Modeling Glucocorticoid-Mediated Fetal Lung Maturation: I.  
Temporal Patterns of Corticosteroids in Rat Pregnancy

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**Running Title:**

Corticosteroid Temporal Patterns in Rat Pregnancy

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**ABBREVIATIONS:** DEX, dexamethasone; CBG, corticosteroid binding globulin; PK/PD, pharmacokinetic/pharmacodynamic; RDS, respiratory distress syndrome; GA,
gestational age; IM, intramuscular; A₀, body weight of a single fetus at time zero set at GA 17; T_D, doubling time

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ABSTRACT

Preterm birth produces neonatal respiratory distress syndrome and dexamethasone (DEX) is administered maternally to induce fetal lung maturation in women at risk of preterm delivery. Antenatal DEX therapy is largely empirical and administering multiple doses of DEX produces undesirable metabolic and developmental effects in the fetus. It is hypothesized that pharmacokinetic/pharmacodynamic (PK/PD) assessment of the maternal/fetal disposition and selected effects of corticosteroids will allow insights into optimal dosing methods. An optimal regimen was defined as a dosing schedule that would reproduce the endogenous prenatal steroid exposure and up-regulation of fetal lung maturational markers precociously. This report focuses on designing such a regimen from a PK standpoint in rats. The temporal profile of endogenous corticosterone in control rats was captured using a radioimmunoassay and showed that maternal and fetal corticosterone increased significantly during the last days of gestation. Six 1 μmol kg⁻¹ intramuscular DEX doses separated by 12 hr intervals were administered maternally starting on gestational age (GA) 18 and PK was captured using a liquid chromatography-mass spectrometry assay. Unbound DEX exhibited a fetal to maternal concentration ratio of 0.55, had a free fraction of 0.2 in maternal and 0.4 in fetal plasma, and declined with a half-life of approximately 3 hr. DEX PK and plasma protein binding were linear during the study and DEX exposure caused severe adrenosuppression. These temporal steroid profiles in the fetal circulation will be used to drive the PD effects reported in a companion paper and an optimal steroid regimen will be proposed.
INTRODUCTION

Endogenous glucocorticoids play a pivotal role in programming the development of the fetus and preparing it for life outside the womb (Liggins, 1994). During most of its gestational life the fetus is exposed to very low levels of corticosteroids. A surge of corticosteroids in late pregnancy turns on fetal lung surfactant production that is necessary for lung maturation. Preterm birth occurs in about 10% of pregnancies where the neonate does not experience the late gestational steroid surge. These prematurely born infants experience respiratory distress syndrome (RDS) and require mechanical lung ventilation, which itself can cause additional lung diseases. RDS is a major cause of infant mortality and is among the most common and costly medical problems afflicting prematurely born infants (NIH Consensus Panel, 1995).

Synthetic fluorinated corticosteroids exhibit transplacental passage when administered to pregnant women because of low affinity for maternal corticosteroid binding globulin (CBG) and poor breakdown by placental steroid metabolizing enzymes (Diederich et al., 1998; Pugeat et al., 1981). DEX, in the form of its soluble phosphate ester prodrug, is administered prenatally to induce fetal lung maturation in women at risk of preterm delivery. This exogenous steroid administration has been shown to reduce the incidence of neonatal RDS and is considered a rare example of medical intervention that improves health care and produces considerable cost saving (NIH Consensus Panel, 1995).

Reports have appeared in the literature that the current empirically chosen dosing regimen of four 6 mg doses of DEX administered every 12 hr reduces the incidence of RDS by only 50% (Newnham, 2001). This has led to the practice of administering multiple doses of corticosteroids, which are administered on a weekly basis for weeks
and even months to women at risk for preterm delivery. Such prolonged exposure has been found to produce adverse cardiovascular, neuronal and developmental effects in the fetus (Newnham, 2001). There is therefore a need for dose optimization for the safe use of these potent drugs. An assessment of glucocorticoid PK and effects in a suitable animal model may therefore provide some insight into the optimal use of steroids in prenatal medicine.

The first objective was to capture the glucocorticoid profile and lung maturation profile in control fetal rats. An optimal regimen was then defined as a DEX dosing schedule that would precociously produce the endogenous prenatal steroid exposure and up-regulation of fetal lung maturational markers. This first report of a two part series focuses on designing such a regimen from a pharmacokinetic standpoint in pregnant rats.

Examination of PK properties during pregnancy in rodents requires measurement of drug concentrations in maternal and fetal plasma and total drug content in fetal tissue (Samtani et al., 2004a). Furthermore, corticosteroid effects during pregnancy are driven by both the endogenous and the exogenously administered corticosteroid. Finally, it is important to assess free corticosteroids in plasma because it is the unbound drug that is accessible to various organs for mediating steroid effects. This article reports extensive data that were obtained to help understand the driving force behind corticosteroid PD effects in pregnant rats.
Materials and Methods

Simulation Study

The aim of this exercise was to design a dosing regimen for DEX that would mimic the endogenous prenatal steroid exposure in the fetus. The following criteria were defined, which are thought to be critical in obtaining optimal steroid exposure: a) The dissociation constant for the fetal lung glucocorticoid receptor is considered to be an efficacy threshold (Samtani et al., 2005). Maintaining fetal free plasma concentrations above this threshold of 4.7 nM (Ballard et al., 1978) in rat might be conducive to mediating fetal lung maturational effects. Interestingly, human and rat fetal lung glucocorticoid receptors have very similar affinity for DEX (Ballard et al., 1978) and thus the pregnant rat is a good animal model for studying prenatal glucocorticoid effects. b) Concentrations of DEX above 100 nM in fetal explant cultures have been observed to have a paradoxical effect in that these high concentrations have an inhibitory effect on the synthesis of certain lung maturational surfactant proteins (Boggaram et al., 1989). Therefore 100 nM was considered the upper safety limit. c) The recommended 4 doses of prenatal DEX therapy is equivalent to the physiologic stress response experienced by premature infants, leads to receptor occupancy in target cells of > 75%, and causes induction of glucocorticoid-regulated genes (Ballard and Ballard, 1995). This therapy produces a peak human fetal free plasma concentration of 30 nM (Ballard and Ballard, 1995) and we therefore targeted this value as our concentration maximum. Surprisingly the half-life of DEX is identical in humans and rats (Samtani and Jusko, 2005a) and by targeting a 30 nM concentration maximum we are attempting to achieve human clinical exposure in fetal rats. d) The route of drug input for simulations was intramuscular injection and the
dosing interval was fixed at 12 hr since this is the clinically used method. e) DEX dosing was simulated to begin on gestational day 18, because sufficient fetal blood for measurement of free and total endogenous and exogenous steroid is available only after this stage of pregnancy. f) Free corticosteroids are elevated in the fetal circulation during late gestation in rats starting on pregnancy day 17 up to day 21 (VanBaeLEN et al., 1977). Thus DEX input into the model continued for up to six doses every 12 hr starting on gestational day 18 and time zero was always defined as gestational day 17.

Antenatal corticosteroids are indicated for threatened preterm labor during the gestational window of 24-34 weeks, which corresponds to 60-85% of human gestation (BallARD and BallARD, 1995). Most studies with larger animals involving antenatal corticosteroids are therefore conducted at 70-80% gestation. However, rats have a short gestational life of only 22 days and the time point selected for DEX input was 18 days (82% gestation) due to the fetal size restrictions. However, a short rodent gestational life also translates into a very late switch on time for critical organs such as fetal lungs and this becomes obvious in the companion paper. Expression of surfactant proteins doesn't start to rise until gestational day 19 and development appears to be completed by 21-22 days gestation. Thus, although 18 days gestation appears to be fairly late in development it still represents a state where the lungs are immature and is a suitable time frame for inducing precocious fetal lung maturation in rats.

To design a regimen that met of all the above criteria we utilized our model for DEX pharmacokinetics in rat pregnancy (Samtani et al., 2004a). The model was developed using some sparse literature data for total DEX concentrations in pregnant rats on gestational day 20. The model as shown in Fig. 1 consists of a two compartment
maternal/fetal exchange model with elimination only from the maternal compartment.

Such a simple system is possible only in rodents, where the fetus lacks drug metabolizing capability (Samtani et al., 2004a). The equations used were:

\[
\frac{dA_{IM}}{dt} = -ka \cdot A_{IM}, \quad A_{IM}(0) = \text{Dose} \quad (1a, b)
\]

\[
\frac{dA_m}{dt} = ka \cdot A_{IM} - \left( \frac{CL_m/F}{V_m/F} + \frac{CL_{mf}/F}{V_m/F} \right) \cdot A_m + \frac{CL_{mf}/F}{V_m/F} \cdot A_f, \quad A_m(0) = 0 \quad (2a, b)
\]

\[
\frac{dA_f}{dt} = -\frac{CL_{mf}/F}{V_f/F} \cdot A_f + \frac{CL_{mf}/F}{V_m/F} \cdot A_m, \quad A_f(0) = 0 \quad (3a, b)
\]

\[
D_f = \frac{A_f}{V_f/F} \quad (4)
\]

where subscript IM refers to the intramuscular drug administration compartment, A refers to amounts including the initial amount \((A(0))\), D refers to DEX concentration as a function of time \((t)\), and the terms expressed as a function of the intramuscular bioavailability \((F)\) refer to apparent pharmacokinetic parameters. Subscripts m and f refer to the mother and fetus, CL and V refer to clearance and volume of distribution, and CL_{mf} and CL_{fm} represent maternal to fetal and fetal to maternal placental transfer clearances.

The parameter values for bioavailability \((F)\) and absorption rate constant \((ka)\) were obtained from a recent publication on intramuscular absorption of this drug in female rats (Samtani and Jusko, 2005a), while other parameter values were from our recent work on PK of DEX in pregnant rats (Samtani et al., 2004a). The assumption was made that pharmacokinetics are not affected by the advancing state of pregnancy except for the parameter \(V_f\), which is expected to rise markedly because of fetal growth that occurs during the last days of gestation. \(V_f\) is expressed as:
\[ V_f = \text{Normalized } V_f \cdot \text{Fetal Body Weight} \cdot \text{Litter Size} \quad (5) \]

\[ \text{Fetal Body weight} = A_0 \cdot e^{\ln 2 \cdot t / T_D} \quad (6) \]

where normalized \( V_f \) is the fetal volume of distribution per gm of fetal tissue from our previous work (Samtani et al., 2004a), \( A_0 \) is the body weight of a single fetus at time zero (gestational day 17), and \( T_D \) is the doubling time from the literature (Schneidereit, 1985). Finally the total fetal concentration from equation 4 was converted to free concentration using a free fraction of 0.14 reported for pregnant rats (Stock et al., 1980) under the assumption that binding in fetal plasma is the same as maternal plasma.

**Animals**

Fifty-four time-pregnant Wistar rats were purchased from Harlan-Sprague-Dawley Inc. (Indianapolis, IN). Animals arrived at 12 days gestation and were housed in our University Laboratory Animal Facility maintained under constant temperature (22°C) and humidity with a controlled 12-hour light/dark cycle. A time period of 5 days was allowed for acclimatization. Rats had free access to rat chow and drinking water. Rats were randomly divided into two groups. Twenty-four rats (Group I) were assigned to the control group and thirty rats (Group 2) were assigned to the treatment group. Studies began either on gestational day 17 (Group 1) or day 18 (Group 2) at which time these animals weighed 330 – 440 gm. This research adheres to Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and was approved by the University at Buffalo Institutional Animal Care and Use Committee.

**Experimental**

**Control Rats.** Rats in Group I were sacrificed at 9:00 AM on GA 17, 18, 19, 20, and 21. Three additional time points at 5:00 PM on GA 18, 19, and 20 were also chosen.
Three animals were sacrificed at each time point. Rats were sacrificed by exsanguination under ketamine/xylazine anesthesia, with maternal blood drained from the abdominal aortic artery and fetal blood collected by neck incision. Individual fetuses were weighed and blood from all the fetuses from a single litter was pooled. Blood was collected in EDTA-containing syringes and capillary tubes, centrifuged immediately at 4°C, and plasma quickly harvested and aliquoted for different assays and ultrafiltration. Samples were frozen at ~20°C until analyzed. One fetus from each litter was frozen in liquid nitrogen and stored at -80°C. Fetal lungs were excised by dissection of the chest cavity, immediately frozen in liquid nitrogen, and stored at -80°C until used for RNA preparation, which is described in the companion paper.

**DEX Treated Rats.** The dose of DEX that met the criteria described in the simulation section was 1 µmol kg⁻¹ (0.4 mg kg⁻¹ DEX). Animals in Group 2 were administered up to six doses of DEX in the form of DEX sodium phosphate (Phoenix Scientific, Inc. St. Joseph, MO). These doses were injected intramuscularly between 8 to 9 AM and between 8 to 9 PM on GA 18, 19, and 20. Three animals were sacrificed at each of the following time points: 1, 6, and 9 hr after the first dose on GA 18; 10 min, 2.5 and 9 hr after the third dose on GA 19; 0.5, 4, and 9 hr after the fifth dose on GA 20; and 12 hours after the sixth dose on GA 21. The methods for sample collection were identical to the control group. The different sacrifice times on the four gestational days will help to illustrate the stationarity of the PK system.

A few methodological issues need to be elucidated further. The use of corticosteroid prodrugs in PK studies poses the risk of overestimation of corticosteroid concentrations due to in vitro hydrolysis of prodrugs after sample collection. The ex vivo prodrug
hydrolysis can be minimized by administering the prodrug via the intramuscular route where a good fraction of the prodrug is activated at the injection site and the steroid can be absorbed directly in the active form (Samtani and Jusko, 2005b). This is the preferable administration route since it is used clinically and produces almost complete and rapid DEX input (Samtani and Jusko, 2005a). To stabilize samples they should be collected in EDTA, kept on ice during processing delays, rapidly spun down at 4°C and stored immediately at −20 to −70 °C (Samtani and Jusko, 2005b). Prodrug hydrolysis is an enzymatic process and is highly temperature dependant (Samtani et al., 2004b). Cooling the samples, therefore, reduces ex vivo generation of DEX.

**Non-pregnant Controls.** Three non-pregnant control female rats were sacrificed before the beginning of the dark cycle, which represents the peak of the circadian cycle for corticosterone in rats (Hazra et al., 2004). Total and free plasma corticosterone concentrations in plasma from these three animals was measured and compared to values from fetal samples.

**Plasma Steroid Assays**

**Preparation of Samples for Measurement of Unbound Steroid Concentrations.** Protein free samples for measurement of unbound corticosterone and DEX concentrations were prepared by the method of ultrafiltration using the Amicon Centrifree Device (Millipore, MA) with a 30 kDa molecular weight cut-off filter. 300-400 µL of fetal plasma was spun at 1000 x g in a fixed angle rotor for 6 minutes, while 0.5 mL maternal plasma was spun for 15 minutes at 37°C. Preliminary experiments indicated that filtration of up to 50% of the sample did not affect binding equilibrium and binding of corticosterone and DEX to the ultrafiltration device was negligible. The ultrafiltration
conditions described above produced a filtrate volume that was 30-40% of the initial plasma used.

**DEX Analysis by Liquid Chromatography Tandem Mass Spectrometry.** DEX was measured in maternal/fetal plasma, plasma ultrafiltrate and fetal tissue. Plasma DEX concentrations were determined by a highly sensitive and specific solid phase extraction liquid chromatography tandem mass spectrometry method as previously described (Samtani et al., 2005). The limit of quantitation was 0.25 nM and the inter-day and intra-day coefficients of variation were less than 20%.

Fetuses from control rats were used for generation of pooled blank fetal tissue homogenate for constructing standards and controls. Entire fetuses were weighed and homogenized (4 mL/g tissue) in ice-cold phosphate-buffered saline (Gibco Invitrogen Corp., Grand Island, NY) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at speed setting 5 with 3 bursts of 10-sec duration with 30-sec intervals. Blank plasma ultrafiltrate was obtained by spinning a 15 mL starting volume of rat plasma in Amicon ultra-15 centrifugal filter units (Millipore, MA) with a 30 kDa molecular weight cut-off filter in a swinging bucket rotor at 1000 x g for 45 minutes at 37°C. Standards and quality controls were prepared by spiking known quantities of steroids into blank matrices and were run on a daily basis for assay calibration.

Sample preparation for the fetal tissue involved addition of 50 µL of the internal standard and 1 mL of methanol to 0.5 mL of tissue homogenate. Addition of methanol caused precipitation of proteins and extraction of steroids upon thorough vortexing. The samples were then centrifuged at 8000g for 20 minutes and the supernatant siphoned off into 50 mL polypropylene tubes to which 20 mL of HPLC water was added to reduce the
content of methanol to < 5%. This mixture was vortexed and subjected to solid phase extraction using Oasis HLB 20cc Vac RC 30 mg cartridges (Waters Corporation, Milford, MA). The extraction procedure is otherwise identical to that described for plasma, except that the funnel shaped Vac RC cartridges can accommodate a 20 mL processed sample instead of the 1 mL processed sample described for plasma.

Sample preparation for plasma ultrafiltrates involved adding 0.75 mL HPLC water to 0.2 mL sample in polypropylene tubes. Sample volume was restricted to 200 µL because the maximum volume of ultrafiltrate obtained during rat PK/PD studies ranges between 100-200 µL. Added was 50 µL of a methanolic stock (1 µg/mL) of prednisolone as internal standard and thereafter the 1 mL processed ultrafiltrate sample was handled exactly identical to the plasma samples.

**Corticosterone Analysis by Radioimmunoassay.** The possibility of simultaneously monitoring corticosterone with DEX during LC/MS/MS analysis was investigated. However, the sensitivity was not satisfactory for corticosterone during PK studies involving DEX due to its strong adrenosuppressive effect. We therefore utilized the commercially available ImmuChem™ Double Antibody ¹²⁵I kit from MP Biomedicals (Costa Mesa, CA) which requires only 5 µL plasma samples. Two minor modifications have been commonly utilized in the literature to improve the sensitivity of this kit (Duffy et al., 2000; Huot et al., 2002). This enhancement in sensitivity becomes necessary when evaluating samples from DEX treated animals that exhibit extremely low corticosterone. We incorporated two additional standards i.e. 36 and 18 nM into the curve by diluting the lowest standard (72 nM) provided with the kit and decreased sample dilution from 1:200 to 1:100. These changes improved the lower limit of quantification from 72 to 9 nM.
using a 5 μL plasma sample. Samples were counted in a 1272 CliniGamma counter from LKB Wallac with a counting time of 2 minutes/tube. Finally, in our experience and in other published reports (Pacak et al., 1995; Taymans et al., 1997) this kit has been found to be suitable for measurement of free corticosterone in plasma ultrafiltrates. To achieve measurable levels of free corticosterone 25 μL of ultrafiltrate samples were diluted 1:10 with assay buffer. The lower limit of quantification with plasma ultrafiltrates was 0.9 nM.

**Mathematical Models**

**Fetal Growth.** Growth curves describing the average body weight of a fetus from a litter in control and DEX treated animals were pooled and equation 6 was fitted to the data to obtain estimates of $A_0$ and $T_D$.

**DEX Pharmacokinetics.** Although protein binding in maternal and fetal plasma was linear the extent of binding was not identical. Differential equations were thus allowed to operate on free rather than total drug concentrations. The DEX PK component of the model presented in Fig. 1 was fitted to free maternal/fetal plasma concentrations using equations 1a to 3b. Five data sets were fitted simultaneously: a) unbound maternal plasma DEX; b) unbound fetal plasma DEX; c) total fetal tissue DEX content expressed as pmol per gm of fetal tissue d) total maternal plasma DEX; and e) total fetal plasma DEX; and the output functions were:

$$D_{m,\text{free}} = \frac{A_m}{V_m/F}$$

$$D_{f,\text{free}} = \frac{A_f}{V_f/F}$$
The additional subscripts free and total refer to unbound and total concentrations in plasma and \( f_{up} \) is the free fraction in plasma. The average fetal body weight in equation 9 was fixed to the fitted curve obtained from analyzing the fetal growth data in the previous section. The average litter size during the study was 14.6. The eight fitted parameters were: \( V_m/F \), Normalized \( V_f/F \) (see equation 5), \( CL_m/F \), \( CL_mF/F \), \( CL_f/F \), \( ka \), maternal \( f_{up} \), and fetal \( f_{up} \).

**Fetal Corticosterone Temporal Patterns in Control vs. Treated Animals.** Our data demonstrated that corticosterone binding in maternal/fetal plasma was highly non-linear and non-stationary and administration of DEX had a marked inhibitory effect on corticosterone secretion. Thus, for the purposes of simplicity and also because the fetal steroid concentrations are of the greatest interest since they drive fetal PD, only the fetal corticosterone data were modeled. The corticosterone inhibition component of the model depicted in Fig. 1 was fitted to the data using:

\[
\frac{dC_{Free,Control}}{dt} = k_o - k_{el} \cdot C_{Free,Control} \cdot \text{ControlFree}_0 \quad C_{Free,Control}(0) = C_{Free,0} \tag{12}
\]

\[
\frac{dC_{Free,DEX}}{dt} = k_o \cdot (1 - \frac{D_{f,Free}}{D_{f,Free} + IC_{50,Free}}) \cdot k_{el} \cdot C_{Free,DEX} \cdot \text{Free}_0 \quad C_{Free,DEX}(0) = C_{Free,0} \tag{13}
\]
Equation 12 and 13 describe the change in fetal free corticosterone concentrations in control and DEX treated animals. The $k_o$ and $k_d$ represent zero-order production and first-order loss rate constants for fetal free corticosterone. The fetal free concentration of DEX ($D_{f,\text{free}}$) obtained from the previous section was fixed and allowed to inhibit the production rate of corticosterone using an IC$_{50,\text{free}}$ term that represents the fetal free DEX concentration causing 50% inhibition of corticosterone synthesis rate. The $C_{\text{Free,0}}$ was the average fetal free concentration of corticosterone observed on gestational day 17.

To rationalize the cause of the non-linearity and non-stationarity observed in the corticosterone temporal profiles, literature data for fetal rat albumin and CBG as a function of gestation were utilized (VanBaelen et al., 1977; Tam and Chan, 1977). Data were recaptured by computer digitalization (Sigma Scan, Jandel Scientific, Corte Madera, CA, USA) and fitted to the following sigmoid functions:

\[
\text{CBG} = \frac{E_{\text{Max},c} - (E_{\text{Max},c} - E_{0,c}) \cdot t^{\gamma_c}}{t^{\gamma_c} + tc_{50}^{\gamma_c}} \quad (14)
\]

\[
\text{Albumin} = E_{0,a} + \frac{(E_{\text{Max},a} - E_{0,a}) \cdot t^{\gamma_a}}{t^{\gamma_a} + ta_{50}^{\gamma_a}} \quad (15)
\]

$E_{\text{Max}}$ and $E_0$ represent the upper and lower plateaus of the sigmoid functions, $\gamma$ values are sigmoidicity parameters, descriptors $a$ and $c$ refer to CBG- and albumin-specific parameters, and $tc_{50}$ and $ta_{50}$ are time parameters where CBG and albumin change by 50%. The following equations describing total corticosterone concentrations in control and DEX treated animals were fitted simultaneously along with equations 12 and 13:

\[
C_{\text{Total,Control}} = C_{\text{Free,Control}} + \frac{C_{\text{Free,Control}} \cdot \text{CBG}}{C_{\text{Free,Control}} + K_{D,\text{CBG}}} + \frac{C_{\text{Free,Control}} \cdot \text{Albumin} \cdot n}{K_{D,\text{Albumin}}} \quad (16)
\]
\[ C_{\text{Total, DEX}} = C_{\text{Free, DEX}} + \frac{C_{\text{Free, DEX}} \cdot \text{CBG}}{C_{\text{Free, DEX}} + K_{\text{D, CBG}}} + \frac{C_{\text{Free, DEX}} \cdot \text{Albumin} \cdot n}{K_{\text{D, Albumin}}} \]  

\[ (17) \]

\( K_{\text{D, albumin}} \) and \( K_{\text{D, CBG}} \) were estimated and refer to the equilibrium dissociation constants for corticosterone binding to its two binding proteins; \( n \) is the number of binding sites per molecule of albumin available for corticosterone binding and was fixed to 2 (Jobin and Perrin, 1974). CBG is a high affinity, low capacity binding protein which exhibits non-linear binding, while albumin is a high capacity, low affinity non-saturable binding protein.

**Data Analysis.** Data from multiple animals were pooled and the ADAPT II program (D'Argenio and Schumitzky, 1997) with the maximum likelihood method was applied for all fittings. The following variance model was used:

\[ \text{Variance} = \text{Coefficient}^2 \cdot Y(t)\text{Power} \]  

\[ (18) \]

where \( \text{Coefficient} \) and \( \text{Power} \) are variance parameters, and \( Y(t) \) represents the model output function. The goodness-of-fit was assessed using correlation coefficients, examination of residuals, visual inspection of the fitted curves, estimator criterion value, sum of squared residuals, and coefficients of variation of the estimated parameters.
Results

Simulation Study Results

Fig. 2 compares the PK results of the simulation (1 umol kg\(^{-1}\) dose) vs. the actual observed fetal exposure to free DEX upon serial IM administration. The figure also provides the desirable lower threshold, the detrimental upper threshold, and the targeted concentration maximum. The simulation correctly predicted the time to reach concentration maximum after each dose, but generally under predicted the fetal exposure to free DEX.

Fetal Growth Curves

The fetal growth curves followed an exponential growth pattern (Fig. 3). Variability in the control group on gestational day 21 (96 h) may be due to the variable litter sizes that were obtained from 2 animals at this time point where one rat had only 8 fetuses, while another had 19 fetuses. Parameter estimates were \( A_0 = 1 \) gm (CV: 3\%) and \( T_D = 1.7 \) day (CV: 3\%). Fig. 4 visualizes the burst of growth that occurs in fetal rats during late gestation.

Maternal/Fetal DEX PK

Total and unbound DEX profiles in the maternal/fetal circulation are shown in Fig. 5. DEX appeared very rapidly in the systemic circulation after IM administration of DEX sodium phosphate. This is evident from the maternal total DEX concentration of 500-750 nM observed at 1 hr, 10 and 30 min after the first, third and fifth doses. Fetal DEX, although lower than maternal concentrations, exhibited rapid transfer across the placenta. DEX could be detected in the fetal circulation at the first sacrifice time point of 1 hr after
the first dose. The fetal tissue DEX contents (ng/gm fetus) were slightly higher than the total fetal plasma concentrations, which indicate that DEX distributes extensively into fetal tissues. On all days of the study, DEX concentrations declined with a half-life of approximately 3 hr in maternal and fetal circulations, which is excellent agreement with our previous results (Samtani and Jusko, 2005a; Samtani et al., 2004a). The free fraction of DEX in the maternal circulation was about 20%, indicating that it is moderately bound to albumin. In contrast the free fraction in the fetal circulation was approximately 40%. DEX PK parameters listed in Table 1 were estimated with high precision, which is evident from the < 35% CV for all parameters. Fig. 6 provides unbound maternal/fetal data from different gestational days plotted against the time since last dose. The plot takes the shape of a simple Bateman function and helps to verify DEX PK stationarity during pregnancy.

Temporal Fetal Corticosterone Patterns in Control/DEX Rats

Total corticosterone in control fetal rats began to rise on GA 17, stayed high up to 19 day GA, and exhibited a shallow decline between GA 19 and 21 (Fig. 7). In contrast the free corticosterone rose to a maximum on day 17 and stayed constant until the end of the study. Total corticosterone in the fetus was somewhat lower than the circadian high observed in non-pregnant controls. However, the free corticosterone in fetal rats closely mirrored the circadian high that occurs in non-pregnant controls after GA 17. Maternal administration of DEX caused marked adrenal suppression, which led to lowering of the total and free corticosterone concentrations (Fig. 7). Corticosterone concentrations in adrenosuppressed fetuses, although fairly low, were still captured by the radioimmunoassay. Parameters describing the release and binding pattern of
corticosterone in fetal rats and inhibition of corticosterone secretion by DEX are provided in Table 2.

**Plasma Protein Binding Stationarity and Linearity**

Binding of DEX in maternal and fetal plasma was found to be linear (Fig. 8). However, DEX was more extensively bound in maternal than in fetal plasma. Thus the common assumption of equal binding in maternal/fetal plasma was found to be inappropriate. Therefore in modeling the data the differential equations were allowed to operate on the free rather than the total drug concentrations. Binding of corticosterone in maternal and fetal plasma in both control and DEX treated groups was non-linear and non-stationary (Fig. 8). The non-stationarity can be attributed to the rapidly declining CBG concentrations in rat plasma during the last few days of gestation (Fig. 9). During this period the albumin concentrations also change but with an increasing trend (Fig. 9). Despite the increasing albumin concentrations, binding to this protein is expected to remain linear due to the large size of this binding pool, which exists at 50-100 times higher concentrations in the fetal rat as compared to CBG. The linearity of this binding system in the fetal rat is illustrated by the binding characteristics of DEX, which binds to albumin alone. The parameter estimates describing the change in fetal plasma binding proteins as a function of time are shown in Table 3.

**Fetal/Maternal Gradients for Corticosteroids**

Fetal/maternal gradients are usually evaluated by assessing the ratio of unbound fetal to maternal concentrations. Binding of steroids to different extents in the two plasma pools can cause differences in total drug concentrations between the mother and the fetus. By estimating the ratio of free drug concentrations the binding effects in the two exchanging
pools can be negated. Assessment of this ratio for corticosterone in control rats shows that this ratio is roughly distributed around one, indicating that corticosterone distributes readily across the placental interface (Fig. 10). However, the ratio for DEX exhibits a value of less than one in all of the thirty steroid-treated animals. This indicates that the placenta creates a barrier for the transfer of DEX from the maternal to fetal circulation (Fig. 10). The possible source of this placental barrier can be understood by comparing the values of Cl_{mf} and Cl_{fm} obtained during curve fitting of the DEX PK data (Table 1). A higher value for Cl_{fm} indicates that the placental DEX transfer occurs much more efficiently in the fetal to maternal direction.
Discussion

The simulation study underestimated the fetal free DEX exposure. This discrepancy can be attributed to an inappropriate assumption (equal maternal/fetal plasma protein binding) made during the simulation study. The fetal plasma protein binding of DEX was found to be lower than that in maternal plasma. This could possibly occur because the fetal plasma protein content is far lower than maternal plasma and because fetal rat albumin starts to rise late in gestation (Tam and Chan, 1977). Despite the disagreement between the simulated and observed profiles, the measured fetal DEX exposure still met the desired criteria and thus the simulation exercise served as a suitable method for designing a prenatal regimen.

DEX did not appear to have an effect on fetal growth and therefore growth curves from both groups were pooled and modeled. The fitted parameters for fetal growth are in good agreement with literature estimates of $A_o = 0.7$ gm and $T_D = 1.5$ day (Schneidereit, 1985).

Binding of DEX, although different in maternal and fetal plasma, was found to be linear in both compartments. This is somewhat unexpected considering that fetal plasma albumin changes dramatically during the last days of gestation (Fig. 9). However the concentration of albumin even in fetal plasma is in the high $\mu$M range. This probably precludes saturation of albumin binding sites by DEX which circulates at nM concentrations after administration of a $1 \mu$mol kg$^{-1}$ dose.

Binding of corticosterone in fetal plasma was found to be highly non-linear and non-stationary. It is interesting to note that although the total concentrations of corticosterone decline in control fetal rats after GA 19, the free concentrations stay unchanged. All these observations can be explained by the sharp fall in CBG concentrations during late
gestation. As CBG concentrations decline, more corticosterone is available for elimination, which causes total corticosterone to decrease but allows the free corticosterone to stay at an elevated plateau. These high fetal free concentrations are close to the circadian peak in corticosterone concentrations that occur daily in control rats. These elevated concentrations mean that more corticosterone is available for distribution to sites such as the fetal lung for mediating PD effects. The declining CBG pool therefore serves as an important mechanism for amplifying glucocorticoid effects. This emphasizes the fact that measuring total steroid concentrations can be misleading and it is the free steroid that is the active species driving PD. Thus, in the companion report, the present fetal free steroid data will be used for driving corticosteroid effects.

There was a marked difference in the distribution of corticosterone and DEX across the placenta. Corticosterone distributed across the barrier readily, while DEX exhibited restricted fetal access. It is generally thought that placental 11-β hydroxysteroid dehydrogenase creates a barrier for maternal endogenous corticosteroids by metabolizing them to their inactive 11-keto metabolites (Yang, 1997). However, it has been recently shown that this placental enzyme shuts down in the rat placenta during the last days of gestation allowing greater access of maternal corticosterone to the fetus (Waddell et al., 1998). This enzyme behavior has been postulated as a possible mechanism for the increased glucocorticoids circulating in the fetal circulation during the last days of gestation. Our data support this hypothesis since the free concentrations of corticosterone appear to equalize in the maternal/fetal circulations after GA 18 in the control group. In contrast DEX access to the fetal circulation was restricted by some placental process. We have recently postulated that placental P-glycoprotein may serve as a barrier to
maternally administered DEX (Samtani et al., 2004a). Most synthetic corticosteroids, including DEX, are excellent substrates for this transporter (Yates et al., 2003) and the placenta expresses high levels of P-glycoprotein where it serves as a pump for effluxing xenobiotics from the fetal to the maternal circulation (Young et al., 2003). The P-glycoprotein argument is further strengthened when corticosterone data are reconsidered. Corticosterone, unlike synthetic corticosteroids, is not a P-glycoprotein substrate (Yates et al., 2003; Karssen et al., 2001) and thus the lack of metabolic and efflux barriers allow this steroid to distribute equally across the placenta. Finally, the importance of computing free fetal/maternal concentration ratios cannot be stressed enough. Due to the lack of available data we previously compared total fetal and maternal DEX concentrations from the literature (Samtani et al., 2004a). This comparison revealed a fetal/maternal gradient of 0.2. It is only now that we understand that an important reason for this gradient is the lower binding of DEX in the fetal circulation. A higher free fraction in fetal plasma allows greater return of DEX from the fetal to the maternal circulation. However, by computing the fetal to maternal ratio of free DEX (average = 0.55 across the duration of the study) it is recognized that plasma protein binding does not completely explain the fetal to maternal gradient and some additional process restricts fetal access, which is probably placental efflux.

It was noted that one animal sacrificed as 81 hr showed markedly lower steroid concentrations (Fig. 5). All five measurements (maternal total and free, fetal total and free, and fetal tissue content) from this particular animal appeared as outliers. This observation rules out assay error since five mismatches cannot arise from the assays. By extrapolating the curves from the previous dose it appears that this animal may not have
received the fifth dose. In performing these labor intensive animal studies, the possibility of such human error cannot be ruled out. The enormity of the study size can be judged from the fact that the 54 pregnant animals humanely killed in this study yielded almost 800 fetuses. The possibly erroneous data points were retained during the modeling process because the use of the maximum likelihood procedure, which is inherently a way of weighting the data, is less susceptible to outliers during the curve fitting procedure (Gabrielsson and Weiner, 1997). This point is emphasized by the fitted curves, which do not appear to be influenced by the five outliers.

The estimation of DEX PK parameters made use of a simple two-compartment maternal/fetal exchange model that assumed stationary PK across the entire duration of the study, except for the parameter Vf. The property of stationary DEX PK is demonstrated in Fig. 6 where unbound maternal/fetal concentrations from different gestational days are plotted against the time since last dose. On gestational days 18, 19, and 20 a trough kill at 9 hr was performed. The overlapping of DEX concentrations at this time point on maternal/fetal profiles in Fig 6. shows that PK are not altered by advancing GA. Furthermore, the data take the shape of a simple Bateman function, which further illustrates stationarity. In constructing Fig. 6, reverse super-positioning (Bauer and Gibaldi, 1983) to strip off contributions of the previous doses during the second to the sixth dosing intervals was not applied. The dosing interval exceeded four elimination half-lives and the contribution of the previous dose would be < 7%, making the correction factor marginal. Fig. 6 also demonstrates that although only ten time points were chosen over the course of a 72 hr study, the judicious choice of sample collection points during this multiple-dosing study helps capture both the disposition and absorption
properties of DEX with good precision (evident from the < 35% CV for all parameters in Table 1).

Modeling the corticosterone data, although yielding parameters with high degrees of precision, produced fitted curves that did not characterize all the data equally well. Examples include GA 18 data for total and free corticosterone in control and DEX treated fetuses (Fig. 7). One possible explanation is that all data in Fig. 7 were fitted simultaneously. It is known that DEX treatment can suppress levels of CBG (VanBaelen et al., 1977) which was not incorporated in the model. This is because quantitative assessments of DEX effects on CBG are sparse or qualitative in nature. Given the lack of complete information, CBG levels were assumed to be similar in control and treated animals. Furthermore, the main purpose of capturing steroid profiles was to ascertain the driving force behind corticosteroid effects. It is generally thought that effects are driven by free steroid concentrations and therefore any discrepancies in fitting the total corticosterone data are not a big concern. In addition, free corticosterone data in the DEX treated group will have a minor contributing effect towards steroid PD because the effects will be primarily driven by the exogenous steroid due to its higher affinity for the glucocorticoid receptor. Thus the most important profile amongst the different curves in Fig. 7 is the fetal free corticosterone in untreated controls and these data were captured relatively well. Finally, the estimates of $K_D$ values in Table 2 confirm that CBG and albumin are high and low affinity binding proteins, which is in good agreement with corticosterone binding properties (Jobin and Perrin, 1974).
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References


JPET #95851


Footnotes

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Figure legends

**Fig. 1**: Model of DEX pharmacokinetics and adrenosuppression mediated by free DEX in fetal plasma. Symbols and differential equations for the model are defined in the text. The dashed arrow and solid rectangle represent inhibition of corticosterone secretion by unbound DEX via an indirect mechanism.

**Fig. 2**: PK results of the simulation (solid curve) vs. the observed fetal exposure to free DEX (filled circles) upon intramuscular administration of 1 umol kg\(^{-1}\) doses. Horizontal lines depict desirable lower and detrimental upper thresholds and the arrow indicates the targeted concentration maximum.

**Fig. 3**: Fetal growth in control (open circles) and DEX treated animals (filled circles) followed the exponential growth model depicted by the solid line.

**Fig. 4**: Photographs of typical rat fetuses on indicated days showing the burst of growth that occurs during late GA.

**Fig. 5**: DEX PK in the mother (top) and fetus (bottom). Open and filled circles symbolize total and free DEX concentrations, while filled triangles represent the amount of DEX in fetal tissue. Solid, dashed, and dotted curves are model fitted results for total concentrations, free concentrations, and fetal tissue content.

**Fig. 6**: Stationary DEX PK is verified by plotting the unbound maternal (open symbols) and fetal (filled symbols) DEX concentrations on different gestational days as a function of time since last dose. Gestational days 18, 19, 20, and 21 are symbolized by triangles, squares, circles, and inverted triangles, respectively.

**Fig. 7**: Fetal corticosterone in untreated controls (top) and DEX treated animals (bottom). Open and filled symbols represent unbound and total concentrations. Triangles represent
data from non-pregnant controls sacrificed at the peak of the circadian corticosterone cycle. The model shown in Fig. 1 was fitted to all the data simultaneously and the curves represent model fittings.

**Fig. 8:** Protein binding of DEX (top) and corticosterone (bottom) in maternal/fetal plasma. Circles represent data from DEX treated animals, while data from controls are represented by triangles. Filled symbols represent maternal data, while open symbols are data from fetal samples. Straight lines are regressions through the maternal/fetal data to demonstrate linear plasma protein binding for DEX.

**Fig. 9:** Fetal plasma CBG (top) and albumin (bottom) as a function of advancing GA. Solid curves represent results of fitting the sigmoid functions to the data.

**Fig. 10:** Unbound fetal to maternal concentration ratio for corticosterone (top) and DEX (bottom). Horizontal lines depict the ratio of 1.
**Table 1.** Pharmacokinetic parameters for DEX in pregnant rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Estimate</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m/F$ (mL)</td>
<td>Apparent maternal volume of distribution</td>
<td>3097</td>
<td>6</td>
</tr>
<tr>
<td>Normalized $V_f/F$ (mL/gm fetus)</td>
<td>Apparent fetal volume of distribution</td>
<td>4.71</td>
<td>7</td>
</tr>
<tr>
<td>$CL_m/F$ (mL/hr)</td>
<td>Apparent elimination clearance</td>
<td>729</td>
<td>5</td>
</tr>
<tr>
<td>$CL_{mf}/F$ (mL/hr)</td>
<td>Apparent maternal to fetal placental transfer clearance</td>
<td>720</td>
<td>31</td>
</tr>
<tr>
<td>$CL_{fm}/F$ (mL/hr)</td>
<td>Apparent fetal to maternal placental transfer clearance</td>
<td>1342</td>
<td>32</td>
</tr>
<tr>
<td>$ka$ (hr$^{-1}$)</td>
<td>Absorption rate constant</td>
<td>8.52</td>
<td>28</td>
</tr>
<tr>
<td>Maternal f$_{up}$</td>
<td>Maternal DEX free fraction in plasma</td>
<td>0.18</td>
<td>7</td>
</tr>
<tr>
<td>Fetal f$_{up}$</td>
<td>Fetal DEX free fraction in plasma</td>
<td>0.39</td>
<td>7</td>
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</table>
Table 2. Parameters for the release and binding of corticosterone in fetal rats and inhibition of corticosterone secretion by DEX

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Estimate</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_o$ (nM/hr)</td>
<td>Zero order production rate of fetal free corticosterone</td>
<td>18.4</td>
<td>14</td>
</tr>
<tr>
<td>$k_{el}$ (1/hr)</td>
<td>First order loss rate of fetal free corticosterone</td>
<td>0.45</td>
<td>9</td>
</tr>
<tr>
<td>$K_{D, CBG}$ (nM)</td>
<td>Equilibrium dissociation constant for corticosterone binding to CBG</td>
<td>107</td>
<td>21</td>
</tr>
<tr>
<td>$K_{D, Albumin}$ (µM)</td>
<td>Equilibrium dissociation constant for corticosterone binding to albumin</td>
<td>21.5</td>
<td>25</td>
</tr>
<tr>
<td>IC$_{50, Free}$ (nM)</td>
<td>Fetal free DEX causing 50% inhibition of corticosterone synthesis rate</td>
<td>0.98</td>
<td>10</td>
</tr>
<tr>
<td>n</td>
<td>Number of binding sites per molecule of albumin available for corticosterone binding</td>
<td>2</td>
<td>Fixed</td>
</tr>
<tr>
<td>$C_{Free,0}$ (nM)</td>
<td>Average fetal free concentration of corticosterone observed on GA 17</td>
<td>9.44</td>
<td>Fixed</td>
</tr>
</tbody>
</table>
Table 3. Parameter estimates describing the change in fetal plasma binding proteins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Parameter Estimates (CV %)</th>
</tr>
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<tbody>
<tr>
<td>Emax (nM)</td>
<td>Upper plateau of the sigmoid function</td>
<td>122$\cdot$10³ (26)</td>
</tr>
<tr>
<td>Eo (nM)</td>
<td>Lower plateau of the sigmoid function</td>
<td>40.0$\cdot$10³ (Fixed)</td>
</tr>
<tr>
<td>t50 (hr)</td>
<td>Time parameter where CBG and albumin change by 50%</td>
<td>46.3 (50)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Sigmoidicity parameter</td>
<td>1.97 (48)</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5

Legend:
- • Maternal (pmol per mL)
- ▲ Fetal (pmol per mL or gm of fetus)

Gestational Age (day)

Maternal (pmol per mL)

Fetal (pmol per mL or gm of fetus)

Time (hr)
Fig. 6
Fig. 7
Fig. 8
Fig. 9

Gestational Age (day)

Fetal Plasma CBG (nM)

Time (hr)

Fetal Plasma Albumin (uM)

0 2 4 6 8 10 12 14 16 18 20 22

17 18 19 20 21 22

0 400 800 1200 1600 2000 2400

17 18 19 20 21 22

0 2 4 6 8 10 12 14 16 18 20 22

17 18 19 20 21 22

0 40 60 80 100 120 140 160 180 200 220 240 260

0 24 48 72 96 120

17 18 19 20 21 22

0 2 4 6 8 10 12 14 16 18 20 22

17 18 19 20 21 22

0 2 4 6 8 10 12 14 16 18 20 22

17 18 19 20 21 22

0 2 4 6 8 10 12 14 16 18 20 22
Fig. 10