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HEPATIC DISPOSITION OF ACETAMINOPHEN AND METABOLITES

PHARMACOKINETIC MODELING, PROTEIN BINDING AND SUBCELLULAR DISTRIBUTION

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Abstract—Successful pharmacokinetic modeling often requires the ability of a simple model to describe a complex series of physiological processes. However, a simple model may be inappropriate. Physiologically-relevant modeling may offer a more appropriate description, but requires further support from *in vitro/in vivo* data. A well-stirred hepatic model with linear processes was proposed to describe *in vivo* disposition of acetaminophen and metabolites after a 100 mg/kg bolus of acetaminophen to vehicle- or phenobarbital-pretreated, renal-ligated rats. Model simulations underpredicted acetaminophen glucuronide (AG) concentrations at early time points in serum, and were inconsistent with AG biliary excretion-rate profiles. Intracellular binding of AG by ligandin was hypothesized, and a cytosolic compartment with reversible binding was incorporated into the model. In this second model, only AG bound in the cytosolic compartment was available for excretion into bile. Model 2 better described the AG biliary excretion rate–time profiles based on calculated Akaike's information criterion values. However, no apparent change was observed in the underprediction of AG serum concentrations. Parameter estimates derived from the two models also were different. The rate constants regulating AG formation and sinusoidal egress were increased significantly after phenobarbital pretreatment according to model 1, while the AG biliary excretion rate constant was decreased significantly. Parameter estimates based on model 2 suggested that phenobarbital pretreatment impaired the cytosolic binding of AG but increased significantly the AG biliary excretion rate constant. The physiologic relevance of model 2 was not supported by a subsequent investigation of the protein binding and subcellular distribution of acetaminophen and metabolites. Acetaminophen, AG and acetaminophen sulfate (AS) were not bound extensively in hepatic cytosol (mean ± SD unbound fractions were 0.90 ± 0.08, 0.97 ± 0.08, and 0.88 ± 0.06, respectively). Phenobarbital pretreatment did not alter significantly the unbound fractions of acetaminophen, AG or AS in hepatic cytosol. Acetaminophen was distributed to a greater extent in lysosomes than in the nuclear, mitochondrial, microsomal and cytosolic fractions. Distribution of AS predominated in cytosolic and lysosomal fractions. AG was detected only in cytosol. Phenobarbital pretreatment decreased the content of acetaminophen, AG and AS in all hepatic fractions. This study demonstrates the utility of pharmacokinetic modeling in exploring mechanistic hypotheses. However, these results underscore the importance of obtaining pivotal data from *in vitro/in vivo* studies to validate hypothesized mechanisms.

Hepatic disposition of endogenous and exogenous compounds is influenced by uptake into the hepatocyte, intracellular transport and binding, metabolism, and subsequent egress into serum or bile. Although many models of hepatic disposition assume apparent first-order processes, regulation of cellular traffic is more complex. Underlying carrier and metabolic systems may be saturable, rate-limiting, and better described by Michaelis–Menten kinetics. Another consideration is the extent of intracellular binding, which may result in nonlinear hepatic disposition of substrates [1, 2]. Levine *et al.* [3] examined biliary excretion of several compounds after phenobarbital pretreatment and concluded that phenobarbital-stimulated formation of new endoplasmic reticulum was not associated with alterations in biliary excretion. Later studies by Levine and Singer [4] implicated a cytosolic link between the formation of a metabolite in the endoplasmic reticulum and its excretion in bile. These authors proposed that a cytosolic protein, ligandin, may be involved in intracellular binding and transport. Binding to cytosolic proteins has been shown to influence the hepatic egress of bilirubin [5], dibromosulfophthalein [6], and steroidal muscle relaxants [7].

Phenobarbital is a prototypic inducer of the cytochrome P450 mixed-fuction oxidase and uridine-5′-diphosphoglucuronyltransferase systems. Although phenobarbital pretreatment increased the formation clearance to acetaminophen glucuronide (AG)§ and increased AG serum concentrations after a bolus dose of acetaminophen, Brouwer and

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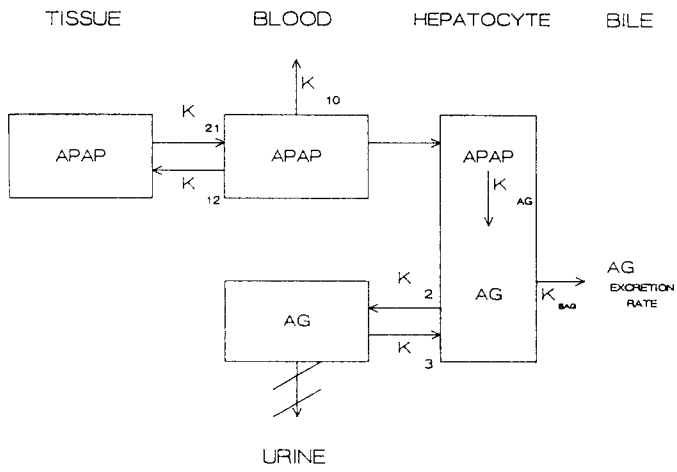
§ Abbreviations: AG, acetaminophen glucuronide; APAP, acetaminophen; AS, acetaminophen sulfate; and AIC, Akaike's information criterion.



$$dA_P/dt = K_{21}*A_T - K_{12}*A_P - K_{10}*A_P - K_{AG}*A_P$$

$$dA_T/dt = K_{12}*A_P - K_{21}*A_T$$

$$dAG_P/dt = K_2*AG_H - K_3*AG_P$$

$$dAG_B/dt = K_{BAG}*AG_H$$

$$dAG_H/dt = K_{AG}*A_P + K_3*AG_P - K_2*AG_H - K_{BAG}*AG_H$$

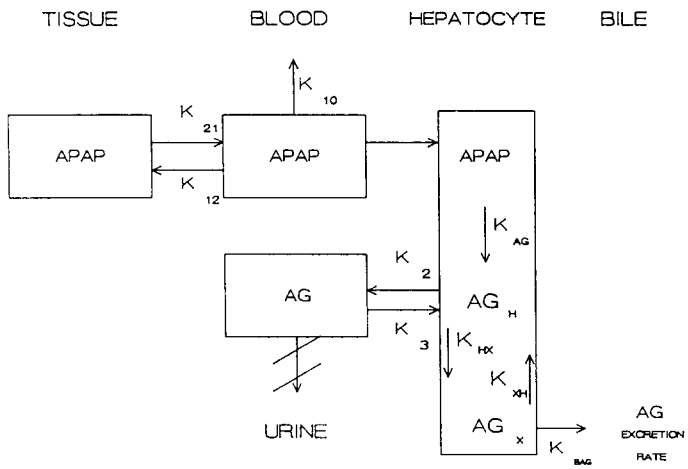
Fig. 1. Representation of (top) Scheme 1 and (bottom) the corresponding model equations describing a well-stirred hepatic model with linear processes (K) for acetaminophen (A; APAP) and acetaminophen glucuronide (AG). The subscripts denote exchange between tissue (T) and blood (P) compartments (12, 21), non-AG elimination (10), AG formation (AG) in the hepatocyte (H), sinusoidal efflux of AG (2), sinusoidal reuptake of AG (3), and AG excretion (BAG) in bile (B).

Jones [8] reported that biliary excretion of AG was impaired in both intact and renal-ligated rats pretreated with phenobarbital. Impaired biliary excretion of AG *in vivo* after phenobarbital pretreatment also has been noted by Loeser and Siegers [9] and Gregus *et al.* [10]. Alterations observed in the hepatobiliary disposition of AG after phenobarbital pretreatment may be due to induction or competitive inhibition. Induction of a cytosolic protein and/or a sinusoidal transport system regulating efflux could reduce the amount of xenobiotic available for biliary excretion. Impairment of AG biliary excretion may be due to competitive inhibition at the canalicular transport site by phenobarbital or a phenobarbital metabolite. Alternatively, phenobarbital-induced synthesis of an endogenous substrate of the canalicular transport sytstem also could result in competitive inhibition of AG biliary excretion. Finally, competition for cytosolic binding sites that are linked physiologically with canalicular transport may alter biliary excretion. The objectives of this investigation were: (1) to develop a pharmacokinetic model to describe the *in vivo* hepatobiliary disposition of acetaminophen and AG in vehicle- and phenobarbital-pretreated, renal-ligated rats, and (2) to examine the validity of the model by quantitating the extent of protein binding in serum and hepatic cytosol, and determining the hepatic subcellular distribution of acetaminophen and metabolites.

METHODS

In vivo *studies.* Concentration–time data were derived from a previous investigation in this laboratory [8]. Briefly, male Sprague–Dawley rats (260–320 g) received either phenobarbital (58 mg/kg/12 hr in 10% ethanol/90% propylene glycol, N = 4) or vehicle (N = 4) by oral gavage for 5 days. Twenty-four hours after the last dose, the rats were anesthetized with urethane (1 g/kg, i.p.), cannulas were placed in the bile duct, right jugular and femoral veins, and the renal pedicles were ligated bilaterally. Acetaminophen (100 mg/kg in 40% propylene glycol/60% 0.9% saline) was administered over 1 min via the femoral vein. A continuous saline infusion (1 mL/hr) was maintained throughout the sampling period. Blood samples (via the jugular vein) and bile were collected over 285 min. Serum was harvested and frozen until assayed. Bile volume was determined gravimetrically (sp. g. = 1.0) and frozen until analysis.

Data analysis. Acetaminophen, AG, and acetaminophen sulfate (AS) concentrations in serum



$$dA_P/dt = K_{21}*A_T - K_{12}*A_P - K_{10}*A_P - K_{AG}*A_P$$

$$dA_T/dt = K_{12}*A_P - K_{21}*A_T$$

$$dAG_P/dt = K_2*AG_H - K_3*AG_P$$

$$dAG_B/dt = K_{BAG}*AG_X$$

$$dAG_H/dt = K_{AG}*A_P + K_3*AG_P + K_{XH}*AG_X - K_2*AG_H - K_{HX}*AG_H$$

$$dAG_X/dt = K_{HX}*AG_H - K_{XH}*AG_X - K_{BAG}*AG_X$$

Fig. 2. Representation of (top) Scheme 2 and (bottom) the corresponding model equations describing a well-stirred hepatic model with linear processes (K) as described previously, and a discrete binding pool of acetaminophen glucuronide within the hepatocyte (AG_X) that is available for biliary excretion. AG_H represents AG within the hepatocyte that is available for binding or diffusion into the systemic circulation. Other subscripts denote AG binding (HX) and AG release (XH).

and bile were quantitated by HPLC by the method of Brouwer and Jones [8]. After addition of the internal standard, *p*-aminobenzoic acid, and protein precipitation with acetonitrile, aliquots of serum and bile samples were injected onto a Shimadzu (Columbia, MD) HPLC system with UV absorbance detection (254 nm) and a C_{18}-5 μm Econosil column (250 × 4.6 mm, Alltech Associates, Inc., Deerfield, IL). The mobile phase consisted of 0.75% glacial acetic acid, 94.25% 0.25 M potassium phosphate buffer (pH 3.1), and 5% acetonitrile flowing at a rate of 0.9 mL/min. Standard curves for acetaminophen, AG, and AS in serum (5–500 μg/mL) and bile (25–2500 μg/mL) were linear ($r > 0.997$) and were prepared daily. Coefficients of variation for acetaminophen and AG were <3% (intraday) and <7% (interday). AG and AS concentrations, corrected for the percentage of conversion as determined by incubation with β-glucuronidase (Type H-2; 120,000 U/mL of β-glucuronidase, 4,200 U/mL of sulfatase, Sigma Chemical Co., St. Louis, MO), were expressed as acetaminophen equivalents. The lower limits of detection of acetaminophen, AG, and AS (expressed as acetaminophen equivalents) were 5, 1.75, and 1.65 μg/mL, respectively.

Kinetic modeling. Two schemes and the corresponding differential equations describing the *in vivo* hepatobiliary disposition of acetaminophen and AG were examined. Model 1 (Fig. 1) describes an acetaminophen bolus, administered to the central compartment (blood), which distributes to peripheral or hepatic tissue. Within the hepatocyte, acetaminophen is metabolized to AG (formation of AS and other acetaminophen metabolites is accounted for by K_{10}). The formed glucuronide may distribute back into the blood or be excreted in bile. Model 2 (Fig. 2) is identical except that it contains a discrete, reversible cytosolic binding compartment for AG that is available for biliary excretion. In this model, AG_H denotes the free cytosolic AG that is available only for sinusoidal efflux or cytosolic binding. The parameter AG_X describes a hypothetical, intracellular compartment of reversibly-bound AG that is available for excretion in bile. Parameters describing the disposition of acetaminophen in serum were constant (dose) or estimated from initial data stripping (K_{12}, K_{21}, K_{10}) with the nonlinear least-squares regression program RSTRIP (MicroMath, Inc., Salt Lake City, UT). Laplace transforms for each set of model equations were derived and fit to the data with the simulation program LAPLACE

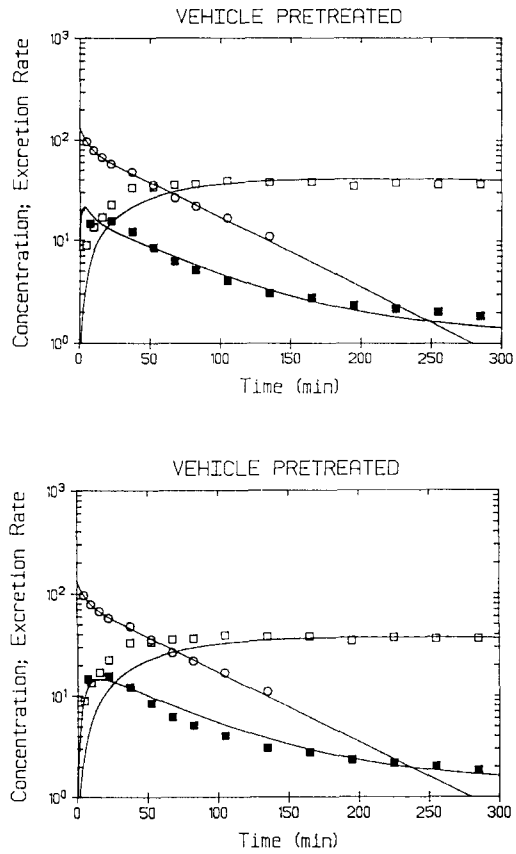


Fig. 3. Representative concentration–time profiles (μg/mL) for acetaminophen (○) and acetaminophen glucuronide (□) in serum, and the excretion rate–time profile (μg/min) for acetaminophen glucuronide (■) in bile in vehicle-pretreated rats. Rats were administered a bolus dose of acetaminophen (100 mg/kg) after vehicle pretreatment p.o. for 5 days. Solid curves denote the computer-generated best fit of (top) model 1 or (bottom) model 2 to the data.

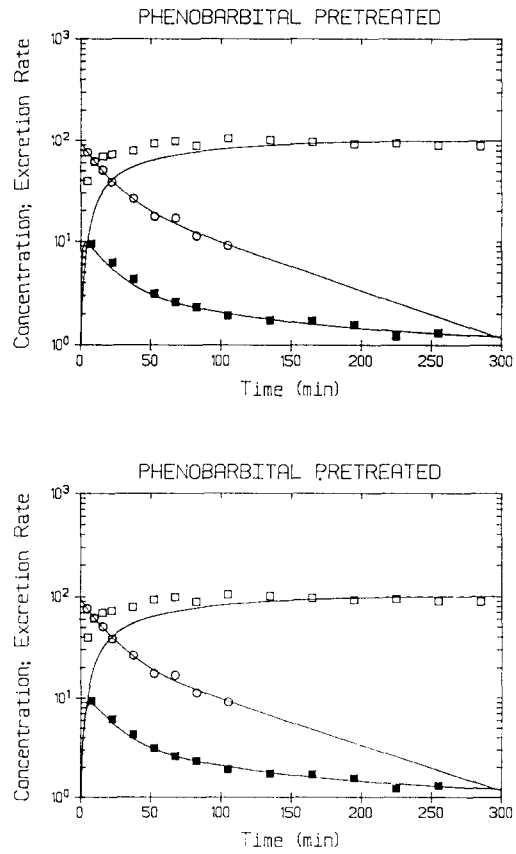


Fig. 4. Representative concentration–time profiles (μg/mL) for acetaminophen (○) and acetaminophen glucuronide (□) in serum, and the excretion rate–time profile (μg/min) for acetaminophen glucuronide (■) in bile in phenobarbital-pretreated rats. Rats were administered a bolus dose of acetaminophen (100 mg/kg) after phenobarbital pretreatment p.o. for 5 days. Solid curves denote the computer-generated best fit of (top) model 1 or (bottom) model 2 to the data.

(MicroMath, Inc.). The goodness of fit for each model was evaluated based on the residual sum of squares, Akaike's information criterion (AIC) [11] and visual inspection of the generated curves relative to the data. The AIC was calculated as:

$$\mathrm{AIC} = n \cdot \ln\left(\sum_{i=1}^{n} w_i(\mathrm{Yobs}_i - \mathrm{Ycal}_i)^2\right) + 2p \quad (1)$$

where n is the number of observations, w_i is the weighting factor for the ith observation, and p is the number of parameters estimated by the model. A weighting factor of 1 was selected for all AIC determinations. The effect due to model revision was calculated as the ratio of the AIC values determined from the revised and initial models. An AIC ratio of less than 1.0 indicates an improvement in the ability of the revised model to describe the data.

A one-way analysis of variance on the generated parameters was performed to examine differences between vehicle and phenobarbital pretreatment within each model. When the F value was significant, *post-hoc* pairwise comparisons were made with Scheffe's test. The criterion for statistical significance was $P < 0.05$.

In vitro *protein binding*. Serum and hepatic cytosol were obtained from naive (N = 4–8 per group) or phenobarbital-pretreated Sprague–Dawley rats. Rat liver was homogenized with 1.5 vol. of 0.15 M KCl, followed by centrifugation of the homogenate for 20 min at 1,200 *g*, rehomogenization, and centrifugation for 60 min at 100,000 *g* (2°) to obtain the supernatant fraction (hepatic cytosol). Various amounts of acetaminophen, AG or AS were reconstituted in 0.8 to 1.0 mL rat serum or hepatic cytosol to achieve concentrations ranging from 2.5 to 100 μg/mL. The samples were centrifuged (1000 *g*) at 37° in Amicon ultrafiltration devices equipped with YMT membranes (Amicon Division, W.R. Grace & Co., Danvers, MA). Centrifugation in a

Table 1. Parameter estimates derived from model 1

Parameter (min^{-1})	Pretreatment	
	Vehicle	Phenobarbital
K_{AG}	0.0022 ± 0.0003	0.0030 ± 0*
K_2	0.4750 ± 0.0957	0.7500 ± 0.1291†
K_3	0.0040 ± 0.0012	0.0029 ± 0.0006
K_{BAG}	0.3938 ± 0.0125	0.1400 ± 0.0406*

Values are means ± SD, N = 4.
* $P < 0.01$.
† $P < 0.05$.

fixed angle rotor varied from 0.5 to 2 min, which depended on the time required to yield approximately 10% of the initial sample volume as ultrafiltrate. Initial studies were conducted to validate that acetaminophen, AG and AS did not bind to the filtration device or membrane.

Subcellular distribution. The localization of acetaminophen, AG and AS in liver subfractions (nuclear, mitochondrial, lysosomal, microsomal and cytosolic) was examined in vehicle- (N = 1) and phenobarbital- (N = 1) pretreated rats (75 mg/kg/24 hr, i.p., ×4 days) in preliminary studies. Liver subfractions were prepared by a modified method [12] of the cell fractionation techniques described by De Duve *et al.* [13]. Acetaminophen (100 mg/kg, i.v. bolus) was administered 24 hr after the last pretreatment dose, and rats were killed 15 min after acetaminophen administration. The liver was homogenized with 3 vol. of ice-cold 0.25 M sucrose (pH 7.0) in a Potter–Elvehjem homogenizer with a teflon pestle. The nuclear fraction was removed by centrifugation at 600 *g* for 10 min. The mitochondrial and lysosomal fractions were obtained by centrifugation for 8.5 min at 6,780 *g* and 9 min at 35,000 *g*, respectively. The microsomal and cytosolic fractions were separated by ultracentrifugation (60 min at 100,000 *g*). Glucose-6-phosphatase, a microsomal marker enzyme, was analyzed by measuring inorganic phosphate production [14]. Activity of succinate dehydrogenase, a mitochondrial marker enzyme, was measured by the method of Shephard and Hubscher [15]. Acid-phosphatase activity (lysosomal marker) was measured with a kit purchased from the Sigma Chemical Co. Protein concentrations were determined by the method of Lowry *et al.* [16].

Table 2. Parameter estimates derived from model 2

Parameter (min^{-1})	Pretreatment	
	Vehicle	Phenobarbital
K_{AG}	0.0028 ± 0.0005	0.0031 ± 0.0002
K_2	0.8125 ± 0.1031	0.8000 ± 0.1414
K_3	0.0026 ± 0.0012	0.0031 ± 0.0002
K_{HX}	2.225 ± 0.8578	0.1262 ± 0.0335*
K_{XH}	0.3978 ± 0.7360	0.0010 ± 0
K_{BAG}	0.0875 ± 0.0250	0.5375 ± 0.1797*

Values are means ± SD, N = 4.
* $P < 0.01$.

RESULTS

Concentration–time profiles for acetaminophen and AG in serum, biliary excretion rate profiles, and the computer-generated best fit of model 1 and model 2 equations to the data in representative rats are depicted after vehicle (Fig. 3) and phenobarbital (Fig. 4) pretreatment. Tables 1 and 2 contain the model-derived parameter estimates for models 1 and 2, respectively. No model-dependent differences were observed in the computer-generated versus measured acetaminophen and AG serum concentration–time profiles. In all cases, predicted concentrations of AG in serum underestimated measured concentrations at early time points (up to approximately 100 min). The AG biliary excretion rate profile was described better by model 2, based on visual inspection of the computer-generated best fit curves to the AG biliary excretion rate–time profiles and calculated AIC values (Table 3). Table 3 also contains the ratio of the AIC values determined from model 2 and model 1. The AG formation rate constant was increased statistically after phenobarbital pretreatment according to model 1. With this model, phenobarbital pretreatment also increased significantly the rate constant for AG egress into blood and decreased significantly the AG biliary excretion rate constant. The parameter estimates based on model 2 indicated that the rate constant describing AG binding to the cytosolic compartment was decreased significantly after phenobarbital pretreatment. In contrast to model 1, with model 2 phenobarbital pretreatment did not alter the AG formation rate constant and increased significantly the estimate of the AG biliary excretion rate constant. The AIC values determined with model 2 were lower than the respective values with model 1 after vehicle and phenobarbital pretreatment.

The *in vitro* protein binding of acetaminophen, AG, and AS in serum (N = 5) and hepatic cytosol (N = 7–8) obtained from naive rats was determined after ultrafiltration. Protein concentrations in serum and hepatic cytosol ranged from 47.7 to 63.9 mg/mL. Protein binding was independent of substrate concentration over the range studied (2.5 to 100 μg/mL for acetaminophen, AG and AS). The unbound fractions (mean ± SD) of acetaminophen, AG and

Table 3. Calculated Akaike's information criterion

	Pretreatment	
	Vehicle	Phenobarbital
Model 1	90.96 ± 20.29	42.62 ± 20.60
Model 2	73.06 ± 14.51	37.62 ± 21.86
Model 2/Model 1	0.814 ± 0.107	0.847 ± 0.150

Values are means ± SD, N = 4.

Table 4. Total protein and subcellular distribution of acetaminophen (APAP) and AS in vehicle (V)- and phenobarbital (PB)-pretreated rats

	Total protein (mg)		APAP (μg/mg protein)		AS (μg/mg protein)	
	V	PB	V	PB	V	PB
Nuclear	1281	1003	0.213	0.049	0.045	0.036
Mitochondrial	79.2	157.2	0.284	0.043	0.085	0.040
Lysosomal	87.6	89.4	0.709	0.404	0.174	0.062
Microsomal	201.6	317.8	0.218	0.023	0.062	0.024
Cytosol	499.8	510.8	0.390	0.062	0.383	0.205

Values were obtained from one vehicle- and one phenobarbital-pretreated rat.

AS in serum were 0.73 ± 0.04, 1.07 ± 0.01, and 0.46 ± 0.03, respectively, and were 0.90 ± 0.08, 0.97 ± 0.08, and 0.88 ± 0.06, respectively, in hepatic cytosol. Phenobarbital pretreatment (N = 3) did not alter significantly the unbound fractions of acetaminophen (0.97 ± 0.03), AG (0.98 ± 0.02) or AS (0.89 ± 0.01) in hepatic cytosol.

Marker enzyme activities in liver homogenate and subfractions were examined to assess the purity of the various fractions [12]. In all cases, adequate separation of the subfractions was demonstrated. The total amounts of acetaminophen and AS were highest in the nuclear and cytosolic fractions. However, when normalized for total protein content in both the vehicle- and phenobarbital-pretreated rats, acetaminophen appeared to be distributed relatively evenly throughout the fractions with a slightly higher localization in lysosomes (Table 4). Phenobarbital pretreatment decreased acetaminophen content by approximately 6-fold in all fractions except for lysosomes, where the decrease was less than 2-fold. AS, normalized for protein content in each fraction, was localized predominantly in cytosol and to some extent in lysosomes. Phenobarbital pretreatment decreased AS content in all fractions approximately 2-fold. The most striking observation was that AG was detected only in the cytosolic fraction in vehicle- and phenobarbital-pretreated rats. AG concentrations in cytosol were decreased approximately 6-fold after phenobarbital pretreatment (from 1.01 to 0.160 μg/mg protein).

DISCUSSION

Pharmacokinetic modeling can be useful in supporting mechanistic hypotheses in conjunction with data from *in vitro/in vivo* studies. Events governing the hepatobiliary disposition of a compound include passive or active uptake, metabolism, binding, and passive or active egress via the blood or bile [17, 18]. Despite these complexities, simple linear models are often sufficient to describe disposition. A well-stirred hepatic model with linear processes was developed to describe the *in vivo* hepatobiliary disposition of acetaminophen and its primary metabolite AG [8]. Attempts were not made to incorporate AS hepatobiliary disposition into the model (other than via K_{10}), thereby focusing on the AG hepatobiliary profile. AG disposition was chosen instead of AS because impairment of biliary excretion after phenobarbital pretreatment was more prominent for AG. The first model described inadequately the concentration–time profile of AG in serum and the biliary excretion rate–time profile of AG. Two hypotheses were proposed to explain the poor fit of the model to the data: (1) carrier-mediated transport of AG exists across the canalicular and sinusoidal membranes, and/or (2) reversible binding of AG occurs within a discrete cytoplasmic pool that was available for excretion into bile. Attempts to incorporate active transport components at various sites in the hepatocyte resulted in an unwieldy model. This, in part, was due to the large number of parameters and the lack of reasonable initial parameter estimates, particularly for the Michaelis–Menten constants. Therefore, the first hypothesis was not pursued further in this study.

The presence of a discrete binding compartment that facilitates the translocation of generated AG from the endoplasmic reticulum to a canalicular transport system was investigated by pharmacokinetic modeling. Conflicting reports [3, 19] have been published regarding the role of the endoplasmic reticulum in the biliary excretion of metabolized and non-metabolized compounds. A subsequent study by Levine and Singer [4] proposed a role for the cytosolic protein, ligandin, in the hepatic egress of compounds. Ligandin (glutathione *S*-transferase B) binds organic anions and is induced after phenobarbital administration [17]. The inclusion of a cytosolic binding compartment in model 2 improved the ability of the model to predict the data, as evidenced by the reduced AIC values. The model yielding the minimum AIC is regarded as most representative of the observed data. The AIC value is dependent on the magnitude of the data points, the number of observations, and the number of parameters [11]. Therefore, model 2, which contained a greater number of parameters compared to model 1 (10 versus 8, respectively), but yielded lower AIC values, provides a more accurate description of the data regardless of pretreatment.

The parameter estimates generated by each model yielded some interesting observations. Estimates of the AG formation rate constant and sinusoidal egress rate constant were increased after phenobarbital pretreatment according to model 1. Phenobarbital pretreatment also decreased the rate constant

governing AG excretion into bile. These data are consistent with the expected induction of uridine-5′-diphosphoglucuronyltransferase, the induction of a sinusoidal carrier system responsible for egress of AG [9], and the impairment of AG excretion in bile, possibly due to a competing phenobarbital metabolite [8]. Parameter estimates determined according to model 2 (including a discrete cytosolic compartment) revealed a decrease in the rate constant regulating intracellular binding of AG, as well as an increase in the AG biliary excretion rate constant after phenobarbital pretreatment. Results based on this model suggested that phenobarbital pretreatment induced the canalicular transport system responsible for AG biliary excretion, but not the AG sinusoidal transport system, and that a concomitant impairment of AG binding in the cytosolic compartment accounted for the decreased AG excretion into bile.

The protein binding of several glucuronide conjugates in hepatic cytosol has been reported [20] and has been correlated with the molecular weight of the conjugates. No cytosolic binding was detected for 2-aminophenyl glucuronide (mol. wt 285) and 4-nitrophenyl glucuronide (mol. wt 315), whereas 15% of phenolphthalein glucuronide (mol. wt 495) was bound in the cytosol. Based on these results, acetaminophen glucuronide (mol. wt 327) may not be expected to bind to cytosolic proteins appreciably, as was observed in the present investigation. Likewise, acetaminophen and AS were not extensively bound to cytosolic proteins. Protein binding of acetaminophen in serum was consistent with a previous study reporting an unbound fraction of 0.73 [21]. AS binding in serum was moderate, whereas AG was not bound to serum proteins. Subcellular localization of acetaminophen, AG and AS in the liver appears to be related primarily to lipophilicity; no unusual compartmentalization was noted after vehicle or phenobarbital pretreatment. Lower hepatic AG concentrations after phenobarbital pretreatment are consistent with the rapid hepatic efflux of AG noted *in vivo*.

The appropriateness of a model, as determined by a reduction in the sum of squares or the AIC, does not guarantee physiological relevance. Despite the decrease in the AIC values compared to values derived from model 1, model 2 is not correct. Subsequent investigations of the cytosolic binding and subcellular distribution of acetaminophen and metabolites were inconsistent with a hypothetical, hepatic cytosolic compartment that binds AG. Interestingly, although visual inspection of Figs. 3 and 4 confirms that the AG biliary excretion rate–time profiles are described better by model 2, no apparent improvement is seen in the description of the AG concentration–time profile in serum. The computer-generated serum AG concentrations at early time points underestimated consistently the actual concentrations. Inclusion of an active transport component for sinusoidal egress of AG, in concert with the linear rate constant may have described the AG serum profile more accurately if additional data were available.

These results stress the importance of mechanistic information in the development of an accurate model. Impaired biliary excretion of AG after phenobarbital pretreatment is not due to alterations in binding of AG within a discrete hepatic compartment. Future studies will focus on the role of carrier-mediated transport (sinusoidal and canalicular) in the impaired biliary excretion of AG by phenobarbital.

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