Modeling Corticosteroid Pharmacokinetics and Pharmacodynamics, Part I: Determination and Prediction of Dexamethasone and Methylprednisolone Tissue Binding in the Rat[S]

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ABSTRACT

The plasma and tissue binding properties of two corticosteroids, dexamethasone (DEX) and methylprednisolone (MPL), were assessed in the rat in anticipation of developing physiologically based pharmacokinetic and pharmacokinetic/pharmacodynamic models. The tissue-to-plasma partition coefficients (K_P) of DEX and MPL were measured in liver, muscle, and lung in vivo after steady-state infusion and bolus injection in rats. Since K_P is often governed by reversible binding to macromolecules in blood and tissue, an attempt was made to assess K_P values of DEX and MPL by in vitro binding studies using rat tissue homogenates and to compare these estimates to those obtained from in vivo kinetics after dosing. The K_P values of both steroids were also calculated in rat tissues using mechanistic tissue composition–based equations. The plasma binding of DEX and MPL was linear with moderate binding (60.5% and 82.5%) in male and female rats. In vivo estimates of steroid uptake appeared linear across the tested concentrations and K_P was highest in liver and lowest in muscle for both steroids. Assessment of hepatic binding of MPL in vitro was severely affected by drug loss at 37°C in male liver homogenates, whereas DEX was stable in both male and female liver homogenates. With the exception of MPL in liver, in vitro–derived K_P estimates reasonably agreed with in vivo values. The mechanistic equations modestly underpredicted K_P for both drugs. Tissue metabolism, saturable tissue binding, and active uptake are possible factors that can complicate assessments of in vivo tissue binding of steroids when using tissue homogenates.

SIGNIFICANCE STATEMENT

Assuming the free hormone hypothesis, the ratio of the unbound drug fraction in plasma and in tissues defines the tissue-to-plasma partition coefficient (K_P), an important parameter in physiologically based pharmacokinetic modeling that determines total drug concentrations within tissues and the steady-state volume of distribution. This study assessed the plasma and tissue binding properties of the synthetic corticosteroids, dexamethasone and methylprednisolone, in rats using ultrafiltration and tissue homogenate techniques. In vitro–in vivo and in silico–in vivo extrapolation of K_P was assessed for both drugs in liver, muscle, and lung. Although the extrapolation was fairly successful across the tissues, in vitro homogenate studies severely underpredicted the K_P of methylprednisolone in liver, partly attributable to the extensive hepatic metabolism.

Introduction

Drugs interact with proteins and other macromolecules in body fluids and tissues. Such binding interactions influence the pharmacokinetics and pharmacodynamics of drugs. Binding to specific carrier proteins, transporters, and enzymes can influence tissue uptake and metabolism, whereas high-affinity binding to receptors influences tissue uptake in some cases (Levy, 1994) and is critical for evoking drug responses.

An appreciable portion of drug in the body may interact reversibly with proteins and cellular constituents in a nonspecific manner. Such nonspecific binding is a major determinant of drug distribution in the body and thus plays an important role in pharmacokinetics. The physiologically based Gillette equation (Gillette, 1971) characterizes the effects of body size and plasma and tissue binding on the steady-state volume of distribution (V_{ss}):

$$V_{ss} = V_p + \sum V_{Ti} \frac{f_{up}}{f_{ut,i}}$$

where V_p is plasma volume, V_{Ti} represents volumes of specific tissues, and f_{up} and $f_{ut,i}$ are the fractions of unbound drug in

ABBREVIATIONS: DEX, dexamethasone; HPLC, high-performance liquid chromatography; MPL, methylprednisolone; MPS, methylprednisolone sodium succinate; PBPK, physiologically based pharmacokinetic.

plasma and in specific tissues (assumed as concentration-independent parameters). The V_{ss}, along with clearance, influences important aspects of pharmacokinetics, including the mean residence time and total tissue concentrations. Simulations and limited experimental evidence suggest a stronger influence of tissue binding on half-life compared with plasma binding (Craig and Welling, 1977; Kurz and Fichtl, 1983). The ratio of f_{up} and f_{ut} defines the tissue-to-plasma partition coefficient (K_P), an important parameter used in physiologically based pharmacokinetic (PBPK) modeling.

Although unbound drug concentrations in plasma can be readily assessed, the study of tissue binding is complicated by methodological factors such as tissue isolation, sample processing, experimental system, and separation of bound versus free drug. In vivo, in vitro, and tissue composition–based computational approaches have been developed. In vivo methods measure drug uptake in tissues once steady state has been established between plasma and equilibrating tissue spaces. Drug in tissue, in excess of that diffused into tissue water, is considered bound to cellular constituents in the absence of metabolism, active transport, and ionization effects (Khalafallah and Jusko, 1984a).

As reviewed extensively (Fichtl et al., 1991; Pacifici and Viani, 1992), tissue binding can be studied ex vivo using perfused organs, tissue slices, and tissue homogenates. Organs such as the liver, kidney, and lungs can be isolated and tissue binding assessed by measuring the steady-state concentration in tissue and the free concentration in the perfusate. Limited duration of organ viability can, however, limit this approach in binding studies. Incubated tissue slices offer the advantage of being technically simple and can be applied to most tissues. However, similar to perfused organs, net drug uptake into slices may represent the sum of several processes (transport, metabolism, and lysosomal trapping), including binding. Tissue homogenates have been successfully applied for generating binding data for numerous drugs in various tissues (Lin et al., 1982; Fichtl et al., 1991; Kalvass et al., 2007; Berry et al., 2010). The advantages of this method include its simplicity and its applicability in most tissues, and results obtained by this technique are not distorted by possible active uptake into cells (Fichtl et al., 1991). However, limitations include the need for tissue dilution and homogenization, which disrupts cellular architecture and may alter the binding characteristics of the tissue.

Mechanistic approaches to predict tissue concentrations and V_{ss} attempt to estimate, based on plasma protein and red blood cell binding, lipophilicity, and pKa, the potential extent of drug partitioning into tissue components such as tissue water, albumin, neutral/charged lipids, and neutral/charged phospholipids (Poulin and Theil, 2000; Berezhkovskiy, 2004; Rodgers et al., 2005; Rodgers and Rowland, 2006). These methods have been fairly successful in predicting V_{ss} for several drugs (Graham et al., 2012), although factors such as active transport, binding to specific proteins, and lysosomal trapping can limit their applicability in some cases.

The synthetic corticosteroids, dexamethasone (DEX) and methylprednisolone (MPL), are frequently prescribed with varying dosage regimens, thus exposing body tissues to a wide range of concentrations. Classic theory holds that, in accordance with the "free hormone hypothesis" (Mendel, 1989), unbound steroids diffuse into intracellular spaces. However, limited experimental evidence indicates a role for active membrane uptake and transporters in steroid disposition (Schinkel et al., 1995; Lackner et al., 1998; Crowe and Tan, 2012). The ubiquitous presence of the glucocorticoid receptor in various tissues may influence steroid uptake in tissues due to high-affinity binding in the cytosol. Although the plasma pharmacokinetics of both steroids have been investigated in rats and humans (Dunn et al., 1991; Hochhaus et al., 2001; Samtani and Jusko, 2005; Hazra et al., 2007), limited information is available concerning their distribution into tissues. Studies in rabbits showed that prednisolone is taken up by several tissues in amounts exceeding the diffusion of free drug into tissue water (Khalafallah and Jusko, 1984a,b).

The aim of this first report of a three-part series (Ayyar et al., 2019a,b) was to determine the binding of DEX and MPL in vitro using tissue homogenates and to compare results with data obtained in vivo in male and female rats. Further comparison was made with predictions based on three mechanistic tissue composition equations of binding in rat tissues. Findings from the in vitro and in vivo studies were used to support the development of a minimal PBPK model of MPL in rats, presented in a companion article (Ayyar et al., 2019a).

Materials and Methods

Reagents and Chemicals

DEX and 6α-MPL ($\geq$98.5% purity) were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)–grade methylene chloride, heptane, and glacial acetic acid were obtained from Fisher Scientific (Pittsburgh, PA). Rats were dosed with methylprednisolone sodium succinate (MPS) (Solu-Medrol; Pharmacia & Upjohn Company, Kalamazoo, MI) via the intramuscular route. Milli-Q water was used (Millipore Corporation, Bedford, MA). DEX sodium phosphate solution (pharmaceutical grade) was purchased from Bimeda Pharmaceuticals (Dublin, Ireland) and standard DEX (purity >98%) was purchased from Sigma-Aldrich. DEX-D_5 (internal standard, purity >98%) was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Animals

Normal male and female Wistar rats were purchased from Envigo Inc. (Indianapolis, IN). The animals were housed in the State University of New York at Buffalo Laboratory Animal Facility and acclimatized under constant temperature (22°C) and humidity with a controlled 12-hour/12-hour light/dark cycle for 1 to 2 weeks. Three female rats were housed per cage, whereas two males were maintained in each cage. In all studies, rats had free access to rat chow and drinking water. The protocols adhered to the Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

In Vivo Distribution. Tissues and plasma harvested from a previously conducted infusion study (Ayyar et al., 2017) were used to assess MPL distribution at steady state. Briefly, male Wistar rats were given a 0.3 mg/kg per hour subcutaneous infusion of MPL for 1 week, and blood and tissues were harvested at various time points. Since steady state in plasma was achieved before 6 hours after the start of infusion, MPL concentrations in plasma, liver, muscle, and

lung were measured in male rats ($n = 3$) infused for 24 hours to determine K_P. The in vivo distribution of MPL in liver was also assessed by measuring the plasma and liver concentration-time profiles of MPL in male and female Wistar rats given a 50 mg/kg intramuscular bolus of drug. An in vivo subcutaneous DEX infusion study was conducted in male Wistar rats ($n = 3$) with the rate of 30 μg/h for 24 hours to achieve steady state, and blood and tissues were harvested for the determination of K_P. In addition, another group of male Wistar rats were given a 2.25 mg/kg subcutaneous bolus of DEX and various tissues and blood were harvested to explore the tissue distribution of DEX in male rats ($n = 3$) at various time points. In all studies, blood was harvested using EDTA as an anticoagulant (4 mM final concentration) to obtain plasma. Harvested tissues were rapidly dissected, frozen in liquid nitrogen, and stored at $-80°C$ until further use.

Tissues for In Vitro Binding. Normal male and female Wistar rats ($n = 2$ to 3 per sex per drug) were euthanized by aortic exsanguination under isoflurane anesthesia. Blood samples were collected into syringes precoated with EDTA and centrifuged immediately at 2000g for 15 minutes at 4°C. Plasma samples were collected and pooled for each animal group and used in plasma protein binding studies. Harvested tissues, except for liver, were rapidly dissected, frozen in liquid nitrogen, and stored at $-80°C$ until further use. Assessments of in vitro stability and tissue binding of DEX and MPL in liver homogenates employed fresh tissue.

Experiments

In Vitro Plasma Binding. Plasma protein binding of MPL and DEX was measured by ultrafiltration using Centrifree micropartition devices (Millipore Corporation) with a 30-kDa molecular mass cutoff filter. Briefly, varying volumes of ethanolic MPL solutions (0.00625, 0.0125, 0.025, 0.005, 0.25, and 1 mg/ml) were added (2.0% volume) to pooled blank plasma samples from each group to yield six plasma samples containing MPL per group (0.125, 0.25, 0.5, 1, 5, and 20 μg/ml). Similar to MPL, different volumes of DEX methanolic stock solutions were spiked into blank plasma samples from individual male and female rats. After incubation at 37°C for 30 minutes, aliquots (500 μl) of plasma of each concentration were transferred into ultrafiltration devices and centrifuged at 2000g for 20 minutes. The filtrates and remaining plasma samples were stored at $-20°C$ until analysis of both free and total steroid concentrations. No degradation was observed upon incubation in plasma samples at 37°C for up to 60 minutes. Preliminary studies indicated negligible nonspecific binding of either steroid to the ultrafiltration device.

In Vitro Tissue Homogenate Binding. Freshly harvested livers were placed on a chilled dish, crudely minced with a pair of scissors, and mixed. The wet weight of livers was determined and added to prechilled 1× PBS (pH 7.4) to prepare 3, 4, 6, and 10× dilutions of tissue and homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at speed setting 5 with two bursts of 15-second duration with 30-second intervals. Stock solutions of DEX or MPL were added (1.0% volume) to each homogenate dilution to produce final concentrations of 1 μg/ml (DEX) and 10 μg/ml (DEX and MPL). A total volume of 500 μl of each homogenate was loaded into ultrafiltration devices, incubated at 37°C for 15 minutes, and then centrifuged at 2000g for 20 minutes at 37°C. The filtrates and homogenates in the donor chamber were stored at $-80°C$ until further analysis. The volume of ultrafiltrate recovered after centrifugation was kept to <10% of the initial homogenate loaded. Measurement of DEX and MPL stability in rat liver homogenates was performed in parallel to liver binding experiments. Aliquots containing 80 μl of the spiked liver homogenates were incubated at 37°C and the reaction terminated at various times (0, 3, 7, 10, 15, 20, and 30 minutes). The samples were stored at $-80°C$ until further analysis. Tissues harvested from control (untreated) male Wistar rats were used for assessment of MPL and DEX binding in lung and muscle. Tissue

binding in both lung and muscle were carried out similar to liver, with the exception that muscle binding was assessed using a 6× dilution only due to technical reasons. The filtrates and homogenates in the donor chamber were stored at $-80°C$ until further analysis. Preliminary experiments confirmed that no depletion occurred in vitro in the lung and muscle homogenates throughout the binding experiment. Total protein content in the tissue homogenates was determined using the Lowry method (Lowry et al., 1951).

Plasma and Tissue Steroid Analysis. MPL was measured in plasma, tissues, and plasma and tissue filtrates using normal-phase HPLC (Haughey and Jusko, 1988) with minor modifications in the extraction procedure for each matrix. A detailed description of the assay is provided in a companion article (Ayyar et al., 2019a). DEX was used as the internal standard for all experiments. Chromatography conditions involved a mobile phase of 585 ml MeCl$_2$, 350 ml heptane, 10 ml glacial acetic acid, and 55 ml ethanol and a Zorbax silica column, a Waters model 1515 isocratic pump (Waters Corporation, Milford, MA), and a Waters model 2487 dual wavelength absorbance detector. The lower limit of quantification of MPL was 10 ng/ml in plasma and filtrates and 50 ng/g in tissue with an intra- and interday coefficient of variation of less than 10%. The quantification of DEX in plasma and filtrates from both tissue and plasma was described previously with minor modifications (Li et al., 2017b). Dried residue was reconstituted in 200 μl of acetonitrile/water [30:70 (v/v)] and formic acid was excluded from the mobile phase. For tissue samples, only the steps preceding solid phase extraction of DEX differed compared with plasma samples. Briefly, powdered tissue was homogenized in PBS at 6× dilution. Appropriate volumes of samples were added into 990 μl methanol. Where necessary, blank tissue homogenate was also added to achieve a total volume of 100 μl homogenate. A volume of 10 μl D$_5$-DEX as the internal standard was added to tubes and vortexed, and the sample was centrifuged at 4°C at 14,000g for 20 minutes. The supernatant was transferred to glass tubes and dried under nitrogen. Methanol (50 μl) was used for reconstitution and vortexed twice for 30 seconds. A total of 450 μl water was added and mixed. Samples (450 μl) were transferred to microfuge tubes containing equal volumes of 4% phosphoric acid and subjected to solid phase extraction using Oasis Prime HLB 1-cm^3 30-mg cartridges (Waters Corporation). The following steps were identical as reported for plasma samples. The lower limit of quantification of DEX was 0.2 ng/ml (or ng/g) in plasma, filtrates, and tissues.

Tissue Data Correction for Residual Blood. Drug concentrations obtained in each tissue were converted from nanograms per milliliter to nanograms per gram of tissue by assuming a tissue density of 1 g/ml. Tissue concentrations were corrected for residual trapped blood as follows:

$$C_t = \frac{C_{t(meas)} \cdot V_{meas} - C_{bl} \cdot V_{meas} \cdot (V_{vasc}/V_t)}{V_{meas} - [V_{meas} \cdot (V_{vasc}/V_t)]} \quad (1)$$

where C_t and $C_{t(meas)}$ are the corrected and measured tissue concentrations, C_{bl} is the measured concentration in blood, V_{meas} is the measured volume of collected tissue, and V_{vasc}/V_t is the fractional vascular volume of blood trapped in tissues as obtained from the literature (Kawai et al., 1998). The corrected tissue concentrations were used for further analysis.

Theoretical and Data Analysis

Calculation of In Vivo K_P. At steady state, the K_P for non-eliminating tissues is given by:

$$K_{P,i} = \frac{C_{i,ss}}{C_{p,ss}} \quad (2)$$

where C is the measured drug concentration, and the subscripts i, p, and ss denote the tissue, plasma, and steady state.

Because the liver is an eliminating organ, $K_{P,hep}$ at steady state is given by :

$$K_{P,hep} = \frac{C_{hep,ss}}{C_{plasma,ss}} \cdot \left(\frac{Q_{hep} + f_{u,p} \cdot CL_{u,int}}{Q_{hep}} \right) \qquad (3)$$

utilizing the hepatic blood flow rate (Q_{hep}), intrinsic clearance ($CL_{u,int}$), and fraction of drug unbound in plasma ($f_{u,p}$), where *hep* denotes liver.

The in vivo distribution of MPL in liver was also compared between both sexes by measuring by the plasma and liver concentration-time profiles of MPL in male and female Wistar rats given a 50 mg/kg intramuscular bolus of drug. Using these data, $K_{P,hep}$ was calculated as :

$$K_{P,hep} = \frac{AUC_{hep,0\to last}}{AUC_{plasma,0\to last}} \cdot \left(\frac{Q_{hep} + f_{up} \cdot CL_{u,int}}{Q_{hep}} \right) \qquad (4)$$

where $AUC_{hep,0\to last}$ and $AUC_{plasma,0\to last}$ represent the area under the curve for MPL from time 0 until the last measured time point. The values used for $CL_{u,int}$ in male rats were 2987 ml/h for MPL (Ayyar et al., 2019a) and 61.8 ml/h for DEX (calculated).

Assessment of K_P from In Vitro Binding. The binding of a drug to protein can be described by a Langmuir-type equation:

$$C_b = \frac{n \cdot P_T \cdot K_A \cdot C_u}{1 + K_A \cdot C_u} \qquad (5)$$

where C_b is the bound drug concentration, C_u is the unbound drug concentration, K_A is the association constant, and $n \cdot P_T$ is the maximum binding capacity. When $K_A \cdot C_u << 1$, then eq. 5 can be simplified to

$$C_b = n \cdot P_T \cdot K_A \cdot C_u \qquad (6)$$

Equation 6 can be rearranged to calculate the binding factor (*BF*), defined as the ratio of C_b and C_u:

$$BF = \frac{C_b}{C_u} = n \cdot P_T \cdot K_A \qquad (7)$$

Since in vitro assessment of drug binding in tissues necessitates preparation of homogenates in buffer, a dilution step is introduced, which results in a reduction of protein concentrations. If the drug binding characteristics are independent of protein concentration, then eq. 7 can be expressed as:

$$BF' = \frac{n \cdot P_T}{dil} \cdot K_A \qquad (8)$$

where *dil* is the fold dilution and $n \cdot P_T \cdot K_A$ is:

$$n \cdot P_T \cdot K_A = dil \cdot BF' \qquad (9)$$

The fraction of unbound drug in an in vitro homogenate ($f_{u,t}$) can be expressed as:

$$f_{u,t} = \frac{C_u}{C_b + C_u} \qquad (10)$$

Dividing by C_u and from eq. 9,

$$f_{u,t} = \frac{1}{(dil \cdot BF') + 1} \qquad (11)$$

and

$$\frac{1}{f_{u,t}} = (dil \cdot BF') + 1 \qquad (12)$$

It will be shown that eq. 12 for estimating in vitro binding in tissue homogenates is equivalent to the definition of in vivo partitioning of unbound drug into a tissue ($K_{P,u}$).

For liver and lung, *BF'* was measured across four tissue dilutions and extrapolated to an undiluted state to compute $f_{u,t}$ using eq. 12. Since binding in muscle was assessed only at a single dilution, $f_{u,t}$ was back-extrapolated to account for dilution as described previously (Kalvass et al., 2007):

$$\text{Undiluted } f_{u,t} = \frac{1/dil}{[(1/f_{u,measured}) - 1] + 1/dil} \qquad (13)$$

Under linear, time-invariant conditions and assumption of the free hormone hypothesis (Mendel, 1989), the in vivo partitioning into a tissue K_P is defined as follows:

$$K_P = \frac{C_{tissue(total)}}{C_{plasma(total)}} = \frac{f_{u,p}}{f_{u,t}} \qquad (14)$$

$K_{P,u}$ represents the partitioning of free drug within a tissue:

$$K_{P,u} = \frac{C_{tissue(total)}}{C_{plasma(total)} f_{u,p}} \qquad (15)$$

Based on eqs. 14 and 15, $K_{P,u}$ can be simplified to:

$$K_{P,u} = \frac{1}{f_{u,t}} \qquad (16)$$

which is equivalent to eq. 12. Finally, in vivo K_P can be predicted as the product of $f_{u,p}$ and $1/f_{u,t}$ measured in vitro (eq. 14).

Prediction of K_P Using In Silico Methods

For comparison, K_P was also calculated according to the tissue composition equations proposed by Poulin and Theil (2002) (referred to as method 1 hereafter) and corrected by Berezhkovskiy (2004) (method 2) and the Rodgers and Rowland method for neutral compounds (Rodgers and Rowland, 2006) (method 3) as programmed in the GastroPlus PBPK Simulator (version 9.6.2; Simulations Plus Inc., Lancaster, CA). These publications provide a detailed description of the approaches.

Results

Plasma Protein Binding

Protein binding studies of DEX and MPL were carried out using plasma from normal male and female Wistar rats. Plasma binding of MPL was assessed over total concentrations ranging from 163 to 22,230 ng/ml ($\sim$0.4–60 μM). Figure 1 (left) demonstrates the lack of concentration dependence of MPL binding in plasma. Plasma binding of MPL in both sexes was essentially identical, with 60.5% $\pm$ 1.6% bound in males and 60.4% $\pm$ 2.3% bound in females. This percentage of bound MPL is comparable to a previously reported estimate in male rats of 63.1% $\pm$ 0.8%, obtained using equilibrium dialysis (Haughey and Jusko, 1991). The linearity of binding indicates a nonsaturable, low-affinity binding to plasma proteins. This finding is consistent with the plasma protein binding of MPL observed in humans and rabbits (Ebling et al., 1986). The percent bound in rats, however, was lower than in humans (77.6%) and rabbits (78.5%), indicating a species difference. The linear binding of these steroids allows calculation of only composite binding affinity constants $N_A \cdot K_A$. Assuming that albumin is the only nonspecific binding protein (550 μM in female rats and 510 μM in male rats) (Rose and Klemcke, 2015; Li et al., 2017a), $N_A \cdot K_A$ ranges between 3.0×10^3 and 3.2×10^3 M. The estimated $N_A \cdot K_A$ for MPL is in close agreement
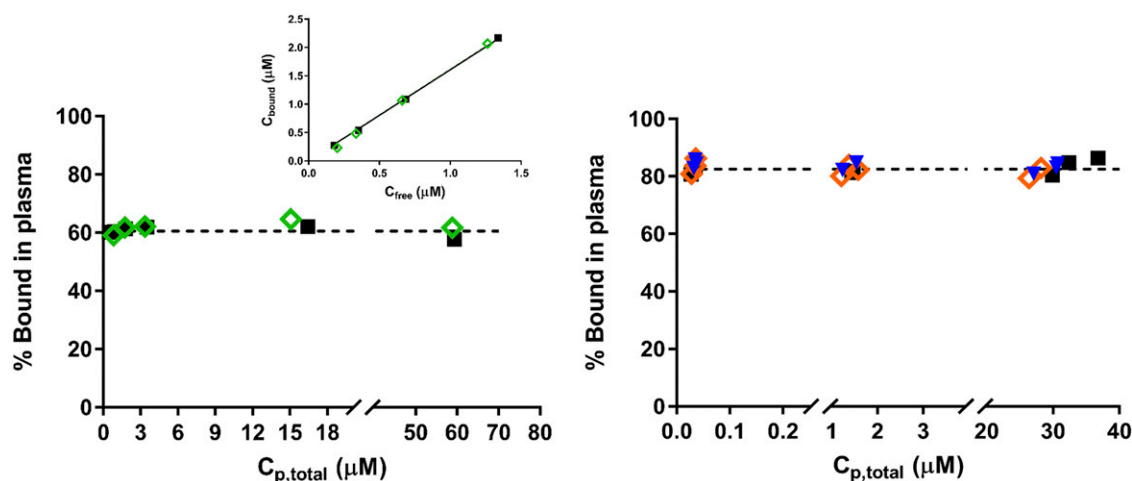
Fig. 1. Percent binding vs. total plasma steroid concentration for MPL (left) and DEX (right) in male (black squares), pooled female (green diamonds), estrus-phased female (orange diamonds), and proestrus-phased female (blue triangles) rat plasma. The inset depicts bound vs. free steroid concentrations of MPL in male and pooled female rat plasma.

to a previously reported value obtained in a hepatocyte incubation medium (inactivated horse serum; 3.15×10^3 M) (Morais and Wagner, 1985). This is also similar to values for prednisolone ($\sim$2.0 $\times 10^3$ M) in male rat plasma (Rocci et al., 1980). Plasma binding of DEX was assessed (Fig. 1, right) over total concentrations ranging from 11 to 12,712 ng/ml ($\sim$0.03–32.4 μM) in male, estrus female, and proestrus female rat plasma. No differences were observed across the three groups. Plasma protein binding of DEX was concentration independent (linear) across the range tested. The extent of plasma binding of DEX was higher compared with MPL at 82.5%, which is comparable to a value reported previously for DEX in male rat plasma (84.7% $\pm$ 0.7%) (Peets et al., 1969).

In Vivo Tissue Distribution

Steady-state tissue-to-plasma distribution of MPL and DEX was measured in liver, muscle, and lung obtained from male Wistar rats. The K_P values for each drug after steady-state infusion were calculated using eq. 1 for muscle and lung and using eq. 2 for liver. The K_P values of DEX in these tissues were also assessed upon subcutaneous bolus injection (2.25 mg/kg) in male rats. The concentration-time profiles of MPL after a 50 mg/kg intramuscular bolus were measured in plasma and liver from both male and female rats, and the resultant K_P values were calculated using eq. 3. The calculated K_P values of MPL and DEX in rat liver, muscle, and lung after bolus injection and/or steady-state infusion are shown in Fig. 2. For MPL, K_P was highest in liver (12.8 $\pm$ 1.2) and lowest in muscle (1.8 $\pm$ 0.4). The K_P values for MPL obtained in male livers via intramuscular bolus and subcutaneous infusion and in female livers after intramuscular bolus were similar (13.4 $\pm$ 0.9 vs. 13.5 $\pm$ 1.4 vs. 11.4 $\pm$ 1.4) after correction for organ extraction using eq. 3. Similar to MPL, the in vivo K_P of DEX was comparable using either subcutaneous bolus or infusion for each of the tissues. The K_P was highest in liver (bolus vs. infusion: 5.5 $\pm$ 0.3 vs. 4.8 $\pm$ 0.5) but unlike MPL, DEX showed similar accumulation in both lung (bolus vs. infusion: 0.53 $\pm$ 0.02 vs. 0.43 $\pm$ 0.05) and muscle (bolus vs. infusion: 0.50 $\pm$ 0.03 vs. 0.49 $\pm$ 0.04).

Stability of Drug in Tissue Homogenates

Stability of MPL and DEX at 37°C was assessed over time in plasma and in tissue homogenates. Depletion of either steroid was minimal throughout the course of the experiment in
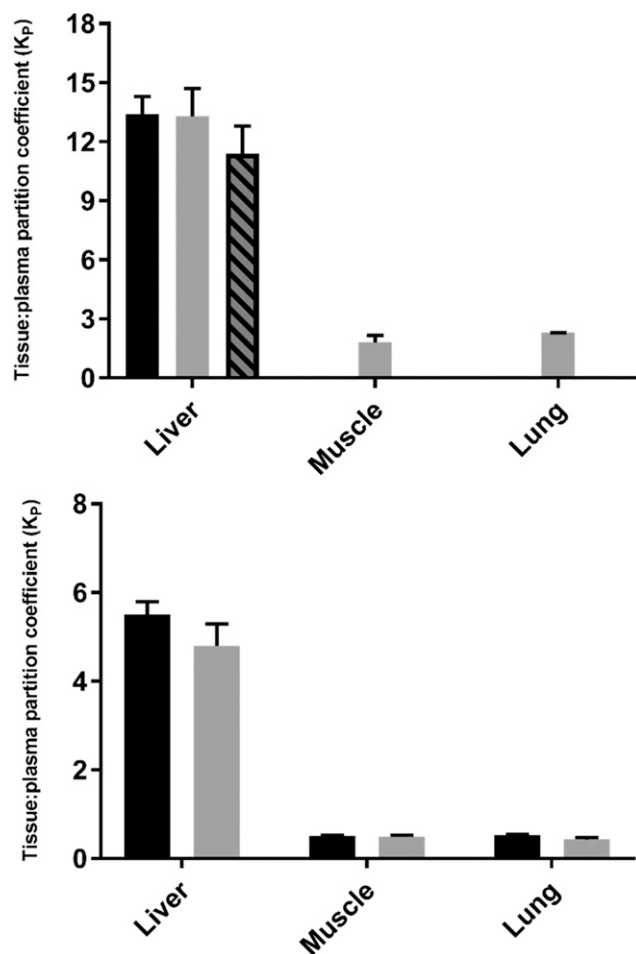

Fig. 2. MPL (top) and DEX (bottom) tissue-to-plasma partition coefficients (K_P) obtained in vivo by bolus injection in male rats (black bars), infusion in male rats (gray bars), or bolus injection in female rats (dashed gray bars). Error bars represent 1 S.D. from the mean.
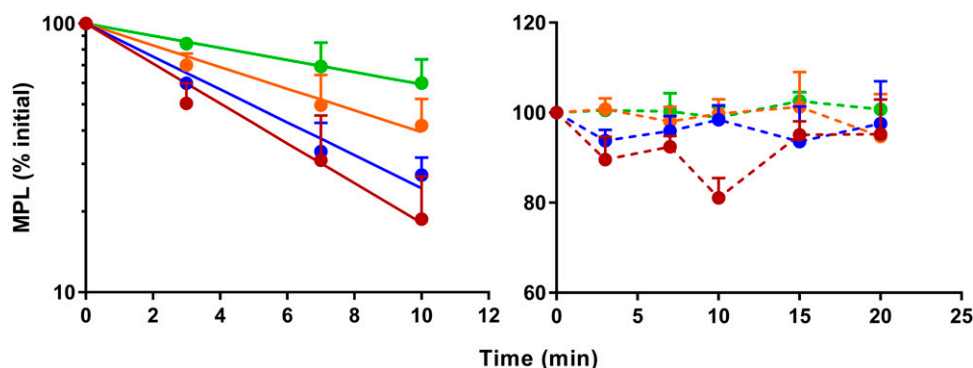
Fig. 3. Time course of in vitro stability of MPL in liver homogenates prepared at $3\times$ (red), $4\times$ (blue), $6\times$ (orange), and $10\times$ (green) dilutions from freshly harvested male (left) and female (right) livers. Symbols represent the mean $\pm$ S.D. ($n = 3$ per time point), the solid line depicts the first-order loss kinetics of MPL in male liver homogenates, and the dashed lines connect the data points.

plasma, muscle, and lung homogenates (data not shown) and did not appear to present any problem in analyzing the binding data. Robust depletion occurred for MPL in male liver homogenates, with the rate and extent of substrate loss inversely proportional to the degree of homogenate dilution. The rate of MPL depletion in the male liver homogenates obeyed first-order loss kinetics for up to 10 minutes after incubation at 37°C (Fig. 3, left), followed by a plateau in concentrations beyond 15 minutes. Of interest, negligible depletion of MPL occurred in female rat liver homogenates, and concentrations remained stable for the duration of the experiment (Fig. 3, right). Preparation of liver homogenates using cryopreserved powdered liver only modestly decreased the extent of MPL loss at 37°C (Supplemental Fig. 1). No significant depletion of DEX occurred in either male or female rat liver homogenates (Supplemental Fig. 2).

In Vitro Binding in Tissue Homogenates

The effect of dilution of tissue homogenates on MPL binding was examined across 3- to 10-fold dilutions of liver and lung homogenates. As shown in Fig. 4 and Supplemental Fig. 3, the extent of MPL binding was directly proportional to the total protein concentration in tissue homogenates. The unbound tissue fractions ($f_{u,t}$) for MPL in liver, muscle, and lung are presented in Table 1, as are the K_P values of MPL calculated on the basis of in vitro binding studies. As shown in Fig. 5, DEX binding was proportional to the total protein concentration in tissue homogenates. The unbound tissue fractions ($f_{u,t}$) for DEX in liver, muscle, and lung are presented in Table 2, as are the K_P values of DEX calculated on the basis of in vitro binding studies.

The $f_{u,t}$ for MPL in liver was estimated as 0.162 ± 0.01 in males and 0.066 ± 0.003 in females, which correspond to in vitro–derived K_P values of 2.5 ± 0.2 and 6.1 ± 0.4. Therefore, the homogenate studies, under the conditions described, significantly underpredicted in vivo K_P of MPL in male liver and, to a lesser extent, in female liver. The $f_{u,t}$ values for MPL in lung and muscle from male rats were estimated to be 0.192 ± 0.01 and 0.298 ± 0.05, corresponding to in vitro–derived K_P values of 2.1 ± 0.1 and 1.4 ± 0.2, in good agreement with their in vivo values. For DEX, the $f_{u,t}$ values in liver were similar in males and females at the two concentrations tested and were comparable to the value obtained for MPL in liver from female rats (~ 0.06). The in vitro–derived K_P values for DEX in liver were about 2-fold lower compared with in vivo estimates. The $f_{u,t}$ value for DEX in lung was 0.115 ± 0.006, and the in vivo K_P was overpredicted by around 3-fold. In muscle, the $f_{u,t}$ of DEX was 0.236 ± 0.03, with an in vitro–derived K_P 0.74 ± 0.1, in relatively good agreement with the in vivo value.

Prediction of Steroid K_P in Rat Tissues

K_P estimates obtained for MPL and DEX from in vivo and in vitro studies were compared with K_P values in rat tissues computed using the tissue composition–based approaches proposed by method 1 (Poulin and Theil, 2002) and corrected by method 2 (Berezhkovskiy, 2004) and method 3 (Rodgers and Rowland, 2006) using the GastroPlus PBPK Simulator. The predicted K_P values for MPL and DEX using these approaches are listed in Tables 1 and 2. All methods underpredicted K_P values severely for both drugs in liver but were somewhat closer for lung and muscle.
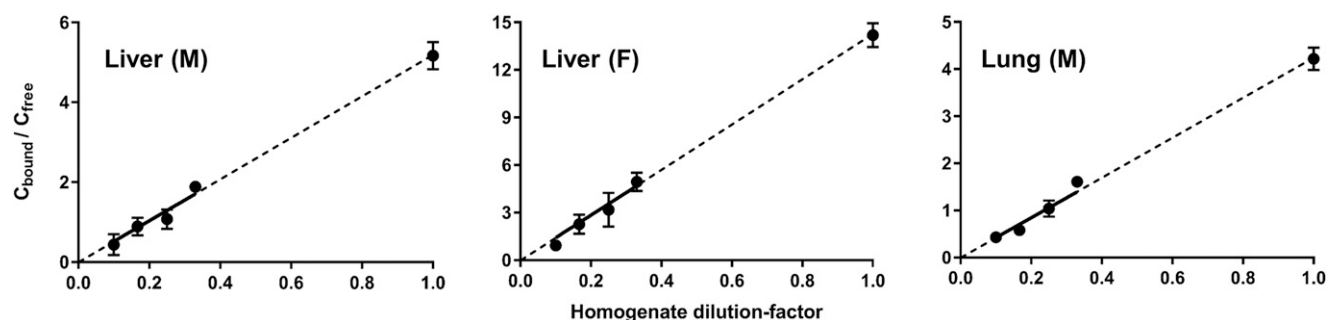

Fig. 4. Binding of MPL in homogenates prepared from male rat liver (left), female rat liver (middle), and male rat lung (right) at initial concentrations of 10 μg/ml. Symbols depict the mean $\pm$ S.D. of the binding factor (C_{bound}/C_{free}) across four dilutions of tissue homogenate. The dashed line represents the best-fit line extrapolated to an undiluted state of tissue ($dil = 1$).

TABLE 1
Comparison of tissue K_P values for MPL determined by different methods Values are listed as the mean ± S.D.

Tissue (Sex)	In Vivo K_P	$f_{u,t\ (in\ vitro)}$	In Vitro K_P	Method 1	Method 2	Method 3
Liver (male)	13.4 ± 0.9[a]	0.162 ± 0.01	2.5 ± 0.2	1.2	0.9	0.8
	13.3 ± 1.4[b]					
Liver (female)	11.4 ± 1.4[a]	0.066 ± 0.003	6.1 ± 0.4			
Lung (male)	2.3 ± 0.0[b]	0.192 ± 0.01	2.1 ± 0.1	1.3	1.0	0.9
Muscle (male)	1.8 ± 0.4[b]	0.298 ± 0.05	1.4 ± 0.2	0.9	0.8	0.6

[a]Intramuscular injection.
[b]Subcutaneous infusion.

Discussion

This study reports tissue binding data for DEX and MPL in the rat, obtained using different methods. A major goal of this work was to examine whether in vitro assessments of drug partitioning and binding using tissue homogenates (along with in vitro plasma binding information) could be used to reliably predict the in vivo tissue distribution of MPL and DEX in selected tissues. For this to be possible, several conditions must be met: 1) nonspecific binding to components of plasma and tissues dominates the tissue distribution process, 2) steroids distribute and bind relatively uniformly within each organ, 3) kinetics of drug uptake into tissues is not rate limiting (i.e., entry is rapid), 4) contributory binding processes are nonsaturating, and 5) active influx or efflux transport does not contribute significantly to distribution. Such assumptions are also operable with use of in silico methods of prediction of K_P.

Technical Considerations

It has been argued that drug binding to (bovine) serum albumin depends on albumin concentration in a complex manner (Shen and Gibaldi, 1974). However, binding of both MPL and DEX to rat plasma and to tissue (liver and lung) homogenates was found to be linearly related to protein concentration (Figs. 4 and 5; Supplemental Fig. 3). This is consistent with other reports demonstrating that drug binding is independent of homogenate protein concentration (Lin et al., 1982; Kurz and Fichtl, 1983; Schuhmann et al., 1987). Some studies have employed equilibrium dialysis for the separation of bound and free drug in homogenate studies. This technique involves lengthy equilibration times during which decay of tissue (Pacifici and Viani, 1992) as well as volume shifts (Boudinot and Jusko, 1984) can occur. Ultrafiltration also presents certain limitations. First, nonspecific binding of drug to the filter and collection apparatus can confound measurements of binding. Second, filtration of greater than 20% of the sample volume can lead to shifts in binding equilibrium (Shen and Gibaldi, 1974). Preliminary recovery experiments confirmed negligible binding of both DEX and MPL to the apparatus. In addition, filtration conditions were optimized such that the filtrate volume was less than 15% of the initial sample volume. To prevent drug metabolism during equilibrium dialysis in diluted tissue homogenates, Lin et al. (1982) conducted binding studies of ethoxybenzamide at 4°C. Although not the case for ethoxybenzamide, the unbound fractions for several other drugs tend to decrease with an increase in temperature (Ballard, 1974). Therefore, the in vitro homogenate studies for DEX and MPL described in this report were performed using ultrafiltration at 37°C. Total drug was stable at this temperature and thus did not present a problem in analyzing binding data, except for MPL in male liver homogenates, where robust depletion occurred upon incubation. Thus, the time course of total MPL concentrations was followed in liver homogenates during the experiment and used to correct for the drug loss. Despite this apparent correction, MPL binding in male liver homogenates underpredicted in vivo estimates by approximately 4-fold. In vitro binding of MPL was assessed at an initial concentration of 10 μg/ml. Low sensitivity of the HPLC assay in tissues (50 ng/g) coupled with small volumes of filtrate (25–60 μl) precluded the examination of concentration-dependent MPL tissue binding at lower concentrations. However, a single validation experiment (data not shown) performed at 1 μg/ml using 4× diluted liver homogenates with filtered volumes pooled from multiple devices yielded a bound/free ratio comparable to that obtained at 10 μg/ml (0.9 vs. 1.1), suggestive of concentration-independent binding within this range. The degree of MPL loss in homogenates was similar at both concentrations. A salient feature of this study was the investigation of in vitro binding of two steroids (DEX and MPL) in three distinct tissues (liver, lung, and skeletal
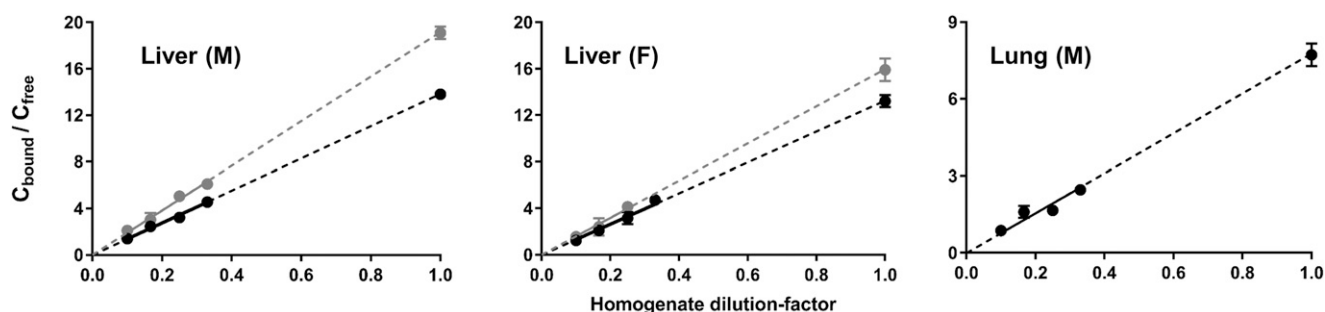
Fig. 5. Binding of DEX in homogenates prepared from male rat liver (left), female rat liver (middle), and male rat lung (right) at initial concentrations of 1 μg/ml (gray) and/or 10 μg/ml (black). Symbols depict the mean ± S.D. of the binding factor (C_{bound}/C_{free}) across four dilutions of tissue homogenate. The dashed line represents the best-fit line extrapolated to an undiluted state of tissue ($dil = 1$).

TABLE 2

Comparison of tissue K_P values for DEX determined by different methods Values are listed as the mean ± S.D.

Tissue (Sex)	In Vivo K_P	$f_{u,t}$ (in vitro)	In Vitro K_P	Method 1	Method 2	Method 3
Liver (male)	5.5 ± 0.3[a]	0.050 ± 0.001	3.6 ± 0.5[c]	1.3	0.7	0.6
	4.8 ± 0.5[b]	0.068 ± 0.001	2.6 ± 0.4[d]			
Liver (female)	—	0.059 ± 0.004	3.0 ± 0.5[c]			
		0.070 ± 0.003	2.5 ± 0.4[d]			
Lung (male)	0.53 ± 0.02[a]	0.115 ± 0.006	1.5 ± 0.3[d]	1.4	0.8	0.6
	0.43 ± 0.05[b]					
Muscle (male)	0.50 ± 0.0[a]	0.236 ± 0.03	0.74 ± 0.1[d]	0.9	0.6	0.34
	0.49 ± 0.04[b]					

[a]Subcutaneous injection.
[b]Subcutaneous infusion.
[c]Low DEX concentration (1 μg/ml).
[d]High DEX concentration (10 μg/ml).

muscle), for which the in vivo K_P values were found to vary across a wide range (0.5–13).

The in vitro homogenate studies examined the tissue binding and stability of MPL in its free alcohol form; therefore, assessments of MPL binding are not confounded by the prodrug conversion. Our in vivo assessments measured total MPL concentrations in rat plasma and tissues after intramuscular and subcutaneous dosing of its water-soluble succinate prodrug, MPS. Since MPS undergoes incomplete conversion (10%–20%) to MPL in rats (Kong and Jusko, 1991), prodrug instability ex vivo can lead to overestimation of MPL concentrations at early time points. Measures taken to minimize possible ex vivo hydrolysis of MPS were as follows: 1) use of EDTA as an anticoagulant when collecting blood, 2) rapid centrifugation of blood after collection at 4°C, 3) storage of plasma and muscle samples at −20°C and −80°C, 4) avoidance of repeated freeze-thaw cycles, and 5) use of a cold temperature (4°C) prior to drug extraction into methylene chloride for measurement by HPLC. After a bolus dose of intramuscular MPS in male rats, a high concentration of MPL at the local injection site (relative to plasma) was measurable within minutes after dosing (Hazra et al., 2007), indicating rapid and significant MPS conversion occurring at the tissue site prior to entry into the circulation. Kong and Jusko (1991) demonstrated using perfused rat livers that only 8%–20% of MPS formed MPL. These findings collectively suggest a minor impact of MPS on the accumulation of MPL in tissues. Pronounced interspecies differences exist in the interconversion of MPS to MPL. The availability of MPL from MPS is incomplete in rats, monkeys, and dogs (10%–20%), whereas it is almost complete (80%–90%) in rabbits and humans (Kong and Jusko, 1991). Multiple studies of MPS in humans indicate a very rapid and extensive conversion of prodrug to active MPL (Derendorf et al., 1985; Al-Habet and Rogers, 1989), which can confound MPL tissue uptake and concentrations at early time points.

Binding to Homogenates Compared with In Vivo Data

There was reasonable agreement between homogenate binding and in vivo distribution in the case of skeletal muscle and lung for both steroids. This permits some speculation concerning the mode of uptake of the drugs into rat tissue. The fact that the extent of in vivo distribution of both steroids in both tissues could be reasonably reproduced in homogenates under the conditions described suggests an absence of active mechanisms at the concentration tested. These observations may be valid for both steroids at lower drug concentrations,

since linear uptake of prednisolone was documented in rabbit muscle slices across a wide range of concentrations (Khalafallah and Jusko, 1984a). Therefore, K_P values obtained using homogenates can be used to inform this parameter for the development of PBPK models in the case of both steroids.

Corticosteroid Binding in the Liver

Total concentrations of DEX were relatively stable throughout the binding experiments in male and female liver homogenates, and in vitro–derived K_P values for DEX in liver from both sexes were in reasonable agreement with in vivo values. On the other hand, in vitro experiments underpredicted the K_P of MPL in both male (4-fold) and female (2-fold) liver homogenates compared with in vivo estimates. Robust in vitro loss of MPL in male homogenates was posited as a reason for the underprediction. It was interesting to find that, in the absence of any discernable loss of MPL in female liver homogenates, there was a 2-fold improvement in, but nonetheless a 2-fold underprediction of, the in vivo K_P. Based on these observations, it is possible that in vitro metabolism only partially explains the disconnect with in vivo binding data. Lackner et al. (1998) provided evidence for the presence of a glucocorticoid-responsive site in highly purified rat liver plasma membranes (free of transcortin and glucocorticoid receptors), which was shown to mediate high-affinity active uptake of endogenous and some exogenous glucocorticoids. Competitive binding studies indicated that prednisolone, a steroid differing by only a –methyl group at the C6 position compared with MPL, showed approximately 3-fold improved specificity compared with corticosterone, whereas other highly potent steroids such as DEX, betamethasone, and cortivazol, all of them bearing an OH or CH_3 group at position C16, exhibited low specificity (Lackner et al., 1998). These observations seem to offer one rational basis for the totality of our findings in regard to in vitro–in vivo prediction of DEX and MPL binding in liver and other tissues using homogenates, a system in which such active import processes would not be operative. Nonlinear tissue uptake of prednisolone observed in vivo and in rabbit liver slices was attributed to either saturable glucocorticoid receptor or transcortin binding (Khalafallah and Jusko, 1984a). The latter mechanism is not applicable because DEX and MPL do not bind to transcortin. The influence of specific high-affinity, low-capacity steroid binding to tissue glucocorticoid receptors is possible in vivo. These receptors are ubiquitous in mammalian tissues and most abundant in liver (Ballard et al., 1974). The argument for hepatic steroid uptake due to receptor binding is further

strengthened based on findings of nonlinear prednisolone binding in rabbit liver slices but linear uptake in heart, skeletal muscle, and fat (Khalafallah and Jusko, 1984a). Assessment of factors such as receptor binding and active transport on hepatic uptake will require more focused experimental testing using cytosolic fractions and very low drug concentrations. The three tissue composition–based methods severely underpredicted K_P values for both steroids in liver, indicating once again that additional mechanisms of hepatic steroid uptake are likely.

In conclusion, the K_P values for MPL and DEX determined from in vitro binding data and calculated from kinetic parameters obtained after administering the drug by two different routes were in reasonable agreement for lung and muscle. This observation suggests that the in vitro binding data obtained can, at least in these two tissues, be used to predict in vivo distribution. Tissue metabolism, saturable tissue binding, and active uptake are possible factors that can complicate assessments of hepatic transport of steroids in vivo when using tissue homogenates. These findings are in general agreement with the statement by Mendel (1989) that, for some steroids, the free hormone hypothesis "is likely to be valid with respect to some tissues, but not with respect to others (in particular, the liver)."

Authorship Contributions

Participated in research design: Ayyar, DuBois, Jusko.

Conducted experiments: Ayyar, Song, DuBois.

Performed data analysis: Ayyar, Song, Jusko.

Wrote or contributed to the writing of the manuscript: Ayyar, Song, DuBois, Almon, Jusko.

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Modeling Corticosteroid Pharmacokinetics and Pharmacodynamics – I: Determination and Prediction of Dexamethasone and Methylprednisolone Tissue Binding in the Rat

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Figure S1. Time-course of *in vitro* stability of methylprednisolone in freshly prepared homogenates from freshly harvested male rat liver (orange), freshly prepared homogenates from cryopreserved male rat liver (blue), and freeze-thawed homogenates from cryopreserved male rat liver (red) at a 3X dilution of tissue. Symbols represent observed values from a single rat.
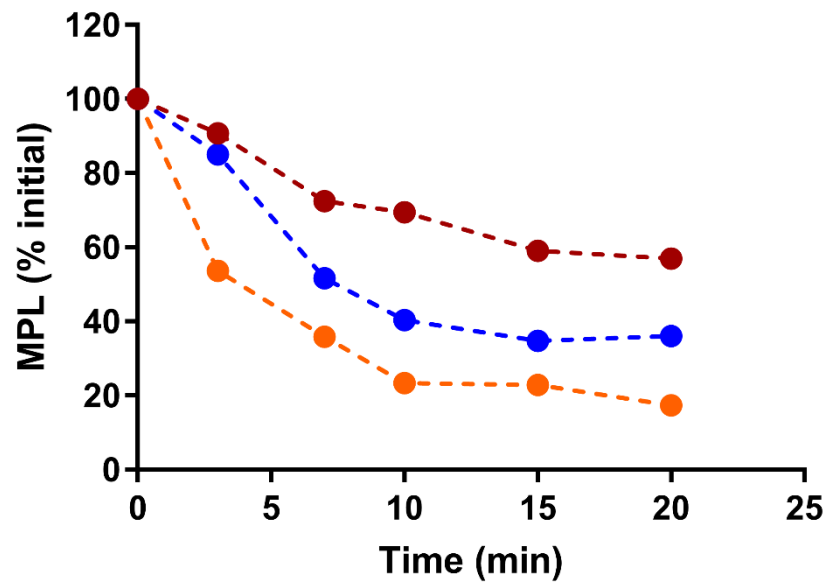
Figure S2. Stability of dexamethasone at initial concentrations of 1 μg/mL and 10 μg/mL in (3X, 4X ,6X, and 10X) diluted rat liver homogenates after incubation at 37 °C for 30 min.
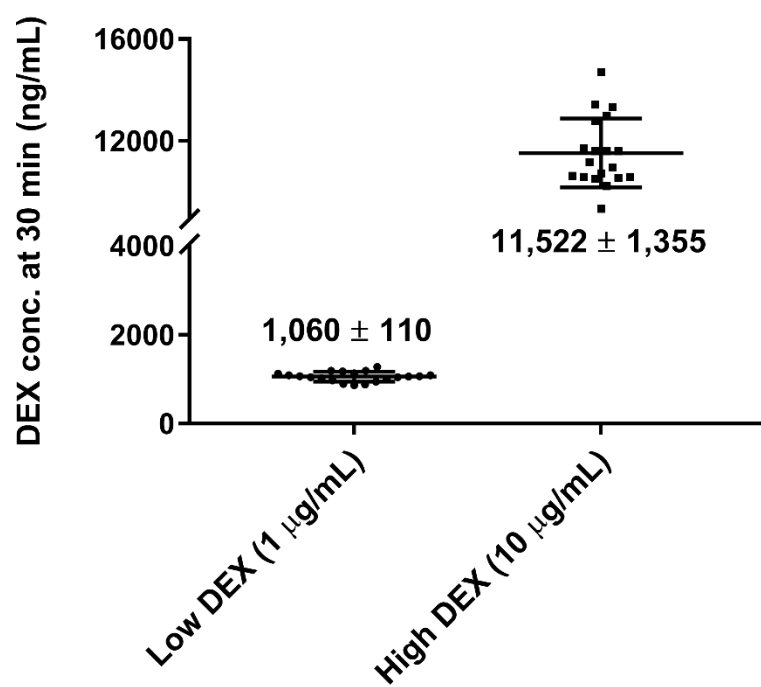
Figure S3. Effect of dilution of male rat livers on the linearity of total protein concentrations in homogenates determined using Lowry assay.