuronide was eluted with 3 ml acetonitrile/10 mM TFA (80:20, v/v). The eluents were then concentrated with a rotary evaporator before injection onto preparative HPLC. The effluent from preparative HPLC containing BNX-G peak was collected on ice and adjusted to pH 2 to 3. After removal of the organic phase, an aliquot of the aqueous solution was taken for analytical HPLC analysis before being lyophilized to dryness and stored at −20°C. The materials isolated exhibited one major peak on HPLC with a purity of 98.5% BNX-G with the remaining as BNX. Cleavage of BNX-G with β-glucuronidase and with specific inhibitor SA also confirmed that it was a β-glucuronide. For electrospray ionization mass spectrometry analysis, lyophilized powder of BNX-G was dissolved in 1% acetic acid/40% acetonitrile (v/v) at a concentration of ~1 μg/ml. Electrospray ionization mass spectrometry of the underivatized BNX-G obtained in the positive ion mode provided ions at m/z 478 and 302 corresponding to BNX-G−H⁺ and BNX−H⁺.

Determination of UGT Activity and Enzyme Kinetic Analysis. UGT activity with BNX as the aglycon was measured by modifying the method described by Spahn et al. (1989). Briefly, microsomal protein was first incubated with Triton X-100 for 15 min at room temperature. The incubation mixture (1 ml) contained 2 mg/ml microsomal protein, 0.05% Triton X-100, 10 mM magnesium chloride, 100 mM Tris buffer, pH 7.4, and 0.05 to 2 mM BNX with and without the addition of inhibitors of hydrolytic enzymes (0.4 mM PMSF and 16 mM SA); it was then warmed to 37°C for 5 min. The reaction (over a 90-min period) was started by adding UDP-glucuronic acid (final concentration, 10 μM). Aliquots of 0.1 ml were taken from the assay mixture at various times and were pipetted into 0.2 ml of ice-cold acetonitrile (including internal standard). The pH of the samples was adjusted to 2 to 4 to further stop the reaction. After centrifugation, the supernatants were dried under nitrogen gas and reconstituted in 0.5 ml mobile phase before injection onto the HPLC. Activities of UGT are expressed as nanomoles of BNX-G formed per minute per milligram of microsomal protein.

The stability of the BNX-G under the same incubation conditions as described above (but without UDP-glucuronic acid) was studied at 20 μM over a 90-min period. Serial samples over time were assayed by HPLC as described above for plasma samples. The apparent first-order degradation rate constant (kₚ) was determined using linear regression analysis.

Kinetic analyses of UGT in the rat liver were carried out by determining initial rates of activity at six different fixed BNX concentrations (0.05, 0.1, 0.2, 0.5, 1, and 2 mM). Two approaches were taken to estimate the true initial metabolic formation rate according to Spahn-Languth and Benet (1993). With the addition of the hydrolytic enzyme inhibitors (PMSF and SA), the true initial BNX-G formation rate was determined simply by using linear regression analysis on the BNX-G formation over time profile. In the case when no inhibitors were present in the incubation mixture, the degradation rate of BNX-G (kₚ) under similar conditions was used to correct the apparent parameters. The formation of BNX-G can be described by eq. 1, which can be rearranged to calculate the true initial formation rate (kₚ). Kinetic constants (Vₘₚₚ and Kᵣ) for UGT were first estimated by plotting the initial rate of activity and substrate concentration on double reciprocal plots and Eadie-Hofstee plots, then determined by fitting the data to the Michaelis-Menten equation (WinNONLIN 1.1). Statistical analyses were performed by general linear model to compare Vₘₚ and Kᵣ between treatment groups and control group. Differences were considered significant at p values less than .05.

\[
\text{BNX-G} = kₚ(1 - e^{-kₚt})/kₚ
\] (1)

Results

Induction of BNX Glucuronidation In Vivo. The concentrations of BNX and BNX-G in both plasma and bile were quantified by HPLC. Figure 2 is the representative data showing the plasma elimination profiles of BNX in control and PH-treated rats. Cₘₚₚₑₚₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑطور

![Figure 2](image)

Solid symbols represent plasma concentrations of BNX; open symbols represent plasma concentrations of BNX-G.

Characterization of Purified Glucuronide Samples. Product stability studies in microsome incubation media (without the addition of UDPGA) in vitro showed that the disappearance of BNX-G followed apparent first order kinetics (Fig. 5). The degradation T₁/₂ₕ was determined to be 33 min (kₚ = 0.0212 min⁻¹). With the addition of both PMSF and SA, BNX-G was stabilized over the 90-min incubation period (Fig. 5). Thus, no correction for apparent glucuronide formation rate was needed when this enzyme-blocked media was used in the study to estimate true rates of BNX-G formation. In addition, the degradation of BNX-G in buffer solution was pH dependent with no apparent degradation measurable in 72 h at pH 5, however, as the pH increased the degradation increased rapidly (J.Q.D. and P.C.S., unpublished data).
Selective Induction of Glucuronidation In Vitro. The CYP enzyme activities of microsomal proteins prepared from treated and control rats were first examined. None of the orally administered nitrogen heterocycles tested significantly induced hepatic microsomal CYP (CYP1A2, CYP2E1, and CYP3A) in vitro compared with those in control animals \((p < .05)\) (Table 1). This confirmed that PH, QO, and DP had no effect on the oxidative metabolism that may occur in the liver by these three representative oxidative enzymes. Figures 5 and 6 are representative data showing the effect of hydrolytic enzyme inhibitors on the degradation and the formation of BNX-G in vitro in rat liver microsomes. Both in the absence (Fig. 7A) and presence (Fig. 7B) of hydrolytic enzyme inhibitors, the formation of acyl glucuronide reached a plateau at 0.5 mM BNX for rat liver microsomes. Two proposed approaches to characterize true metabolic formation rates of acyl glucuronide gave comparable results and DP had no effect on the oxidative metabolism that may occur in the liver by these three representative oxidative enzymes.

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**Fig. 3.** Effects of orally administered PH, QO, and DP on \(C_{\text{max}}\) and AUC\(_{0-\infty}\) for BNX-G in rats.

Results represent the mean ± S.D. \((n = 4)\). Asterisks indicate statistically significant difference \((p < .05)\) from control.

**Fig. 4.** Effects of orally administered PH, QO, and DP on A\(_{\text{et,bile,0-3h}}\) and Cl\(_{\text{et,bile,app}}\) for BNX-G in rats.

Results represent the mean ± S.D. \((n = 4)\). Asterisks indicate statistically significant difference \((p < .05)\) from control.

TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CYP 1A(^d)</th>
<th>CYP 2E(^e)</th>
<th>CYP 3A(^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.41 ± 0.019(^d)</td>
<td>1.09 ± 0.10</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>PH</td>
<td>0.52 ± 0.037</td>
<td>0.80 ± 0.074</td>
<td>0.27 ± 0.068</td>
</tr>
<tr>
<td>QO</td>
<td>0.45 ± 0.011</td>
<td>0.74 ± 0.33</td>
<td>0.24 ± 0.064</td>
</tr>
<tr>
<td>DP</td>
<td>0.58 ± 0.078</td>
<td>1.14 ± 0.12</td>
<td>0.38 ± 0.047</td>
</tr>
</tbody>
</table>

\(^d\) Acetanilide 4-hydroxylase activity.

\(^e\) p-Nitrophenol hydroxylase activity.

\(^f\) Erythromycin \(N\)-demethylase activity.

Parameters determined are given as the mean ± S.D. \((n = 4)\).
as shown in Fig. 7. When the data of Fig. 7 was analyzed, it was apparent that doses of PH and DP caused major changes in the rate of glucuronidation of BNX in vitro. At the substrate concentration of 0.5 mM, the UGT activities toward BNX was elevated 4- and 2-fold by PH and DP, respectively. QO treatment did not alter the in vitro BNX glucuronidation rate significantly (Fig. 7). Michaelis-Menten parameters ($V_{\text{max}}$ and $K_M$) were determined using the corrected metabolic formation rates (Table 2). $V_{\text{max}}$ of BNX glucuronidation was increased up to 5-fold by PH, however, neither QO nor DP caused a significant increase of $V_{\text{max}}$ although the mean value for DP suggested a greater effect than for QO. $K_M$ was not significantly modified by any of the three nitrogen heterocycles studied.

**Discussion**

Previous studies looking at the induction response to some simple diaryl pyridines indicated that the position of the nitrogen in the ring(s) strongly influenced the induction profile (Franklin, 1991; Vargas et al., 1997). 2-Substituted pyridines, such as DP, were selective inducers of only phase II enzymes; 4-substituted isomers also induce CYP isozymes (Franklin, 1991; Vargas et al., 1997). Several phenanthrene derivatives containing two heterocyclic nitrogen atoms (such as PH) were found to selectively increase phase II drug-metabolizing enzyme activities without inducing CYP (Franklin et al., 1993; Vargas et al., 1997). The phase selectivity was independent of the location of the nitrogen atoms in the aromatic system. Rats treated with QO also showed induction of conjugation enzyme activities without inducing either CYP concentration or CYP1A-, CYP2B-, CYP2E-, and CYP3A-selective activities (Le and Franklin, 1997). In the present study, we confirmed that CYP1A2-, CYP2E1-, and CYP3A-selective activities of rat microsomes were not altered by any of the three nitrogen heterocycles (PH, QO, and DP) (Table 1).

BNX glucuronidation in vivo in rats were enhanced by all three induction reagents. To further examine the induction mechanism, in vitro microsomal incubation studies were conducted to investigate the effect of nitrogen heterocycles on UGT activities. There appeared to be a trend of increase in the $V_{\text{max}}$ of BNX glucuronidation in rat liver microsomes by all three nitrogen heterocycles; however, the induction was only significant with PH (Table 2) probably due to small sample size ($n = 4$). Interestingly, PH also demonstrated the highest induction effect in vivo. $K_M$ was not noticeably altered by any of the three reagents. This suggests that the UGT protein levels may be induced by nitrogen heterocycles without affecting binding affinity. It is known that UGTs expressed in both human and rat liver microsomes belong to two gene families ($UGT1$ and $UGT2$), each containing at least four members. Human UGT 2B7 has been demonstrated to glucuronidate primarily compounds containing an aliphatic carboxylic acid function. These compounds include many nonsteroidal anti-inflammatory drugs such as BNX, diflunisal, and zomepirac (Jin et al., 1993). The specific rat UGT isozyme responsible for BNX glucuronidation has not yet been identified.

Figure 6 illustrates the extent of underestimation of the initial metabolic rates when neglecting potential product loss by the labile
nature of acyl glucuronides in complex matrices such as microsomes that likely contain numerous esterases. The two procedures used in this study to characterize the true metabolic formation rates of the labile BNX-G were recommended by Spahn-Languth and Benet (1993). Both techniques have their limits. The use of hydrolytic enzyme inhibitors to block the decomposition of glucuronidation product may potentially change the physical characteristics of the incubation medium. Correcting the apparent formation parameter with a separately determined degradation rate constant of acyl glucuronide in the absence of inhibitors, however, requires the availability of the glucuronide to permit stability testing in the same medium in which formation is determined. Both approaches were used in the present study to determine BNX glucuronidation in rat liver microsomes. Comparable mean values for $K_M$ and $V_{max}$ were obtained by both methods.

The induction of BNX glucuronidation in vivo in rats agreed with the findings from in vitro studies. PH proved to have the highest effects on increasing both the systemic and biliary exposure to BNX-G in vivo (Figs. 3 and 4). Although AUC of BNX-G was induced at least 3-fold by three nitrogen heterocycles tested, the increase of biliary exposure was no more than 2-fold. This suggested that the biliary transport of BNX-G might have been saturated, leading to enhanced glucuronide being presented to blood. Whether glucuronides are excreted from the body in bile or urine depends in part on the size of the aglycone (parent compound or phase I metabolite). Molecular weight cutoffs for the preferred route of excretion vary among mammalian species (Hirom et al., 1976). Rats have lower molecular weight cutoffs for the preferred route of excretion (Hirom et al., 1976). Although no oxidative metabolite of BNX has been identified, Mohri et al. (1997) identified a BNX taурine conjugate in rat bile and showed that approximately 2.5% of the BNX dose was excreted as this conjugate in bile over a 12-h period.

In conclusion, all three nitrogen heterocycles were inducers of BNX glucuronidation in vivo in rats with PH probably more effective than QO and DP. This method provides a means to modulate acyl glucuronide exposure in vivo. There appeared to be a trend of increase of the $V_{max}$ of BNX glucuronidation in rat liver microsomes by all three nitrogen heterocycles; however, the induction was only significant with PH probably due to small sample size. $K_M$ for glucuronidation of BNX was not noticeably altered by any of the nitrogen heterocycles. Three measured hepatic microsomal CYP activities were unchanged by the nitrogen heterocycles documenting the selectivity of the induction. Comparable mean values for $K_M$ and $V_{max}$ were obtained by the two methods to characterize true metabolic formation rates of the labile BNX-G.

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