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Title: **Placental transfer and fetal elimination of morphine-3-β-glucuronide in the pregnant baboon**

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List of nonstandard abbreviations:

Cl	Clearance
HPLC	High-performance liquid chromatography
M3G	morphine-3-β-glucuronide
P1M	Model using data from paired infusions with single maternal compartment and single fetal compartment
P2M	Model using data from paired infusions with central and tissue maternal compartments and single fetal compartment
P2MNFC	Model using data from paired infusions with central and tissue maternal compartments and single fetal compartment with no direct fetal clearance
S1CPT	Model using data from single infusion with one compartment
S2CPT	Model using data from single infusion with central and tissue compartments
SPE	Solid Phase Extraction

Abstract

The glucuronide metabolites of several widely used drugs are detected in fetal plasma following maternal drug administration. However, the disposition of these metabolites is poorly understood and clinical concerns have been raised about accumulation of active metabolites in the fetus. For this reason, morphine-3-β-glucuronide (M3G), an active metabolite of morphine, was studied to provide quantitative data on disposition. Maternal, fetal, and bi-directional placental clearances of M3G were measured in 3 pregnant baboons. During maternal infusion of M3G to steady-state, the glucuronide metabolite readily appeared in fetal plasma achieving a mean (± SD) fetal-to-maternal concentration ratio of 0.79 ± 0.04. In paired maternal and fetal infusions, steady-state clearances (mean ± SD) were 53 ± 3 (maternal), 1.5 ± 0.5 (maternal-to-fetal), 2.6 ± 0.1 (fetal-to-maternal), and -0.70 ± 0.6 ml·min^{-1} (fetal). These clearance values support bidirectional transfer of M3G across the placenta and indicate negligible direct clearance from the fetus. The clearance of M3G across the placenta is more than twenty-fold less than that of morphine. Despite this low index of permeability, placental transfer contributes significantly to the glucuronide pool in the fetus. Placental transfer emerges as the major clearance pathway for the glucuronide from the fetus and suggests a component of active efflux. What is more, the results do not support the concept of sequestration in the fetal intestine as a significant route of clearance. Together these results clarify the distribution and clearance of glucuronides in the pregnant primate and facilitate prediction of fetal exposure to active metabolites.

Introduction (715)

Glucuronidation is a major drug elimination pathway. The glucuronide metabolites of several drugs have been detected in fetal plasma following maternal drug administration raising the question of their origin. It has been shown that the sheep and primate fetus can contribute to the presence of glucuronide metabolites through metabolism (Wang et al., 1986; Olsen et al., 1988; Wang et al., 1990; Garland et al., 2005; Garland et al., 2006). In sheep, glucuronide metabolites have extremely low placental permeability suggesting little contribution from the mother (Wang et al., 1985; Olsen et al., 1988). However, the contribution from placental transfer has not been determined in primates. The information that is available suggests that the primate placenta is more permeable than that of the sheep. The similarity of fetal and maternal metabolite concentrations under steady-state conditions following maternal drug or metabolite administration in human and nonhuman primates supports a considerable transplacental contribution from the mother to the fetal metabolite pool (Gerdin et al., 1990a; Gerdin et al., 1990b; Garland et al., 1998b; Garland et al., 1998c; Garland et al., 2001).

The limited consideration given to metabolite disposition in pregnancy may be accounted for by the fact that the metabolites of many drugs are inactive and the commonly held concept that fetal metabolism has little impact on overall drug disposition. However, the glucuronide metabolites of morphine, morphine-3-β-glucuronide (M3G) and morphine-6-β-glucuronide (M6G) have demonstrated distinct activities that may contribute to the effects of the parent drug (Labella et al., 1979;

Pasternak et al., 1987; Skarke et al., 2005). Furthermore, recent studies in the pregnant baboon demonstrate that fetal metabolism not only significantly decreases fetal drug concentrations, but can result in concentrations of metabolites that exceed those in the mother (Garland et al., 2001). The extensive parallels between human and nonhuman primates suggest that studies of fetal metabolism and exposure to metabolites in baboons are relevant to the human fetus (Garland et al., 1996b; Garland et al., 1998a; Caspersen et al., 2007).

Olsen and coworkers (1988) first raised the possibility that drug metabolites in the fetus could exceed those in the mother due to formation by the fetus and limited clearance from the fetus. In prior studies, the fetal baboon was shown to metabolize morphine to M3G and M6G (Garland et al., 2005). As in the adult, M3G was the primary metabolite and accounted for approximately 30 percent of the clearance of morphine from the fetus. This suggested that the formation of metabolite by the fetus was not negligible (Garland et al., 2005; Garland et al., 2006). In addition, when M3G and M6G were infused directly to the fetus, they were eliminated at a relatively low clearance rate (Garland et al., 2005). Although the mechanism of elimination was not delineated, it was concluded that clearance most likely occurred across the placenta.

The extent to which metabolite concentration in the fetus exceeds the concentration in the mother at steady-state during maternal administration can be understood by examining the mathematical expression of the fetal-to-maternal metabolite ratio derived from the equations of the two-compartment model at steady-state (Garland et al.,

1996a). However, to predict fetal metabolite concentration in relationship to the maternal metabolite concentration, it is necessary to have reliable estimates for placental transfer and direct fetal metabolite clearance, in addition to the currently available data on fetal metabolism. Estimates of clearance parameters for the metabolite can be obtained by the same methodology that has been used for drugs combining data from both maternal and fetal infusions of the compound in the same animal (Szeto et al., 1982b; Garland et al., 1998b). This methodology allows for the calculation of placental clearances in both directions and direct fetal clearance.

The objective of the present study was to identify the major routes of elimination of the primary morphine metabolite, M3G, from the fetus, determine the contribution of placental transfer to the presence of the metabolite in the fetus, and quantify the contributions of placental and nonplacental clearance pathways. This study is part of a comprehensive evaluation of the disposition and effect of drugs and their metabolites in the pregnant baboon using morphine as a model agent.

Methods

Animal model

The animals were studied using a system of individualized backpack and tether. This system, along with the methods for maintenance, breeding, preconditioning, anesthesia, surgery, and postoperative care, were described in detail in a previous report and are summarized here (Stark et al., 1989). Animals were maintained in accordance with all regulations for the care and use of laboratory animals by the National Institutes of Health, U.S. Department of Agriculture, and American Association for the Accreditation of Laboratory Animal Science. Research protocols were approved by the Institutional Animal Care and Use Committee at Columbia University.

Breeding colony A breeding colony of baboons (*Papio* species) housed in the Institute of Comparative Medicine, Columbia University, New York, was available for study. Gestational age was calculated from the date of conception. This date was estimated as the midpoint of the period of timed mating (±3 days). Term gestation (~175 days) was estimated from the average length of pregnancy in the colony. Ultrasound was performed at 70 – 110 days to confirm singleton pregnancy, fetal size, and placental location. Lights in the colony room were cycled (0700 h on, 1900 h off) and feeding times (0800 and 1600 h) were constant.

Surgery Surgery was scheduled between 120 and 145 days. Approximately two weeks prior to surgery, the animal was fitted to a backpack and sham tethered. This allowed proper adjustment of the backpack straps and assessment of acceptance of the animal to the tether system. Only those animals who readily adapted to the system were studied. Surgery was performed under general anesthesia (isoflurane and nitrous oxide) using sterile surgical techniques. Vascular catheters were placed in the maternal femoral artery and vein and in the fetal carotid artery and jugular vein. An amniotic fluid catheter was sutured to the neck region. Electrophysiological electrodes and a tracheal catheter were also placed to monitor fetal well-being and drug effects. The uterus was closed in two layers taking care to appose amniotic membranes. After closure of the rectus sheath and prior to skin closure, the catheters and electrodes were tunneled under the skin to the mid-scapular region of the mother where they exited the skin to the backpack.

Tether system The backpack housed the pressure transducers and the catheter and electrical connectors. It was attached to a stainless steel tether cable that connected to a freely rotating pole on the top of the cage. The catheters passed through this cable and were connected to peristaltic infusion pumps and solutions that were attached to the pole (P720, Instech Laboratories, Inc., Plymouth Meeting, PA). The maternal and fetal vascular catheters were continuously infused with normal saline containing heparin (1 $U \cdot ml^{-1}$) at rates of 5 and 2 $ml \cdot h^{-1}$ respectively to keep catheters patent. Catheters were accessible at the top of the cage to obtain blood and amniotic fluid samples. An electrical swivel at the top of the pole allowed the whole apparatus to turn freely with the

activity of the animal while providing electrical power for pumps and signal transmission of physiological data.

Post-operative analgesia and monitoring of pregnancy Post-operative analgesia was supplied with a continuous infusion of morphine sulfate (50-150 $\mu g \cdot kg^{-1} \cdot h^{-1}$) to the mother. This was reduced stepwise as the mother resumed normal activity in the 2 – 4 days after surgery. No tocolytic agents were used. Cefazolin was administered daily for 3 days to the mother and into the amniotic fluid and then only as indicated. The mothers were maintained in their home cages near other familiar animals. A minimum of 6 days post-surgery and at least 48 h following cessation of post-operative morphine were allowed for stabilization prior to the start of infusion protocols.

Mothers were monitored for signs of labor assessed by maternal behavior and pressure recordings from the amniotic fluid catheter (Cobe low-level pressure transducers, Cobe, Boulder CO; Hewlett-Packard 8805-B Amplifier, San Diego, CA). Physiologic stability of the fetus was assessed by fetal heart rate, blood pressure, arterial acid base status, and blood gas values (Hewlett-Packard 3040A heart rate monitor, San Diego, CA; Radiometer ABL735, Radiometer America, Westlake, OH). This animal model allowed the pharmacokinetic studies to be performed during the last trimester of a stable pregnancy without the need for anesthesia or undue restraint.

Pharmacokinetic protocol

Drug doses and preparation Morphine-3-β-D-glucuronide (M3G) was obtained from the National Institute of Drug Abuse (NIDA), Division of Neuroscience and Behavioral Research, Bethesda, MD, USA. All M3G solutions were prepared from a 2 $mg \cdot ml^{-1}$ stock solution in normal saline and stored at -70°C until use. The planned doses were 2.5 $mg \cdot h^{-1} \cdot 15kg^{-1}$ to the mother and 0.1 $mg \cdot h^{-1}$ to the fetus. These doses were based on the plasma concentrations achieved in a nonpregnant animal for the mother and a dose kinetic study for the fetus (Garland et al., 2005). The goal was to obtain similar maternal concentrations across animals during maternal infusion and to obtain fetal concentrations during fetal infusions similar to those in the fetus during infusion of morphine to the mother. This was done to avoid issues of potentially saturating fetal elimination processes confounding clearance calculations; although, a previous study had indicated linear dose kinetics for M3G in the fetus (Garland et al., 2005).

The infusion solutions were prepared in a single batch. The maternal solution was prepared first by diluting the stock solution with normal saline to a concentration of 0.5 $mg \cdot ml^{-1}$. An aliquot of this was diluted 1:10 to prepare the fetal solution (0.05 $mg \cdot ml^{-1}$). This was done to minimize variability between maternal and fetal doses based on dose preparation. Heparin (sodium injection, USP, 5000 $U \cdot ml^{-1}$, American Pharmaceutical Partners, Inc., Schaumburg, IL, USA) was added during preparation to achieve a concentration of 0.5 $U \cdot ml^{-1}$ for the fetal solution and 1.0 $U \cdot ml^{-1}$ for the maternal solution. The solutions were filtered through 0.22 µm, 25mm MCE filters (Fisher Scientific,

Pittsburg, PA, USA) into sterile Viaflex infusion bags (Baxter-Clinitech Division, Dearfield, IL). The infusion bags were wrapped in aluminum foil to protect from light and stored at –20°C until they were ready to be used on the animal.

Infusion and sampling protocols All infusions were administered through the maternal superficial femoral or fetal internal jugular venous catheters and all samples were obtained through maternal superficial femoral and fetal carotid arterial catheters. The order of the infusions was randomized to control for gestational age and time since surgery. The two infusions (maternal and fetal) were scheduled approximately one week apart. The bag of infusion solution was thawed overnight at room temperature and the bag weighed prior to being installed. After a baseline sample, the infusion was started at the appropriate rate using a precalibrated peristaltic infusion pump (P720, Instech Laboratories Inc., Plymouth Meeting, PA). The maternal solution would deliver 2.5 $mg \cdot h^{-1}$ at 5 $ml \cdot h^{-1}$. Rate adjustments were made for each animal based on their weight so the mother would receive 2.5 $mg \cdot 15\ kg^{-1} \cdot h^{-1}$. The fetal infusion would deliver 0.1 $mg \cdot h^{-1}$ when infused at 2 $ml \cdot h^{-1}$. These rates of infusion were used as the drug solutions replaced the normal saline infusions used to maintain vascular patency.

The duration of infusion was 52 h with samples obtained at 6, 24, 30, 48, 50, and 52 h from the beginning of the infusion in both the mother and fetus. The drug solution was covered with an opaque bag throughout. The infusion was stopped and timed samples were obtained from the infusion recipient (mother or fetus) during the elimination phase. For the mother these were at 15, 30, 45, 60, 90, 120, and 180 minutes after stopping

the infusion. For the fetus, samples were obtained at 15, 30, 45, 90, 120, and 180 minutes after stopping the infusion. Maternal samples were not taken during fetal elimination as the concentrations would be too low to be of value and fetal samples were not taken following maternal infusion to minimize the volume of blood obtained from the fetus.

The infusion solution bag was reweighed following the infusion. The difference between the weight of the solution bag from the beginning and end of the infusion divided by the total time of the infusion was used to determine a final infusion rate. Although all pumps were precalibrated to deliver 5 or 2 $ml \cdot h^{-1}$, some differences in rates were noted once the pumps were infusing to the animal, presumably due to changes in the resistance to flow.

Blood samples were obtained by drawing back the dead space of the catheter plus 0.5 – 1 ml blood for the fetus and a minimum of 3.0 ml of blood for the mother. The sample volume of 0.6 ml was then obtained for drug analyses. The extra blood withdrawn was returned and the line flushed with 3 ml of normal saline which was sufficient to clear the line. Blood was placed in heparinized microtainer tubes (Becton Dickinson, Franklin Lakes, NJ), gently mixed, and then separated by centrifugation. Plasma was transferred to plastic vials and stored at –70º C until analysis. A sample of infusion solution was also obtained.

HPLC method for analysis of M3G in plasma samples

The HPLC method used was similar to that previously reported for measuring morphine, M3G, and M6G except only fluorometric detection was used as M3G is not electrochemically active (Garland et al., 2005; Garland et al., 2006). The method uses an automated Waters Alliance 2695 HPLC system (Waters, Milford, MA) with fluorometric and/or coulochemical detection.

Extraction procedure for plasma samples Sample preparation was performed using Solid Phase Extraction (SPE) columns (1 ml Bond Elute C18 50 mg columns, Varian Inc., Sugarland, TX) on a Visiprep-DL Solid Phase Vacuum Manifold (Model 5-7250) followed by sample evaporation under nitrogen with a Visidry drying attachment (Model 5-7124, Supelco Inc., Bellefonte, PA). The columns were conditioned under vacuum with 2 x 1 ml methanol (HPLC grade, Mallinkrodt, Fisher Scientific, Pittsburgh, PA), 1 ml purified water (Milli-Q NanoPure Water Filtration System, Millipore, Bedford, MA), and 1 ml 0.01M ammonium bicarbonate buffer, pH 9.3 (HPLC grade, Mallinkrodt, Fisher Scientific). The SPE columns were then loaded by gravity with 200 µl of ammonium bicarbonate buffer (pH 9.3) followed by 100 µl of sample plasma or, for standards, 100 µl of blank pooled plasma with 20 µl of 5X standard, 100 µl of the internal standard nalorphine HCl (800 $ng \cdot ml^{-1}$, Sigma, St. Louis, MO), and finally an additional 200 µl of ammonium bicarbonate buffer (pH 9.3). After equilibration, the columns were washed with 1 ml of 0.01M ammonium bicarbonate (pH 9.3) and dried under vacuum. The samples were eluted with 2 x 150 µl methanol (HPLC grade, Fisher Scientific), dried

under nitrogen, and reconstituted in 200 µl of mobile phase. All samples were extracted in duplicate.

Chromatographic conditions The separation was performed isocratically on a Waters Alliance 2695 HPLC system using a Spherisorb C18 column (ODS2, 3µm, 4.6 × 100 mm I.D., Waters, Milford, MA) at ambient temperature with a flow rate of 1.5 ml·min^{-1}. The mobile phase was 10 mM sodium phosphate monobasic (Fisher Scientific), 1.5 mM sodium dodecyl sulphate (ultraPURE, Gibco BRL, Grand Island, NY), and 24% acetonitrile (HPLC grade, Fisher Scientific) adjusted to pH 2.1 with o-phosphoric acid 85% (HPLC grade, Fisher Scientific). After injection of 50 µl of sample, the eluting compounds were measured by fluorescence detection (excitation 210 nm and emission 340 nm) on a Linear Model LC305 detector (Linear Instruments, ESA, Bedford, MA). The lower limit of detection was taken as the lowest point on the standard curve which was 5.0 ng·ml^{-1} for M3G. Values for a control sample included in each extraction had a mean value of 260 ng·ml^{-1} and coefficient of variation of 12.3% in 25 extractions over a period of 4 years.

Pharmacokinetic and statistical analyses

Determination of steady-state M3G concentrations at each time point during M3G infusion were visually inspected and the early time points were expressed as a percentage of the mean values from samples taken 48 to 52 h after the onset of

infusion. The mean of appropriate samples was then used to define a mean steady-state concentration for each animal and infusion route.

Steady-state clearances The total (Cl_{Mt} and Cl_{Ft}), placental (Cl_{MF} and Cl_{FM}) and direct or nonplacental (Cl_{Md} and Cl_{Fd}) clearances of M3G for the maternal and fetal compartments were determined using the method of Szeto *et al.*, (1982) using the following equations (note: *c* is used for steady-state concentrations of metabolite during maternal infusion and *c'* during fetal infusion; R_M *and* R_F *are* the rates of infusion to the mother and fetus).

Equation 1

$$Cl_{Mt} = \frac{R_M}{c_M - c_F\left(\frac{c'_M}{c'_F}\right)}$$

Equation 2

$$Cl_{Ft} = \frac{R_F}{c'_F - c'_M\left(\frac{c_F}{c_M}\right)}$$

Equation 3

$$Cl_{MF} = Cl_{Ft}\left(\frac{c_F}{c_M}\right)$$

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Equation 4

$$Cl_{FM} = Cl_{Mt}\left(\frac{c'_M}{c'_F}\right)$$

Equation 5

$$Cl_{Md} = Cl_{Mt} - Cl_{MF}$$

Equation 6

$$Cl_{Fd} = Cl_{Ft} - Cl_{FM}$$

Compartmental analyses Concentration-time values during and following infusion for each route of infusion in each animal were visually inspected and fit to one, two, or three compartment models, as appropriate, using WinNonlin (Pharsight Corporation, Mountain View, CA). Following this, the data from the paired infusions was fit to user defined models that simultaneously fit the data from the two infusions. Selection for model with best fit was determined by visualization, standard errors of parameter estimates, correlation coefficients, and Akaike and Schwartz criteria.

Statistical analyses Model data are presented as parameter estimate ± SE. Data for all animals are presented as mean ± SD. The small number of animals precludes reasonable statistical comparisons, although differences between maternal and fetal

concentrations, concentration ratios, and placental clearances were compared by paired t-test or one sample t-test. Alpha of .05 was considered significant. All statistical analyses were done using Systat statistical package (Systat Software, Inc., Point Richmond, CA).

Results

Data from paired maternal and fetal infusions of M3G from 3 pregnant animals were analyzed. The gestational ages of the animals at surgery, infusion, and delivery are provided in **Table 1**. Fetuses had normal blood gas values and normal heart rate patterns as estimated from prior research (Daniel et al., 1992). All fetuses survived for at least 7 days beyond the time of study.

Steady-state concentrations The concentration-time curves graphically represented in **Figure 1** suggest that steady-state for M3G is achieved in both maternal and fetal compartments by 24 h, if not by 6 h. However, the variance in maternal concentrations during the maternal infusion in two animals suggested that the rate of infusion of metabolite varied making it difficult to define steady-state numerically. For this reason, only the 48 – 52 h concentration time points were used to define steady-state concentrations.

The calculated rates of drug delivery and mean steady-state values from 48 – 52 h are provided in **Table 2**. During maternal infusion, fetal steady-state concentrations are similar to but slightly less than maternal concentrations ($p < 0.05$). The similarity between fetal and maternal concentrations supports transfer of the metabolite across the placenta. On the other hand, the difference, reflected in the fetal-to-maternal concentration ratio being less than one (**Table 2** and **Figure 2**), implies either a direct clearance pathway on the fetal side of the placenta, or that fetal-to-maternal clearance

exceeds maternal-to-fetal clearance. During fetal infusion, maternal concentrations are well below those in the fetus. The difference between fetal and maternal concentrations during fetal infusion reflects the high maternal clearance and/or low placental clearance. The stability in fetal-to-maternal ratios during both maternal and fetal infusions provides further support that steady-state was achieved relatively early (**Figure 2**).

Noncompartmental steady-state model The clearances calculated from the mean steady-state concentrations are presented in **Table 3**. As expected, the maternal clearance was by far the highest. With regard to the placental clearances, the fetal-to-maternal clearance was consistently higher than the maternal-to-fetal clearance ($p < 0.06$). This difference suggests the presence of active efflux of metabolite from the fetus. Overall, less than 4 percent of the dose of M3G administered to the mother crossed the placenta. The calculated direct fetal clearances gave values less than zero that support no direct loss of metabolite from the fetus other than to the mother.

Compartmental models A stepwise approach was taken to develop a compartmentally based model of maternal-fetal distribution and clearance (Figure 3). The models were used to estimate the volumes of the maternal and fetal compartments, to provide information on elimination half-life of M3G from the fetus, and to refine estimates of clearances. To address the issue of the variability in maternal infusion rate early on, a subset of the data set was analyzed from 48 to 54 h in addition to the entire data set. Parameter estimates were similar for both data sets; however, the limited data set was

associated with smaller errors. Parameter estimates for stepwise modeling of the 48 to 54 h data set are presented in **Table 4** and discussed below.

Inspection of the elimination phases of the maternal and fetal data sets suggested that a one compartment model would be sufficient to describe the system in most cases (**Figure 1**). The maternal and fetal data sets were independently fit to 1-, 2-, and 3-compartment models using a constant infusion input and clearance parameters (WinNonlin). Initial parameters were program defined. Several weighting schemes were applied but none improved estimations. Reported results used uniform weighting with Gauss-Newton minimization. Estimates were derived for one and two compartment models there being insufficient late time points available to discern a third compartment. These models are referred to as S1CPT and S2CPT designated as maternal or fetal to reflect single infusion with one or two compartments (**Table 4**). In general, there was reasonable fit to both one and two compartment models. For the maternal infusion, the maternal samples slightly favored the two compartment model; although, standard errors for the volumes of the two maternal compartments and the tissue clearance were moderately large. Although not constrained by the model, the clearance from central to deep compartment was not different from the clearance from deep to central compartment. This suggested passive exchange between these two compartments. For the fetal infusion, the one compartment model provided the most reliable fit across animals, although the data from animal 275 were perhaps better described by two compartments.

Elimination half-lives were generated from the one and two compartment models for the mother and fetus to provide additional support for the time to reach steady-state (**Table 5**). The maximum estimate for half-life from the fetus was less than 2.5 hours suggesting that no more than 12 hours is needed to approximate steady-state.

Based on these initial results with compartmental modeling, two user defined maternal-fetal models were set up in WinNonlin that simultaneously fit the maternal and fetal concentrations during the paired maternal and fetal infusions for each pregnancy (**Figure 3**). The first model was maternal one-compartment – fetal one-compartment (P1M) and the second was maternal two-compartment – fetal one-compartment (P2M). Maternal-fetal exchange was through the central maternal compartment. Both of these models included a direct fetal clearance component. For exchange between the maternal central and deep compartment, a single clearance parameter was used to minimize overall number of parameters required. This is in keeping with passive diffusion as suggested above. Again, after evaluating different weighting schemes, uniform weighting and Gauss-Newton minimization was used. Initial parameters were based on the estimates from the independent one and two compartment models and the steady-state clearances. Various initial conditions were explored to assess the robustness of the model. For instance, the initial premise of no difference in the bidirectional clearances across the placenta and the presence of a direct fetal clearance component to account for the fetal-to-maternal difference in concentrations were used as initial conditions with minimal effect on final estimates. The model with a maternal tissue compartment provided the best fit (**Figure 4**); however, error in the estimates of

the size of the maternal compartments was large (**Table 4**). Observed concentrations differed from predicted concentrations by 2 % at steady-state to 10 – 20 % at very early and very late time points. Simulations were run using the parameters from animal 267 with varying amounts (2 – 10%) of normally distributed random error added to assess the effect of number of data points. Parameters were then estimated by applying the models to these new data sets. Increasing the data points significantly reduced error.

Models incorporating data from both infusions allow separation of direct fetal clearance from fetal placental clearance. The estimates for fetal-to-maternal placental clearance determined from the paired infusions were remarkably similar to those for overall fetal clearance determined from the single infusions (Table 4). Furthermore, the estimates for direct fetal clearance determined from the paired infusions were negligible. To further assess the validity of absence of direct fetal clearance, simulations with parameter estimates for animal 267 were rerun with the fetal-to-maternal placental clearance reduced to equal the maternal-to-fetal clearance and direct fetal clearance added to equal the amount of the reduction. With 10 % error added to simulated data, limiting time-points to the times of observed data, and setting initial conditions to no direct fetal clearance so that all fetal clearance was placental, the model gave estimates that were not different to those used in the simulation. This supports that the model can distinguish between direct fetal clearance and active placental transport despite the limited number of time-points in the data set.

Based on this finding, a final model was set up that eliminated the direct fetal clearance component (P2MNFC). This model yielded a more robust estimate of placental clearances and permitted more reasonable comparison of the bidirectional placental clearances supporting that fetal-to-maternal clearance is greater than maternal-to-fetal clearance ($p < 0.05$). This comparison is presented as the ratio of the fetal and maternal placental clearances in **Table 4**. The support for the presence of an active clearance process from the fetus to the mother is strengthened by the finding that this clearance ratio is greater than one (1).

Discussion

The objective of this research was to identify the major routes of elimination of glucuronide metabolites from the fetus, determine the contribution of placental transfer to the presence of the metabolite in the fetus, and quantify the contributions of placental and nonplacental pathways for metabolite clearance. Under steady-state conditions during maternal infusion the concentrations of M3G in the fetus closely approximate those in the mother and, as such, demonstrate significant transfer of this major glucuronide metabolite of morphine across the primate placenta. The results from the paired maternal and fetal infusions support the conclusion that the small but consistent difference between fetal and maternal concentrations seen during maternal infusion are due to enhanced efflux of the glucuronide from the fetus and not due to direct clearance mechanisms from the fetus. The addition of this new knowledge enhances the ability of clinically relevant pharmacokinetic models to predict fetal exposure to drugs and their metabolites during maternal therapy.

The appearance of morphine glucuronide in fetal plasma following maternal administration provides clear evidence that glucuronide metabolites can cross the primate placenta and result in significant fetal concentrations. Fetal levels reach a plateau at concentrations that are 70 to 90 percent of maternal concentrations by 24 hours. The fetal-to-maternal clearance calculated for M3G (1.5 $ml \cdot min^{-1}$) is considerably lower than that of the parent drug morphine and of zidovudine (56 $ml \cdot min^{-1}$ and 30 $ml \cdot min^{-1}$ respectively), the two other drugs that have been evaluated in this animal

model (Garland et al., 1998b; Garland et al., 2001). The lower clearance of the glucuronide metabolite no doubt reflects the low lipid solubility of this relatively large polar molecule. It is important to note that while the placental clearance of the glucuronide is low the half-life in the fetus is not overly prolonged. This finding is also a reflection of low lipid solubility of the metabolite leading to a lower volume of distribution. No values are available for volume of distribution and clearance of M3G in humans; however, morphine-6-glucuronide has been studied in adult humans. Values for clearance and volume of distribution standardized to weight (2.2 $L.min^{-1}.kg^{-1}$ and 0.12 $L.kg^{-1}$) are similar to those in the adult baboon (Lotsch et al., 1998).

The finding of a lower concentration of M3G in the fetus than in the mother under steady-state conditions is robust despite technical problems with the stability of the maternal infusion rate in two animals (Figure 1 and 2). Under steady-state conditions, it is expected that the concentrations of a compound in the mother and fetus will be equal when the compound administered to the mother is passively transferred across the placenta and there is no direct clearance from the fetus (Szeto et al., 1982a; Wang et al., 1986; Garland et al., 2005). Hence, the finding of a fetal-to-maternal concentration ratio less than one (1) for M3G leads to the conclusion that total fetal clearance (fetal-to-maternal clearance plus direct fetal clearance) is in excess of the maternal-to-fetal clearance.

The question then becomes how much of the excess clearance is direct fetal clearance and how much is active transport from the fetus to the mother. The clearance estimates

from both noncompartmental steady-state analyses and from compartmental pharmacokinetic modeling suggest that this excess can be accounted for entirely by active transport from the fetus to the mother and that there is no measurable contribution from other direct fetal clearance mechanisms.

It is possible that variance in the dose of M3G caused by initially inconsistent infusion rates could influence the estimates for both fetal-to-maternal clearance and direct fetal clearance. Examination of the steady-state equations (Equations 1 – 6) indicates that maternal-to-fetal clearance is independent of the maternal dose because the fetal-to-maternal concentration ratio during maternal infusion is dose independent. The remainder of the data used to calculate maternal-to-fetal clearance is from fetal infusions. This is also true for calculation of total fetal clearance. Fetal placental clearance, on the other hand, is directly proportional to maternal infusion rate and thus the distribution of the total fetal clearance between placental or direct fetal clearance remains at issue.

Examination of the individual data sets allows further clarification. For animal 273 (Table 2 and Figure 1), the mean maternal infusion rate would underestimate the actual dose being received at the time of measurement of steady-state concentrations because the maternal concentrations at 48 h were higher than those obtained from 6 – 24 h. In turn, this would underestimate the fetal placental clearance. However, fetal placental clearance cannot exceed total fetal clearance. Thus, the conclusion that there is no direct fetal clearance remains likely for this animal. In contrast, for animal 275 the mean

infusion rate would overestimate total fetal clearance and might obscure a direct fetal clearance component. To address the issue of variability of maternal infusion rate in this animal a secondary analysis of the data was performed. A parameter that would correct for a linear change in dose was included in the model. With this correction of infusion rate, the model estimates of fetal-to-maternal clearance remained greater than maternal-to-fetal clearance with no direct fetal clearance. This secondary exploration of the data increases confidence in the finding that there is no direct fetal clearance and that the difference in maternal and fetal concentrations is due to an active transport process from the fetus to the mother.

The finding of an absence of any direct fetal clearance of the metabolite is quite striking and was unexpected. In the human, drug metabolites are known to be present in amniotic fluid and in fetal and newborn meconium. Based on the impermeable nature of drug metabolites, it had been assumed that metabolites are excreted in fetal urine and cleared by fetal swallowing with subsequent sequestration in the intestinal tract until passage of meconium after birth. In the current mass balance studies, the absence of evidence for direct fetal clearance would indicate that any metabolite excreted into the amniotic fluid was swallowed and reabsorbed intact and/or directly transferred across the amnion and chorion into the maternal circulation. The implication of the present study is that there is no support for a direct fetal clearance pathway such as deconjugation of M3G to morphine (that is, removal of the glucuronide moiety) or sequestration of M3G in meconium.

The finding of enhanced efflux of the glucuronide from the fetus was not wholly unexpected. With consideration of the chemical structure of glucuronides, paracellular transport mechanisms, transfer through syncytial pores, or the assistance of transport carrier proteins would be required for passage through lipid membranes (Faber et al., 1992). While the mechanisms by which glucuronide metabolites cross the placenta are unclear, it is known that transporters are abundant in the placenta and several may have an affinity for glucuronides (St-Pierre et al., 2000; Pascolo et al., 2003; van de Wetering et al., 2007). P-glycoprotein, MRP2, and BCRP are ATP-dependent efflux transporters predominantly expressed on apical maternal facing membranes of the syncytiotrophoblast (St-Pierre et al., 2000; Maliepaard et al., 2001; Nagashige et al., 2003). MRP2 has affinity toward M3G, P-glycoprotein does not, while BCRP affinity toward other glucuronides has been shown (Xie et al., 2000; Adachi et al., 2005; Zamek-Gliszczynski et al., 2006; van de Wetering et al., 2007). MDR3 and MRP1 are present on the basolateral surface of syncytiotrophoblast and fetal capillary endothelium; however, their affinity toward M3G is unknown (Nagashige et al., 2003; Evseenko et al., 2006). MRP3, located predominantly in fetal capillary endothelium transports M3G (St-Pierre et al., 2000; van de Wetering et al., 2007). Less is known about transporters that allow access of M3G to the interior of the endothelial and trophoblastic cells. Organic acid transport peptides seem the most likely candidates and are known to be expressed in the placenta (Nishino et al., 1999; Nishimura and Naito, 2005). Notably, fetal clearance of M3G across the placenta is not saturable at levels expected clinically (Garland et al., 2005). From this study it is reasonable to propose that placental passage of glucuronides has at least a component of active transport

superimposed on other transfer mechanisms. Active transport could also occur at the level of the amnion, chorion, and decidua, though it is unlikely to have a significant impact. For zidovudine, transfer at the level of the amnion was estimated to be less than 3 percent of the transfer occurring at the level of the placenta (Tuntland et al., 1998). Furthermore, exchange seemed to occur in both directions and more likely reflected passive diffusion.

It is reasonable to assume that metabolite transfer between the maternal and fetal compartments, when it occurs, is primarily at the level of the placenta. The placenta is highly specialized for transfer of substances between the mother and fetus. However, in the model "placental" clearances would include *any* transfer of drug between the maternal and fetal compartments. For example, drug that crosses directly from the mother to the amniotic fluid and is then swallowed and absorbed by the fetus would be represented in Cl_{MF}. On the other hand, drug excreted in fetal urine and then crossing directly from amniotic fluid to the maternal circulation would be included in Cl_{FM}. Direct fetal clearances (Cl_{Fd}) would reflect any secondary metabolic processes occurring in the fetus or placenta (such as β-glucuronidase activity leading to deconjugation) and any excretion of metabolite in fetal urine that fails to reappear in either the fetal or maternal circulation. The latter includes sequestration of the metabolite in the fetal intestinal tract, diffusion directly into the maternal gut or bladder, and vaginal amniotic fluid leak.

There are limited reports on the permeability of the placenta towards glucuronide metabolites, although transfer had been suspected based on the levels of glucuronide

metabolites found in the fetus following parent drug administration (Gerdin et al., 1990b; Garland et al., 1998b; Garland et al., 2001). M3G administered to pregnant rhesus monkeys was detected in fetal plasma by percutaneous fetal sampling (Gerdin et al., 1990a). Pilot studies from this laboratory with morphine and zidovudine glucuronides provided further support for transfer of glucuronide metabolites from the mother to the fetus in the baboon (Garland et al., 1998c). The most direct support for placental transfer of glucuronides comes from the human placental perfusion system where the rate of transfer of zidovudine glucuronide was noted as half that of zidovudine (Liebes et al., 1993). These studies are in stark contrast to research in pregnant sheep which conclude that glucuronide metabolites have extremely limited placental permeability (Wang et al., 1985; Olsen et al., 1988; Olsen et al., 1989).

The structural differences between the primate and sheep placenta may account for some of these observed differences in placental permeability (Schroder, 1995; Enders and Carter, 2004). The sheep has an epitheliochorial placenta with four distinct membranes intervening between the fetal and maternal circulations; whereas, the maternal spiral arteries of the primate hemomonochorial placenta end directly in lacunae where maternal blood bathes the fetal syncytiotrophoblast that is almost fused with the walls of the fetal villous capilliaries to form two closely opposed layers. Although each placental type evolved to minimize the diffusional distance, the permeability for hydrophilic substances in the sheep is markedly reduced for a substance with a molecular radius greater than 0.45 nm (Faber et al., 1992; Enders and Carter, 2004). The guinea pig placenta is also hemomonochorial in nature and

permeability similar to that of the primate placenta might be expected. However, it is difficult to draw direct comparisons with the data that is available. Olsen et al. (1989) did find that the permeability for M3G was in keeping with an inverse relationship with molecular weight. However, very little transfer of M3G was evident.

The overall goal of this research is to build a reliable and usable model of drug and metabolite distribution in pregnancy. The steady-state approach provides a reliable estimate of clearance from a limited number of blood samples – a critical factor for studies of the fetus. The use of clearance parameters is also useful as they provide a measure of physiological processes. In the present study, fetal-to-maternal and maternal-to-fetal clearances are likely measures of placental function. For the metabolite, direct maternal clearance most likely reflects maternal renal clearance. The steady-state method has been extensively used in the pregnant sheep model to examine aspects of placental transfer and fetal metabolism (Szeto et al., 1982a; Wang et al., 1986; Yeleswaram et al., 1993; Krishna et al., 2002). Primate studies are more limited and have mainly been directed toward addressing the disposition of anti-HIV medications in pregnancy (Odinecs et al., 1996; Garland et al., 1998b; Tuntland et al., 1998). Predictive models require delineation and acquisition of estimates for the major factors contributing to disposition. With information on a small number of model drugs, it is likely that comparative in vitro studies of fetal metabolism and placental transfer (passive and/or active) will be predictive of fetal disposition.

More complex compartmental models can be developed; however, the ability to discriminate between models becomes more difficult as the number of parameters and the estimates of error increase. Using a stepwise approach as exemplified in this study allows inclusion and exclusion of parameters at each step keeping the number of parameters, and hence error, to a minimum. The ability to both infuse and sample compartments of interest, in this case the mother and fetus, defines the transfer between the two compartments. Although, the term compartment is still applied to the maternal and fetal space, the transfer between these two spaces is independent of additional compartments in either the mother or fetus. As such, measurements of transfer between compartments are noncompartmental although the specific pathway may not be defined. This methodology could be extended for confirmation of the absence of direct fetal clearance by comparing concentrations in the fetus during infusions to the fetus with fetal concentrations obtained during infusions into the fetal intestinal and/or amniotic cavity.

In conclusion, the permeability of the primate placenta for M3G is low in keeping with the physicochemical properties of the compound. However, the rate of placental transfer is sufficient for clinically relevant concentrations of M3G to be present in the fetus. Furthermore, the permeability is sufficiently limited that when the metabolite is produced by the fetus, fetal concentrations of the metabolite are likely to exceed those in the mother. Finally, the placenta appears to be the primary route for clearance of the glucuronide metabolites from the fetus with little, if any, sequestration in deep fetal compartments. This new knowledge on the disposition of glucuronide metabolites will

be applied to metabolite concentrations obtained in the mother and fetus during drug infusion for the development of pharmacokinetics models relevant to primate pregnancy.

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Footnotes

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Figure Legends

Figure 1. Steady-state M3G infusions to the mother and fetus. Individual M3G concentration-time plots in maternal (circle, solid line) and fetal (triangle, dashed line) plasma for each animal during continuous infusion of M3G to the mother (left hand column) and fetus (right hand column). Animal 267, top row; 273, middle row; 275, bottom row.

Figure 2. Stability of FM ratios of M3G during constant infusion. Fetal-to-maternal (FM) concentration ratios during maternal (circle) and fetal (triangle) infusion.

Figure 3. Pharmacokinetic models. Concentration data were fit to models in a sequential fashion. A) Data from single infusion data sets (maternal and fetal) were first fit to standard 1 and 2 compartment models (S1CPT and S2CPT). Estimates from these data were used for parameter estimates in subsequent steps. B) Data from paired maternal and fetal infusions were simultaneously fit to each maternal-fetal model. (See text for model descriptions.)

Figure 4. Model fitting of paired infusion data. Predicted plasma concentrations from P1M (left hand column) and P2M (right hand column) models (see text and Table 4 for model description). Observed concentrations in the mother (circle) and fetus (triangle) during maternal (filled symbol) and fetal (open symbol) infusion were simultaneously fit to each model. Lines represent predicted concentrations with solid lines during maternal

infusion and dotted lines during fetal infusion. Animal 267, top row; 273, middle row; 275, bottom row.

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Table 1 Study animals

		Event		Infusion		
	Weight	Surgery	Delivery	Maternal	Fetal	Comment
	kg	*Gestational age (days post conception)*				
267	13½	132	165	153	146	No labor
273	14¾	143	173	149	156	No labor
275	15½	126	145	139	132	No labor

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Table 2. Average (±SD) dose ($mg \cdot h^{-1}$) and steady-state concentrations ($ng \cdot ml^{-1}$) of M3G

Animal	Dose		Concentration during maternal infusion			Concentration during fetal Infusion		
	M	F	M	F	F/M ratio	M	F	F/M ratio
267	2.23	0.105	692±34	579±13	0.84±0.04	39.9±3.0	872±18	22±2
273	2.02	0.086	625; 697[1]	509±22	0.81; 0.76[1]	31.2±1.7	615±3	20±1
275	2.35	0.112	813±35	605±26	0.74±0.02	82±17	1632±1	20±4
Mean ± SD					0.79±0.04			21±1

[1] Two values only

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Table 3. Clearances ($ml \cdot min^{-1}$) calculated using steady-state model

Animal	Cl_{Mt}	Cl_{Ft}	Cl_{MF}	Cl_{FM}	Cl_{Md}	Cl_{Fd}
267	55.7	2.08	1.74	2.55	54.0	-0.47
273	53.0	2.42	1.86	2.69	51.2	-0.27
275	50.1	1.18	0.88	2.53	49.2	-1.3
Mean	52.9	1.89	1.49	2.59	51.5	-0.7
SD	2.9	0.64	0.53	0.09	2.4	0.6

Table 4. Comparison of volume of distribution (ml) and clearance (ml·min^{-1}) estimates from compartmental pharmacokinetic models (mean ± SE)

			Single or Central Compartment					Tissue Compartment		
		Animal	S1CPT	S2CPT	P1M	P2M	P2MNFC	S2CPT	P2M	P2MNFC
Volume	Maternal	267	3100 ± 330	1610 ± 500	2980 ± 240	1600 ± 450	1600 ± 440	1810 ± 450	1660 ± 390	1660 ± 380
		273	2660 ± 430	920 ± 400	2500 ± 260	900 ± 300	893 ± 290	1970 ± 370	1810 ± 270	1810 ± 260
		275	3030 ± 410	1220 ± 270	2950 ± 460	1220 ± 780	1210 ± 760	2280 ± 270	2180 ± 790	2190 ± 760
	Fetal	267	267 ± 13	266 ± 690	265 ± 18	265 ± 13	265 ± 12	0.87 ± 680		
		273	368 ± 8		368 ± 39	367 ± 20	367 ± 19			
		275	131 ± 16	52.0 ± 1.5	130 ± 11	131 ± 10	131 ± 10	103 ± 3		
Clearance	Maternal	267	55.0 ± 1.8	53.6 ± 1.1	54.8 ± 1.6	53.5 ± 1.0	53.5 ± 0.9	50 ± 34	50 ± 32	50 ± 32
		273	53.7 ± 2.9	51.0 ± 1.3	53.2 ± 2.1	51.0 ± 1.1	51.0 ± 0.9	55 ± 27	56 ± 22	56 ± 21
		275	50.1 ± 2.0	48.2 ± 0.7	49.9 ± 2.5	48.1 ± 2.3	48.1 ± 2.0	50 ± 16	48 ± 46	48 ± 44
	Fetal[1]	267	2.01 ± 0.02	2.01 ± 0.03	0.0003 ± 1.1	0.003 ± 0.36		0.12 ± 190		
		273	2.31 ± 0.01		0.002 ± 1.6	0.005 ± 0.80				
		275	1.17 ± 0.04	1.14 ± 0.003	0.0004 ± 1.3	0.0001 ± 1.1		1.07 ± 0.06		
	Placental		Fetal-to-maternal			Maternal-to-fetal			Fetal/Maternal	
			P1M	P2M	P2MNFC	P1M	P2M	P2MNFC	P2MNFC	
		267	2.1 ± 1.1	2.07 ± 0.38	2.07 ± 0.03	1.78 ± 0.08	1.73 ± 0.05	1.73 ± 0.05	1.20 ± 0.04	
		273	2.4 ± 1.6	2.39 ± 0.82	2.39 ± 0.03	1.93 ± 0.12	1.84 ± 0.06	1.84 ± 0.05	1.30 ± 0.04	
		275	1.2 ± 1.3	1.19 ± 1.1	1.19 ± 0.02	0.92 ± 0.08	0.89 ± 0.07	0.89 ± 0.06	1.35 ± 0.10	

Model descriptions: S1CPT, single infusion 1 compartment; S2CPT, single infusion 2 compartment; P1M, paired infusion 1 maternal compartments; P2M, paired infusion 2 maternal compartments; P2MNFC, paired infusion 2 maternal compartments no direct fetal clearance

[1] Corresponds to total fetal clearance for single analysis and direct (nonplacental) fetal clearance for paired analysis respectively.

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Table 5. Elimination half-lives (h)

	Animal	S1CPT	S2CPT
Maternal	267	0.65 ± 0.06	1.0 ± 0.2
	273	0.57 ± 0.07	1.0 ± 0.2
	275	0.70 ± 0.08	1.2 ± 0.2
	Mean ± SD	0.64 ± 0.06	1.1 ± 0.2
Fetal	267	1.5 ± 0.1	1.5 ± 0.1
	273	1.8 ± 0.1	
	275	1.3 ± 0.1	2.4 ± 0.1
	Mean ± SD	1.6 ± 0.3	2.0 ± 0.6

Model descriptions: S1CPT, single infusion 1 compartment; S2CPT, single infusion, 2 compartment

Figure 1

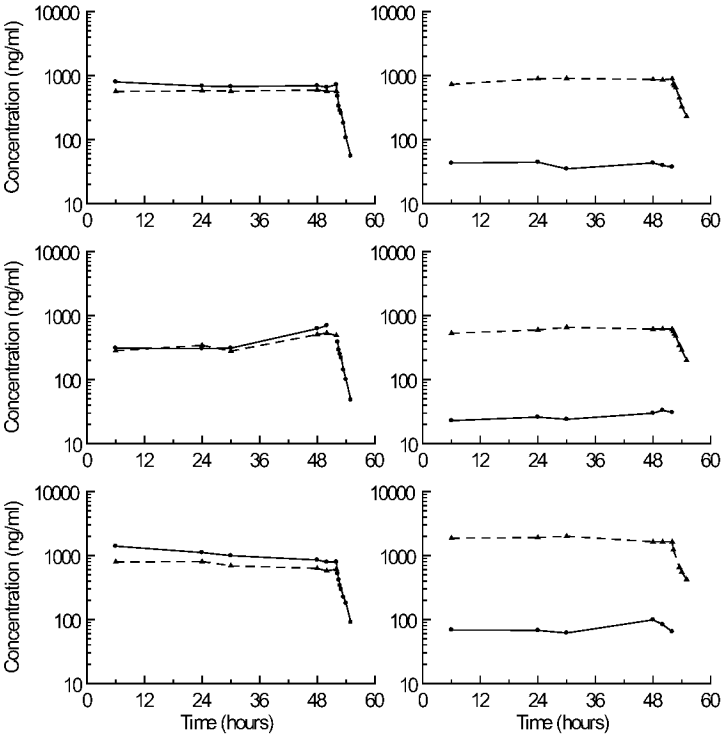
Concentration (ng/ml)
10000
1000
100
10
0
12
24
36
48
60
Time (hours)

Figure 2

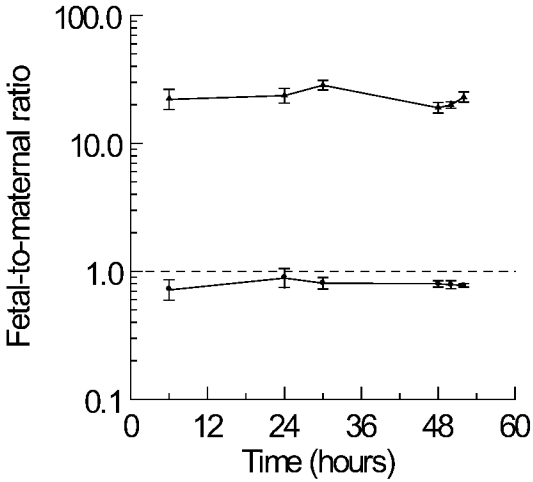
100.0
10.0
1.0
0.1
Fetal-to-maternal ratio
0
12
24
36
48
60
Time (hours)

Figure 3

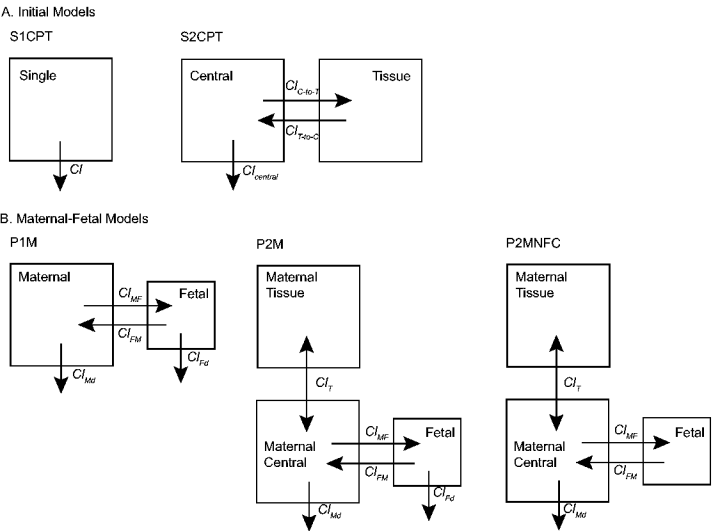
A. Initial Models
S1CPT
Single
Cl
S2CPT
Central
Tissue
Cl C-to-T
Cl T-to-C
Cl central
B. Maternal-Fetal Models
P1M
Maternal
Fetal
Cl MF
Cl FM
Cl Fd
Cl Md
P2M
Maternal Tissue
Cl T
Maternal Central
Fetal
Cl MF
Cl FM
Cl Fd
Cl Md
P2MNFC
Maternal Tissue
Cl T
Maternal Central
Fetal
Cl MF
Cl FM
Cl Md

Figure 4