Metabolic Rate Constants for Hydroquinone in F344 Rat and Human Liver Isolated Hepatocytes: Application to a PBPK Model

Torka S. Poet,*† Hong Wu,* J. Caroline English,‡ and Richard A. Corley*

*Battelle, Pacific Northwest Division, Center for Biological Monitoring and Modeling, P.O. Box 999, Richland, Washington 99352; †Health and Environmental Laboratories, Eastman Kodak Companies, Rochester, New York 14652

Received April 13, 2004; accepted July 15, 2004

Hydroquinone (HQ) is an important industrial chemical that also occurs naturally in foods and in the leaves and bark of a number of plant species. Exposure of laboratory animals to HQ may result in species-, sex-, and strain-specific nephrotoxicity. The sensitivity of male F344 versus female F344 and Sprague-Dawley rats or B6C3F1 mice appears to be related to differences in the rates of formation of key nephrotoxic metabolites. Metabolic rate constants for the conversion of HQ through several metabolic steps to the mono-glutathione conjugate and subsequent detoxification via mercapturic acid formation were measured in suspension cultures of hepatocytes isolated from male F-344 rats and humans. A mathematic kinetic model was used to analyze each metabolic step by simultaneously fitting the disappearance of each substrate and the appearance of subsequent metabolites. An iterative, nested approach was used whereby downstream metabolites were considered first, and the model was constrained by the requirement that rate constants determined during analysis of individual steps must also satisfy the complete, integrated metabolism scheme, including competitive pathways. The results from this study indicated that the overall capacity for metabolism of HQ and its mono-glutathione conjugate is greater in hepatocytes from humans than in those from rats, suggesting a greater capacity for detoxification of the glutathione conjugates in humans. Metabolic rate constants were applied to an existing physiologically based pharmacokinetic model, which was used to predict total glutathione metabolites produced in the liver. The results showed that body burdens of these metabolites will be much higher in rats than in humans.

Key Words: physiological model; bioactivation; nephrotoxicity; species-specific.

Hydroquinone (HQ) is present in natural products such as wheat and some types of fruit, and it is used in photographic developing solutions, in rubber and vinyl monomer manufacturing (Deisinger et al., 1996), and in naturopathic remedies as an antioxidant (Wittig et al., 2001). In addition, HQ is a metabolite of the industrial solvent, benzene, as recently reviewed by Lovern et al. (1999). A consequence of the industrial, consumer, and natural sources of HQ is a high potential for low-level human exposures.

Two chronic bioassays (NTP 1989; Shibata et al., 1991) with HQ have indicated increases in the severity of chronic nephropathy and renal adenomas in male F-344 rats in regions of the kidney that were significantly affected by chronic progressive nephropathy (Wynnser et al., 1995; Hard et al., 1997). Marked gender and species differences in the nephrotoxicity of HQ have been noted. Long-term exposure studies indicate that the kidneys of male F-344 rats are more susceptible to adverse effects associated with HQ exposure than kidneys in female rats, other strains of rats, mice, or dogs (Boatman et al., 1996; English et al., 1994; NTP 1989; Shibata et al., 1991). In a retrospective study on human mortality from subjects working in an HQ manufacturing plant, no increase in mortality was attributable to renal disease or tumors (Pifer et al., 1995).

The mechanism of HQ-induced nephropathy and the gender, strain, and species differences in susceptibility have not been fully elucidated. The gender difference may simply be due to spontaneous nephropathy progressing more rapidly in male than female rats (Sandberg et al., 2003; Seely et al., 2002). In addition, species differences in metabolism may be a factor in the extent of nephrotoxicity resulting from HQ exposures. Metabolism to reactive intermediates is clearly a factor in the renal toxicity and exacerbation of chronic progressive nephropathy associated with HQ ingestion in laboratory animals. While glucuronide and sulfate conjugation are the primary pathways for detoxification, accounting for as much as 90–99% of the administered dose in rats, the minor glutathione conjugation pathway appears to be responsible for renal toxicity and, ultimately, tumorigenicity (DeCaprio 1999). Other studies have indicated that benzoquinone might represent a reactive and mutagenic metabolite, but the processing of DNA damage appears to be different in human and mouse cells (Nakayama et al., 2000). Gender-specific enzyme induction after repeated dosing may also be a factor (Boatman et al., 1996).

The complex metabolism and associated toxic potential of specific HQ metabolites epitomizes the importance of understanding the balance between species-, strain- and gender- specific activation versus detoxification pathways. A simplified
metabolism scheme for HQ is shown in Figure 1. Toxicity associated with the glutathione pathway is attributed to either protein binding or redox cycling of hydroquinone–benzoquinone glutathione conjugates. Each of the steps leading to the formation of the mono-glutathione conjugate followed by either additional glutathione conjugations or metabolism to the nontoxic mercapturic acid is catalyzed by enzymes that have been demonstrated with other substrates to have tissue, sex, strain, and species differences. If such differences apply to HQ, they will significantly improve assessments of the nephrotoxic and tumorigenic potential of HQ. Therefore, the purpose of the study was to determine the rate constants for the metabolism of HQ to the mono-glutathione conjugate and its subsequent detoxification via the mercapturic acid pathway in male F-344 rats and humans, using isolated hepatocytes in suspension culture (steps 1–3, Fig. 1). This is a different approach than commonly taken, following the presumably nontoxic conjugation pathway to the mercapturic acid rather than following the mono-glutathione conjugate to the toxic di-, tri-, and (non-toxic) tetra-glutathione conjugates. The mercapturic pathway was investigated in these studies to determine species differences in nontoxic metabolites, metabolism to which will compete for production of the toxic moieties and may show important species differences.

A physiologically based pharmacokinetic (PBPK) model was previously developed to quantify the potential differences in metabolism of HQ in different species and to integrate those differences with mode of action to improve risk assessments (Corley et al., 2000). Metabolic rate constants determined from the current study were incorporated into the PBPK model to replace assumed values for the metabolism of hydroquinone via the glutathione pathway to decrease the uncertainties associated with simulations of blood and urinary excretion of HQ and its metabolites in rats and humans.

**MATERIALS AND METHODS**

**Study design.** An iterative, nested, in vitro metabolism study design coupled with pharmacokinetic modeling was used to determine apparent metabolic rate constants for the conversion of HQ and subsequent metabolites in hepatocytes isolated from male F-344 rats and human accident victims. Isolated hepatocytes were used because (1) they contain all of the enzymes associated with the metabolism of HQ, (2) they should have less nonspecific protein binding than homogenates (cellular compartmentalization is maintained), and (3) there are data indicating that hepatocytes may be more predictive of in vivo systems and therefore more useful for whole-body extrapolations (see reviews by Houston 1994; MacGregor et al., 2001).

Using the nested approach to assess metabolic rate constants involved the initial determination of the terminal metabolic rate constant, after which each rate constant was fixed as each “upstream” metabolite was sequentially used as a substrate in hepatocytes incubations. Thus, the rate constants associated with the last (furthest downstream) step in the formation of the mercapturic acid (N-acetyl cysteine conjugate of HQ; HQ-NAcCys), the reversible acetylation/de-acetylation reactions of HQ-Cys to HQ-NAcCys (step 1 in Fig. 1), were determined first. Once these constants were estimated, studies were conducted to calculate the rate constants for the formation of the cysteine conjugate of HQ (HQ-Cys) from the mono-glutathione conjugate of HQ (HQ-SG). These rate constants were determined by analysis of the simultaneous loss of HQ-SG and the appearance of HQ-Cys and its metabolites, using the rate constants for the acetylation/de-acetylation reactions previously determined, to account for the further metabolism of HQ-Cys (step 2 in Fig. 1).

This process of adding a specific metabolite as a substrate and simultaneously determining its disappearance with the appearance of downstream metabolites and analysis with a pharmacokinetic model was repeated at each step moving sequentially upstream to HQ (ending with step 3, Fig. 1). The pharmacokinetic model was further constrained by the requirement that rate constants determined during analysis of individual steps 1–3 in the metabolism of hydroquinone via the mono-glutathione conjugate pathway must also satisfy the complete, integrated metabolism scheme, including competitive metabolic pathways such as the formation of di-, tri-, and tetra-glutathione conjugates and the major pathways of glucuronide and sulfate conjugation of HQ, although the metabolism to multi-conjugated species was not measured and only the di-conjugate was modeled in vitro. For human hepatocyte studies, samples from the same individuals were used to determine parameter values for each sequential step.
**Isolation of rat hepatocytes.** Hepatocytes were isolated from anesthetized male F-344 rats using a two-step *in situ* perfusion with collagenase (McQueen, 1989). Viability was assessed based on plasma membrane integrity by trypan blue dye exclusion, with only cell preparations with >90% viability used in subsequent experiments. Rat weights and cell yields for the isolation of hepatocytes were used for extrapolation of rate constants for use in *in vivo* PBPK models and are given in Table 1.

**Human samples.** Cryopreserved isolated hepatocytes from human donors were purchased from In Vitro Technologies (Baltimore, MD). Cryopreserved hepatocytes have recently become a viable option for human hepatocyte metabolism studies because they are easier to obtain and use in suspension cultures than fresh hepatocytes, and because studies have demonstrated the validity of metabolic parameters determined in these cell preparations (Hengstler et al., 2000; Hewitt et al., 2001; Li et al., 1999; Naritomi et al., 2003). Samples from six individual male donors were evaluated. The hepatocytes were supplied with some demographic information such as gender, pathogen screen, and some enzyme activity profiles, but they were not identified in any manner with the tissue donor. The Battelle Institutional Review Board reviewed the procurement process and determined the project to be exempt from review in accordance with Federal Regulation 10 CFR 745.101(b) (4). Samples were identified in these studies as provided by In Vitro Technologies. Demographic data for each sample are given in Table 2.

**Test material.** Photographic grade HQ (99% pure) was obtained from Aldrich Chemical Co (Milwaukee, WI). The mono-glutathione conjugate of HQ (HQ-GSH: 2-(glutathione-S-yl)hydroquinone, 99.8% purity), the cysteine conjugate (HQ-CYS: 2-(L-cysteine-S-yl)hydroquinone, 99.6% purity), and the N-acetylcysteine conjugate (HQ-NAC: 2-(N-acetylcystein-S-yl)hydroquinone, >99.9% purity) were kindly provided by Eastman Kodak Company (Health and Environmental Laboratories, Rochester, NY). All other chemicals were obtained at the highest grade possible from commercial sources.

**Incubation procedures.** To experimentally and mathematically isolate the steps in the metabolism of HQ (Fig. 1), a nested, sequential metabolite incubation approach working from the terminal steps upstream to HQ was designed to regulate the temperature (72 °F ± 3 °C), relative humidity (50 ± 20%), airflow and 12 h photocycle, with 15 to 20 air changes/h. Deionized water (reverse osmosis) and PMI 5002 Certified Rodent Diet (Animal Specialties, Inc., Hubbard, OR) were provided *ad libitum*. Animal cages were washed regularly in accordance with good husbandry practices. All cages contained a pressure-activated, stainless-steel water nipple. Each animal was observed for morbidity and mortality daily.

### TABLE 1
Hepatocyte Isolations from Male F-344 Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Avg</th>
<th>SD</th>
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<tr>
<td>Body weight</td>
<td>200</td>
<td>234</td>
<td>256</td>
<td>276</td>
<td>256</td>
<td>244.4</td>
<td>28.9</td>
</tr>
<tr>
<td>Viability</td>
<td>89</td>
<td>92</td>
<td>92</td>
<td>89</td>
<td>91</td>
<td>90.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Cells/ml (10⁶)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>160</td>
<td>173</td>
<td>150</td>
<td>169</td>
<td>166</td>
<td>163.6</td>
<td>9.0</td>
</tr>
<tr>
<td>Total viable cells</td>
<td>4.8 × 10⁶</td>
<td>5.2 × 10⁶</td>
<td>4.5 × 10⁶</td>
<td>5.1 × 10⁶</td>
<td>5.0 × 10⁶</td>
<td>4.91 × 10⁶</td>
<td>2.7 × 10⁶</td>
</tr>
<tr>
<td>Viable cells/g liver</td>
<td>4.9 × 10⁷</td>
<td>5.3 × 10⁷</td>
<td>4.6 × 10⁷</td>
<td>5.2 × 10⁷</td>
<td>5.1 × 10⁷</td>
<td>5.02 × 10⁷</td>
<td>2.7 × 10⁷</td>
</tr>
<tr>
<td>Viable cells/kg</td>
<td>9.60 × 10⁷</td>
<td>1.21 × 10⁸</td>
<td>1.15 × 10⁸</td>
<td>1.40 × 10⁸</td>
<td>1.27 × 10⁸</td>
<td>1.20 × 10⁸</td>
<td>1.62 × 10⁸</td>
</tr>
<tr>
<td>Incubations</td>
<td>HQ, inhibitors, HQ-Cys</td>
<td>HQ, inhibitors, HQ-Cys</td>
<td>HQ, HQ-SG, bromoheptane</td>
<td>HQ, HQ-SG, HQ-Cys</td>
<td>HQ-HQ-SG, bromoheptane</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Viable cells/g liver were calculated based on a presumed liver weight of 40 g liver/kg BW (Brown et al., 1997).*

### TABLE 2
Demographic for Male Human Hepatocyte Donors

<table>
<thead>
<tr>
<th>Lot</th>
<th>Age</th>
<th>% Viabilityᵃ</th>
<th>% Viabilityᵇ</th>
<th>Cause of death</th>
<th>Tobacco useᶜ</th>
<th>Alcohol use</th>
<th>Substance use</th>
<th>Medical history</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEC</td>
<td>48</td>
<td>86</td>
<td>82</td>
<td>Intracranial hemorrhage</td>
<td>Yes</td>
<td>Yes</td>
<td>THC</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>CYE</td>
<td>52</td>
<td>73</td>
<td>73</td>
<td>Intracranial bleed</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>ENR</td>
<td>72</td>
<td>73</td>
<td>76</td>
<td>Intracranial bleed</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Kidney stone</td>
</tr>
<tr>
<td>EQB</td>
<td>72</td>
<td>88</td>
<td>86</td>
<td>Intracranial hemorrhage</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Prostate remove</td>
</tr>
<tr>
<td>IEM</td>
<td>54</td>
<td>85</td>
<td>77</td>
<td>Stroke</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Hypertension</td>
</tr>
<tr>
<td>NQT</td>
<td>53</td>
<td>88</td>
<td>87</td>
<td>Cerebrovascular accident</td>
<td>Yes</td>
<td>Yes</td>
<td>cocaine</td>
<td>Colon polyps</td>
</tr>
</tbody>
</table>

ᵃViability as reported by supplier.
ᵇViability measured in laboratory immediately prior to incubation.
ᶜNo = none reported.
ᵈThis sample exhibited different kinetics than observed in any other sample and was only used for HQ incubations (see text for further details).
employed. The incubation procedures followed those outlined by Anari et al. (1996). The cells were preincubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) with 12.5 mM Hepes for 20 min under an atmosphere of 1% O₂/5% CO₂/94% N₂ in rotating round-bottom flasks in a 37°C water bath. In some cases, the pathway of interest was not the only potential route of metabolism. Isolation of the metabolic rate constants of interest was achieved by monitoring both the loss of the substrate and the appearance of the next metabolite of interest, and assuming that any differences were due to competing metabolism, and, where possible, by using specific metabolic inhibitors. In some preparations, GSH was depleted by including 200 μM bromoheptane in the preincubation (Anari et al., 1996). In other preparations, glucuronidation was inhibited by preincubating with 4 mM D-galactosamine (Carrera et al., 1998).

Pilot studies were conducted for each set of reactions to determine the optimal number of hepatocytes, volumes, and substrate concentrations. Isolated hepatocytes from human or rat liver were incubated with 2–5 concentrations of substrate (HQ-Cys, HQ-GSH, or HQ) for 5–75 min. The time course data were used in the mathematical model as described below. Control incubations with heat-inactivated hepatocytes to measure non-enzymatic changes were also included.

Analytical methods. Analytical methods for simultaneously detecting HQ and its metabolites in suspension cultures of isolated hepatocytes were developed using high-performance liquid chromatography (HPLC). Aliquots of each hepatocyte suspension (50 μl) were placed into small polyethylene microcentrifuge tubes and centrifuged (10,000 rpm, 10 min, 5°C) and the supernatants were removed for analysis. Aliquots of each supernatant (5 to 15 μl) were analyzed by HPLC under the following conditions: an HPLC equipped with a Supelcosil LC-18 column (5 μm, 15 cm × 4.6 mm internal diameter) with pre-column; column oven temperature of 40°C; eluent, 1% (by volume) trifluoroacetic acid, and a ramp of ethanol from 0–99% over 20 min; flow rate of 1.0 ml/min; and a diode array detector. Preliminary methods development indicated that all metabolites could be successfully detected at a wavelength of 308 nm. Retention times were approximately 5.6, 7.1, 9.0, and 10.5 min for HQ, HQ-SG, HQ-Cys, and HQNaCys, respectively.

Mathematical model. The metabolic rate constants were calculated using a mathematical model written for SIMUSOLV (registered trademark of the Dow Chemical Company, Midland, MI), a FORTRAN-based computer program containing a numerical integration, optimization, and graphical routine, which was used to fit the metabolic parameters to the data. The initial equations assumed Michaelis-Menten kinetics, and related rate equations were used to estimate metabolic rate constants from time-course experiments by simultaneously fitting the loss of the parent and the formation of the metabolites by both visual inspection and maximum log likelihood estimation techniques. The mathematical model was expanded after completion of each metabolic step to include the new, upstream metabolic step. For example, for the conversion of HQ-SG to HQ-Cys, the rate constants for the subsequent metabolic step of HQ-Cys, were determined first and held constant (see equations below). The initial glutathione S-transferase-mediated conjugation of HQ was investigated last. Sample equations for the N-acetylation of HQ-Cys, and the concurrent de-acetylation (Eq. 1), the loss of HQ-SG as it is metabolized to HQ-Cys (Eq. 2), and the concurrent formation of HQ-Cys (Eq. 3) are given:

\[
\frac{d[\text{HQ} \cdot \text{Cys}]}{dt} = - \left( \frac{V_{\text{max}} \cdot [\text{HQ} \cdot \text{Cys}]}{K_{\text{m}_{\text{Ac}}} + [\text{HQ} \cdot \text{Cys}]} \right) + \left( \frac{V_{\text{max}_{\text{Ac}}} \cdot [\text{HQ} \cdot \text{NaCys}]}{K_{\text{m}_{\text{NaCys}}} + [\text{HQ} \cdot \text{NaCys}]} \right)
\]

(1)

\[
\frac{d[\text{HQ} \cdot \text{SG}]}{dt} = \left( \frac{V_{\text{max}} \cdot [\text{HQ} \cdot \text{SG}]}{K_{\text{m}_{\text{SG}}} + [\text{HQ} \cdot \text{SG}]} \right)
\]

(2)

\[
\frac{d[\text{HQ} \cdot \text{Cys}]}{dt} = \left( \frac{V_{\text{max}_{\text{Ac}}} \cdot [\text{HQ} \cdot \text{SG}]}{K_{\text{m}_{\text{SG}}} + [\text{HQ} \cdot \text{SG}]} \right) - \left( \frac{V_{\text{max}} \cdot [\text{HQ} \cdot \text{Cys}]}{K_{\text{m}_{\text{Cys}}} + [\text{HQ} \cdot \text{Cys}]} \right) + \left( \frac{V_{\text{max}_{\text{Ac}}} \cdot [\text{HQ} \cdot \text{NaCys}]}{K_{\text{m}_{\text{NaCys}}} + [\text{HQ} \cdot \text{NaCys}]} \right)
\]

(3)

Similar equations to describe the disappearance of substrate (HQ) and the appearance of metabolite (HQ-SG) were used for other incubations. These equations included the rate constants from previous incubations. Apparent rate constants were determined because rate constants were estimated using this iterative, nested approach, rather than by direct determination (which was not experimentally feasible).

Incorporation of apparent metabolic rate constants into PBPK model. Metabolic rate constants for the conversion of HQ to HQ-gluc and HQ-SG, and for HQ-SG to HQ-Cys determined in vitro were allometrically scaled from the in vitro V_{max} using measured parameters of body weight, scaled liver weight (Brown et al., 2000), and raised to 0.74 power and applied to the PBPK model for HQ developed by Corley et al. (2000). As is standard practice, Km values were not extrapolated in any way from the in vitro system to describe the in vivo situation. Using these measured rate constants and maintaining all other parameters used in the original model, only absorption rate constants were adjusted, if such an adjustment was necessary to fit the data.

### Table 3
Comparison of Activities for HQ-Cys and HQ-NaCys Metabolism Measured in the Present Study and from a Previous Report

<table>
<thead>
<tr>
<th>Sample</th>
<th>Current study acetylation</th>
<th>Current study deacetylation</th>
<th>Barber et al.(^a) acetylation</th>
<th>Barber et al.(^a) deacetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max}</td>
<td>nmol/min/10^6 cells(^c)</td>
<td>nmol/min/10^6 cells</td>
<td>nmol/min/1.1 mg</td>
<td>nmol/min/1.1 mg</td>
</tr>
<tr>
<td>Male F-344 kidney microsomes or cytosol(^b)</td>
<td>1.71</td>
<td>0.533</td>
<td>1.5</td>
<td>ND</td>
</tr>
<tr>
<td>Male F-344 isolated hepatocytes</td>
<td>10.8</td>
<td>4.57</td>
<td>6.8</td>
<td>0.23</td>
</tr>
</tbody>
</table>

\(^a\)Barber et al., 1996.

\(^b\)Acetylation was determined in microsomes and deacetylation in cytosol.

\(^c\)The estimated yield of isolated hepatocytes is 5.02 × 10^7 cells/g liver (Table 1). That of microsomes is 45 mg protein/g liver (Houston, 1994), therefore, 1 × 10^6 cells should be similar to approximately 1.1 mg microsomes.
RESULTS

Metabolism of HQ-Cys to HQ-NacCys in Rat Liver Hepatocytes

The furthest downstream metabolic step of interest was the metabolism of the cysteine conjugate, HQ-Cys to the N-acetyl cysteine (step 1, Fig. 1). This reversible reaction is mediated by N-acetylase and deacetylase enzymes, which have previously been shown to have species and gender differences (Barber et al., 1996). Isolated hepatocytes were incubated with target concentrations of 8.9, 110, or 220 μM HQ-Cys, and the media concentrations of HQ-Cys and HQ-NacCys were monitored at timepoints ranging from 5 to 75 min (Fig. 2). Parent HQ-Cys concentrations declined slightly, whereas HQ-NacCys concentrations rapidly reached a peak by 15 min of incubation.

The mathematical model was used to estimate the apparent Michaelis-Menten rate constants by simultaneously fitting the model to the concentrations of HQ-Cys and HQ-NacCys over time. Initial estimates of species-specific metabolic rates previously determined by Barber et al. (1996) in rat and human liver microsomes (Table 3) were used as starting parameters for mathematical model optimization. Apparent K_m's and V_max's are summarized in Table 4.

Metabolism of HQ-SG in Rat Liver Hepatocytes

Isolated hepatocytes were incubated with 24, 120, or 1200 μM HQ-SG, and the media concentrations of HQ-SG, HQ-Cys and HQ-NacCys were monitored in aliquots sampled at times from 5 to 75 min by HPLC (Fig. 3). Bromoheptane was initially used to inhibit the conjugation of additional glutathione side-chains, but this inhibition was incomplete and nonspecific, so it was not possible to completely isolate the metabolism of the monoglutathione metabolism pathway. The incomplete inhibition was verified in isolated hepatocytes incubated with HQ and bromoheptane. In these samples, the production of HQ-SG was approximately half the production without bromoheptane (data not shown). Therefore, metabolic rate constants for the conversion of HQ-SG to the di-conjugate were optimized from the HQ-SG loss from the system while compensating for the loss of HQ-SG, leading to HQ-Cys and HQ-NacCys to maintain mass-balance.

The HQ-SG levels visibly dropped over the course of the incubation as the initial HQ-SG was metabolized. Levels of HQ-Cys reached approximately 1.5 nmol/ml and concentrations of HQ-NacCys reached approximately 0.12 nmol/ml by 75 min of incubation in the highest HQ-SG incubations. At the two lower starting concentrations, both metabolites leveled out after approximately 20 min. In hepatocytes exposed to the highest concentration, metabolite levels continued to increase over the course of the incubation.

The Michaelis-Menten parameters for the metabolism of HQ-Cys to HQ-NacCys were optimized based on the metabolism of HQ-Cys (Fig. 2) at K_m = 8.8 μM, V_max = 1.71 nmol/min/10^6 cells. However, this did not result in an acceptable fit to the data, so the K_m was optimized from this experiment. A substantial improvement to the fit was obtained by holding all parameters for the metabolism of HQ-Cys and HQ-NacCys constant from the HQ-Cys exposures, except the K_m for the metabolism of HQ-Cys, which was optimized to be 1.14 μM. Metabolic rate constants are given in Table 4.
Isolated hepatocytes were incubated with 45, 91, 454, 910 μM HQ, and the media concentrations of HQ, HQ-SG, HQ-Cys, and HQ-NacCys were monitored in aliquots sampled at times from 5 to 75 min (Fig. 5). The amount of HQ-SG produced was dependent on the initial concentration of HQ. Pre-incubation with bromoheptane resulted in twofold lower production of HQ-SG in these samples, but it did not completely inhibit the formation of HQ-SG (data not shown). The levels of HQ-Cys were approximately 1/20 those of the HQ-SG. At the lowest exposure concentration, no HQ-Cys was detected. At the lower two exposure concentrations, HQ-NacCys could not be detected in the media. The limit of detection for HQ-NacCys in these samples was approximately 0.04 nmol/ml.

The mathematical model was used to estimate rate constants by fitting to the concentrations of HQ and HQ-SG over time. Rate constants for the conversion of HQ-SG and HQ-Cys were held constant from incubations with the different starting material. The final set of metabolic rate constants for all metabolites of interest is shown in Table 4.

### Metabolism of HQ-Cys to HQ-NacCys in Human Donor Hepatocytes

Cryopreserved human hepatocytes from five different donors were incubated with 11 or 110 μM HQ-Cys and aliquots were removed for analysis by HPLC at 5–75 min of incubation. The decline in HQ-Cys concentrations was barely perceptible, but concentrations of HQ-NacCys increased over time (Fig. 6). Maximal amounts of HQ-NacCys from the higher exposure concentration varied from a high of 3.4 nmol/ml (sample CEC) to a low of 1.1 nmol/ml (sample ENR; data not shown). For simplicity, data from two representative male donor samples are shown (CEC and EQB).

As with the determination of metabolic rate constants for acetylation/deacetylation of HQ-Cys in rats, the metabolism rates previously determined in microsomes by Barber...
et al. (1996) were scaled to hepatocytes and used as initial estimates for model optimization to the data from this study (Table 3). Rate constants optimized for the metabolism of HQ-Cys in cryopreserved human hepatocytes are shown in Tables 5 and 6.

**Metabolism of HQ-SG in Human Donor Hepatocytes**

Cryopreserved human hepatocytes from five different donors were incubated with 12, 60, or 240 μM HQ-SG, and aliquots were removed for analysis by HPLC at 5–75 min of incubation. HQ-SG levels dropped perceptibly, and HQ-Cys levels steadily increased over the 75 min of incubation. In these samples, the

![Figure 3](https://example.com/figure3.png)

**FIG. 3.** Metabolism of HQ-SG (A) to HQ-Cys (B) and HQ-NacCys (C) in hepatocytes isolated from male F-344 rats. Initial HQ-SG concentrations were as follows: 1200 (circle), 120 (square), and 24 nmol/ml (diamond). There were $3 \times 10^6$ viable cells/ml. Each data point represents a determination in $N = 5$ hepatocyte isolations from individual rats. Lines represent the pharmacokinetic model simulations.

![Figure 4](https://example.com/figure4.png)

**FIG. 4.** Metabolism of HQ to HQ-SG, HQ-Cys, and HQ-NacCys in hepatocytes isolated from male F-344 rats with and without galactosamine. Galactosamine was used to inhibit glucuronidation. For simplicity, only initial HQ concentration of 450 nmol/ml in samples preincubated with 4 mM galactosamine (diamonds) or without galactosamine (squares) are shown. Additional samples were incubated with 45, 90, and 910 μM HQ. There were $3 \times 10^6$ viable cells/ml. Each data point represents a determination in $N = 6$ hepatocyte isolations from two individual rats. Lines represent the pharmacokinetic model simulations. (A) HQ parent, (B) HQ-SG production, (C) HQ-Cys production, (D) HQ-NacCys.
maximal production of HQ-Cys was fairly consistent, but the maximal production of HQ-NacCys varied from less than 0.5 nmol/ml to more than 2 nmol/ml (Fig. 7).

Metabolic rate constants were determined in these samples using the mathematical model. Just as had been observed in rat isolated hepatocytes, the $K_m$ optimized for the conversion of HQ-Cys to HQ-NacCys did not result in an acceptable fit to the data when the starting material was HQ-SG; lower $K_m$ values were required. The average optimal $K_m$ determined from the HQ-Cys incubations was $4.163 \pm 1.045 \mu M$, five times higher than the value needed to fit the data with metabolism of HQ-SG (Table 5). An example of the effect of the different $K_m$ values on the fit of the data is shown in Figure 8. All other metabolic rate constants for the conversion of HQ-Cys were held constant from previous experiments with HQ-Cys as the starting material.

The loss rate of HQ-SG was not fully explained by the appearance of HQ-Cys; to achieve mass-balance, the rate constants were optimized for the conversion of HQ-SG to the di-glutathione conjugate (Table 5).

In an attempt to inhibit GSH transferase-mediated production of the di-GSH, we included bromoheptane in some samples during that initial 20-min preincubation period. A mass-balance of the metabolite production data indicated that this inhibition was incomplete. In later incubations with HQ, preincubation with bromoheptane resulted in a twofold lower production of HQ-SG, but it did not completely inhibit the production of HQ-SG. In addition, the production of HQ-Cys in samples containing bromoheptane was nearly five times lower than in samples without bromoheptane, indicating that the inhibitor was nonspecific (data not shown).

**Metabolism of HQ in Human Donor Hepatocytes**

Cryopreserved hepatocytes prepared from six different donors were incubated with 45, 227, or 2270 nmol/ml HQ, and aliquots were analyzed by HPLC, at times ranging from 5 to 75 minutes. All three metabolites of interest were detected in these samples, with the exception of HQ-NacCys in hepatocytes suspensions from the lowest dosing concentration from samples CEC (Fig. 9) and ENR (data not shown). Peak Levels of HQ-SG ranged from 60 to 100 nmol/ml (Fig. 9). Peak levels of each downstream metabolite were approximately tenfold lower than the metabolite from which they were produced.

All rate constants determined in the nested metabolism studies were held constant. Some samples contained D-galactosamine to inhibit glucuronidation. With the exception of sample CYE, a single set of metabolic rate constants to describe the production of the glucuronide was determined to result in an acceptable fit to the data in these inhibited samples (Table 5).

Sample CYE exhibited markedly higher loss of HQ with a concomitant decline in the levels of HQ-SG recovered (Fig. 10). These results were explained by using a $V_{\text{max}}$ of 33.4 nmol/min/mg for the conversion of HQ to the glucuronide, two times higher than the $V_{\text{max}}$ determined to fit to the data from the other donor.

**FIG. 5.** Metabolism of HQ (A) to HQ-SG (B), HQ-Cys (C), and HQ-NacCys (D) in hepatocytes isolated from male F-344 rats. Initial HQ concentrations were: 910 (circle), 450 (triangle), 91 (square), and 46 (diamond) nmol/ml. There were $3 \times 10^6$ viable cells/ml. Each data point represents a determination in $N = 5$ hepatocyte isolations from individual rats. Lines represent the pharmacokinetic model simulations. HQ-Cys metabolite data for the 46 nmol/ml concentrations were below detection limits. HQ-NacCys metabolite data for the 91 and 46 nmol/ml concentrations were below detection limits.
samples. Because of the variability in apparent glucuronidation in this sample, incubations with more downstream metabolites (HQ-SG and HQ-Cys) were not conducted. To reduce the number of variables as much as possible, the average $K_m$ for all metabolic steps was used to optimize for $V_{\text{max}}$ in this sample. This sample apparently exhibited much higher metabolism for the di-conjugated glutathione pathway as well ($V_{\text{max}}$ of 12.6 nmol/min/10^6 cells). Like the results with D-galactosamine

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**TABLE 5**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (nmol/min/10^6 cells)</th>
<th>Intrinsic clearance ($V_{\text{max}}/K_m$)</th>
<th>$V_{\text{max}}^C$ (mg/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ → HQ-SG</td>
<td>277 ± 38.8</td>
<td>5.60 ± 0.81</td>
<td>20.2</td>
<td>29.6</td>
</tr>
<tr>
<td>HQ-SG → HQ-Cys</td>
<td>14.9 ± 3.46</td>
<td>0.55 ± 0.055</td>
<td>37.2</td>
<td>11.3</td>
</tr>
<tr>
<td>HQ-SG → HQ-diSG</td>
<td>162 ± 50.5</td>
<td>3.60 ± 0.52</td>
<td>22.3</td>
<td>73.0</td>
</tr>
<tr>
<td>HQ-Cys → HQ-NacCys</td>
<td>767 ± 333</td>
<td>10.8 ± 4.79</td>
<td>14.1</td>
<td>127.4</td>
</tr>
<tr>
<td>HQ-NacCys → HQ-Cys</td>
<td>38.9 ± 11.0</td>
<td>4.57 ± 2.61</td>
<td>117</td>
<td>78.0</td>
</tr>
<tr>
<td>HQ → HQ-Gluc</td>
<td>46.0</td>
<td>15.2</td>
<td>330</td>
<td>84.3</td>
</tr>
</tbody>
</table>

$^a$Values are mean ± SD of 5 male human donor samples. Range for $K_m$ is given in parentheses; for $V_{\text{max}}$ see Table 6.

$^b$V_{\text{max}}C was allometrically scaled from the average in vitro $V_{\text{max}}$ using estimated parameters of body weight 82.5 (Roy et al., 2003), scaled liver weight (Brown et al., 1997), and measured number of isolated hepatocytes/g liver (Table 3) and raised to 0.74.

$^c$HQ-diSG = HQ di-glutathione conjugate of HQ.

$^d$The rate of metabolism of HQ to the glucuronide was optimized starting from the data of Seaton et al. (1995) by fitting to the loss rate of HQ in samples preincubated with and without inhibition of glucuronidation.

$^e$For this reaction is given as used for metabolism of HQ parent; the $K_m$ used to fit the metabolism of HQ-Cys was higher (see text).
D-galactosamine. 

metabolism of HQ-SG to HQ-Cys is also inhibited by treatment in rat isolated hepatocytes, it appears that the mechanism involving the renal toxicity of HQ or its metabolites does not follow the sulfate conjugates (Deisinger et al., 1999; NTP 1989; Whysner et al., 1988). High levels of the sulfate and glucuronide metabolites identified in Figure 1 that will be important in elucidating potential species differences in the production of renally toxic metabolites. To best isolate each individual step in the sequential metabolism of HQ, hepatocytes were incubated with HQ or individual metabolites (HQ-SG and HQ-Cys), and changes in media concentrations were measured over time. Metabolic rate constants for each step were determined using a pharmacokinetic model to fit Michaelis-Menten equation rate constants to the metabolite loss and production data simultaneously. Rate parameters were held constant between different incubation conditions.

In vivo and in vitro evidence suggests that HQ causes kidney tumors through a cytotoxicity/compensatory cellular regeneration-based promotional mechanism, not via direct DNA affects (English et al., 1994; NTP 1989; Whysner et al., 1995). Although the renal toxicity of HQ or its metabolites does not follow the mechanism involving β-lyase cleavage of the cysteine conjugate to produce reactive thiols (Monks et al., 1988), intraperitoneal injections with HQ metabolites have shown that the most toxic form is the tri-GSH conjugate. In vivo, the primary metabolites of HQ in Sprague-Dawley rats are the glucuronide and sulfate conjugates (Deisinger et al., 1999; Divincenzo et al., 1984; English et al., 1988). High levels of the sulfate and glucuronide metabolites are excreted in the urine (Whysner et al., 1995), but the glutathione conjugate is selectively reabsorbed by the kidney tubule (Monks and Lau 1990; Whysner et al., 1995).

Although there are other potential mechanisms such as increases in macromolecular binding (Boatman et al., 1996), it has been proposed that multi-glutathione conjugated HQ may exert its nephrotoxic effects via redox cycling (Lau et al., 1988; Whysner et al., 1995). Regardless of the exact mechanism of action, metabolism is clearly a factor in the renal toxicity of HQ. The balance between bioactivation, potentially through the multi-conjugated glutathione metabolites, and deactivation, potentially through the GGT pathway forming mercapturic acids, is likely to be central to the species- and gender-specificity of observed toxicity.

Due to the complexities associated with the metabolism and disposition of HQ, an integrated physiologically based pharmacokinetic model can be an important tool for evaluating dose-, route- and species-dependent kinetics of HQ. Corley et al. (2000) previously developed a PBPK model that described the disposition of HQ in rats and a human male subject. The metabolic rate constants for the conversion of HQ to HQ-gluc and HQ-SG determined in the present study were applied to this model. In addition, the model was modified to include a metabolic description for the further conversion of HQ-SG to the di-GSH conjugate; all other rate constants and parameters from the original model were held constant. Data from the literature from Hill et al. (1993) were used to optimize the intraperitoneal absorption rate of 1.82 h⁻¹ (changed from 1 h⁻¹ in the original model). The intratracheal absorption rate constant of 75 h⁻¹ optimized in the original model from the data of Deisinger and English (1999) was not changed for the model fits because of the updated metabolism rate constants. The fit of the model to the available data did not change appreciably from simulations reported by Corley et al. (2000), confirming earlier assumptions used to estimate metabolic rate constants. Using the rate constants measured in vitro, the model predicts a slightly higher peak blood concentration for reversibly bound and unbound HQ and slower elimination after intratracheal administration (Fig. 11) and a slightly faster rate of total elimination of urinary and biliary conjugated moieties (Fig. 12).

The paper by Corley et al. (2000) also contained a data set from a male human volunteer administered 275 mg HQ orally. The oral absorption rate constant for the human subject was optimized following inclusion of the new rate constants, and the optimization rate was 0.75 h⁻¹, lower than the 1.75 h⁻¹ used in the original model (Fig. 13). Glucuronide conjugation is the initial favored step in the metabolism of HQ in both rats and mice, with humans having a significantly higher capacity (Vmax) and intrinsic clearance (Vmax/Km) than do rats. In human hepatocytes, an overall higher capacity for metabolism was also observed in the relative rates of metabolism of the mono-glutathione conjugate to HQ-Cys, indicating a greater potential for detoxification than that observed in the rat. Human hepatocytes appeared to have a higher capacity (Vmax) for the acetylation/de-acetylation reactions, although rat enzymes appeared to have a higher affinity (lower Km’s). This resulted in higher intrinsic clearances (Vmax/Km) in rat hepatocytes than in human hepatocytes, with the balances favoring acetylation in rat hepatocytes and de-acytelyation in humans. The maximum rate (Vmax), however, favors acetylation over deacetylation in both species. The significantly higher Km’s in human hepatocytes indicate that,

| Table 6: Individual Vmax (nmol/min/10⁶ cells) Values for the Six Human Donor Samples |
|---------------------------------|----------------|--------|---------|--------|--------|--------|
|       | CEC  | CYE a | ENR    | EQB    | IEM    | NQT    |
| HQ → HQ-SG | 6.0  | 2.9   | 5.2    | 6.2    | 4.2    | 4.9    |
| HQ-SG → HQ-Cys | 0.60 | 0.30  | 0.49   | 0.57   | 0.48   | 0.56   |
| HQ-SG → HQ-diSG b | 3.3  | 12.6  | 3.4    | 4.4    | 3.0    | 3.4    |
| HQ-Cys → HQ-NacCys | 10.0 | 11.0  | 12.1   | 8.2    | 18.0   | 7.0    |
| HQ-NacCys → HQ-Cys | 4.6  | 5.9   | 3.6    | 5.2    | 10.3   | 4.9    |

*This sample was not used in the averages reported in Table 5. For an explanation, see the text.

HQ-diSG – HQ di-glutathione conjugate of HQ.
to saturate this pathway, higher concentrations of HQ-Cys and HQ-NacCys would be needed in humans than in rats.

The model was modified by adding the relevant metabolic rates reported in Tables 4 and 5 to best estimate the levels of HQ-Cys produced in the liver of rats and humans (Fig. 14A).

Consistent with the reports that the mercapturate is excreted by HQ-exposed humans at relatively high concentrations compared to experimentally exposed rats, HQ-Cys liver concentrations are predicted to be considerably lower in rats than humans. This is due to the significantly higher intrinsic
clearance of HQ-SG conversion to HQ-Cys estimated in the isolated hepatocytes of humans compared to rats (Tables 4 and 5). The PBPK model indicates that competing acetylation/deacetylation pathways are much less important drivers of the final HQ-Cys concentrations.

By comparison, the rat may be at greater risk for producing the more toxic di-glutathione conjugates than are humans since the intrinsic clearance ($V_{max}/K_m$) for the production of the di-glutathione was significantly greater in rat hepatocytes than in human hepatocytes, whereas the metabolism of HQ-SG to

FIG. 8. A comparison of the effect of $K_m$ on mathematical model predictions of metabolism using human donor CEC sample as an example of (A) production of HQ-NaCys in incubations with HQ-Cys and (B) production of HQ-Cys and HQ-NacCys in incubations with HQ-SG. The low $K_m$ was used in the model to describe metabolism of HQ-SG and HQ (Table 5).
HQ-Cys is lower in rat isolated hepatocytes than in human isolated hepatocytes (Tables 4 and 5). This suggests that the balance of deactivation to bioactivation may be very different in rat and human liver. Indeed, the production of the putative proximal toxicant, HQ-triSG, would be expected to be much higher in F-344 rat liver than in human liver. However, the tissue-specific concentrations of HQ-triSG will depend on stability and translocation of the tri-conjugate and on the stability and

FIG. 9. Metabolism of HQ to HQ-SG, HQ-Cys, and HQ-NacCys in hepatocytes isolated from two representative human male subjects. Initial HQ concentrations were as follows: 2270 (circle), 227 (square), and 45 (diamond) nmol/ml. There were $1.5 \times 10^6$ viable cells/ml. Each data point represents a determination in $N = 3$ hepatocyte incubations from the same individual. Lines represent the pharmacokinetic model simulations.
FIG. 10. Metabolism of HQ (A) to HQ-SG (B), HQ-Cys (C), and HQ-NacCys (D) in hepatocytes isolated from human male subject CYE with and without galactosamine. Galactosamine was used to inhibit glucuronidation. An example of data from incubations with an initial HQ concentration of 227 nmol/ml is shown in samples preincubated with 4 mM D-galactosamine (diamonds) or without D-galactosamine (squares). There were 1.5 $\times$ 10^6 viable cells/ml. Each data point represents a determination in N = 3 incubations from the same individual. Lines represent the pharmacokinetic model simulations.

FIG. 11. Data and simulation of the concentrations of (A) free HQ and (B) reversibly bound HQ in venous blood of male Sprague-Dawley rats administered [14C]HQ by intratracheal administration (Deisinger and English, 1999). The solid lines represent model simulations as parameterized by Corley et al. (2000); dotted lines are simulations using rate constants determined in the present study.

FIG. 12. Cumulative combined amounts of glutathione-conjugated HQ eliminated in the bile and N-acetyl cysteine conjugates eliminated in the urine of male Sprague-Dawley rats given 200 mg/kg intraperitoneal doses of HQ (Hill et al., 1993). The solid lines represent model simulations as parameterized by Corley et al. (2000); dotted lines are simulations using rate constants determined in the present study.
translocation of HQ-diSG substrate and subsequent tissue-specific metabolism to HQ-triSG.

The PBPK model was used to estimate the total amount of di- and higher glutathione conjugates produced from the mono-glutathione conjugate in the liver of an F-344 rat compared to a human after oral absorption of 0.01 to 5000 mg/kg (Fig. 14B). Based on model-predicted oral absorption rates and in the scaled metabolic rate constants, total di- and higher glutathione conjugate moieties are predicted to be considerably greater in rats than in humans. With the currently available information indicating that the di- and tri-glutathione conjugates of hydroquinone are the toxic metabolites, total production of di- and higher glutathione conjugates from the mono-glutathione conjugate represents an improved dose metric for risk assessment. Although the PBPK model currently compensates for the metabolism of the mono-glutathione conjugate of hydroquinone to the cysteine conjugate, there is no information available on the relative rates of metabolism of the di-glutathione conjugate to the tri- or tetra-glutathione conjugate, nor on the relative rates of metabolism of di- and tri-glutathione conjugates to their respective mercapturic acids. Furthermore, no information is available on the comparative rates of metabolism of the glutathione conjugates in kidney tissues. Such information, when available, may further improve estimates of the internal dose surrogate for species differences in renal toxicity.

Seaton et al. (1995) reported a threefold variation in the V_{max} for the microsomal glucuronide conjugation of HQ in human liver samples. In the present study, we found that a single set of rate constants resulted in an acceptable fit to the data for all human samples except CYE, which had a much greater rate of metabolism to the glucuronide. Likewise, the metabolism of 7-hydroxy coumarin to the glucuronide (as reported by In Vitro Technologies) was higher in this sample than in the other five. Although the six human donor samples were chosen at random, other than CYE, they had comparable marker substrate metabolism of 7-hydroxy coumarin glucuronide (Fig. 15). Donor information for CYE also indicates that this was the only donor with no reported alcohol, tobacco, or substance use (Table 2).

Although research using rodent species is of practical necessity for hazard determination, this study once again highlights the importance of taking into account species differences in renal toxicity.
metabolism. The use of an in vitro hepatocyte model allowed a direct comparison between rat and human metabolic parameters, and it suggests a greater flux of HQ through the bioactivation pathway in the rat. These results also demonstrate the potential importance of multiple metabolic steps and the balance between HQ deactivation, predominating in the human, and activation, predominating in the rat. In the future, these rate constants will provide valuable information to continue the evolution of the PBPK model for HQ developed by Corley et al. (2000), to include species-, gender-, and tissue-specific parameters to define internal dose surrogates that are more directly associated with nephrotoxicity and tumorigenicity.

ACKNOWLEDGMENTS

The authors thank Tamnie Guerin for the synthetic preparation of HQ-SG, HQ-Cys, and HQ-NacCys. The project was sponsored by the HQ Panel of the American Chemistry Council.

REFERENCES


