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Quantification of dexamethasone and corticosterone in rat biofluids and fetal tissue using highly sensitive analytical methods: assay validation and application to a pharmacokinetic study

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Abstract

A sensitive, specific, accurate and precise LC/MS/MS method was developed for the simultaneous measurement of dexamethasone and corticosterone in rat plasma. The method was extended to dexamethasone analysis in rat plasma ultrafiltrate and fetal tissues. Samples were processed using SPE involving Oasis HLB cartridges, which offered complete extraction recovery for the analytes. Samples were subsequently analyzed using LC/MS/MS. A structurally related corticosteroid, prednisolone, was used as the internal standard. Using a 500 μ L plasma sample, limits of quantification of 0.2 and 2.0 ng/mL were achievable for dexamethasone and corticosterone. This level of sensitivity allowed characterization of maternal/fetal dexamethasone profiles after administration of multiple doses of dexamethasone sodium phosphate to rats. However, this sensitivity was not satisfactory for corticosterone during pharmacokinetic studies involving dexamethasone due to its strong adenosuppressive effect. This led us to investigate the suitability of a commercially available radioimmunoassay kit, which through extensive testing and minor modifications was found to offer extremely sensitive, specific, accurate and precise analysis of corticosterone. Knowledge of the steroid profiles captured using these highly sensitive analytical tools may potentially help in the optimization of corticosteroid therapy during pregnancy.

Keywords

corticosteroid; LC/MS/MS; pharmacokinetics; radioimmunoassay; SPE

INTRODUCTION

The synthetic corticosteroid dexamethasone is administered maternally to induce fetal lung maturation in women at risk of preterm delivery. Such treatment has been shown to reduce the incidence of neonatal respiratory distress syndrome and is considered medical intervention that improves health care and produces considerable cost saving (NIH

Consensus Panel, 1995). However, the dose and duration of dexamethasone therapy has remained largely empirical and giving multiple doses during pregnancy produces adverse cardiovascular, neuronal and developmental effects in the fetus (Newnham, 2001). It is surmised that pharmacodynamic assessment of dexamethasone properties during pregnancy will allow insights into optimal methods of corticosteroid use in prenatal medicine. The characterization of drug pharmacodynamics requires knowledge of the pharmacokinetic profile, which serves as the driving force for drug effects. Examination of pharmacokinetic properties during pregnancy in rodents requires measurement of drug concentrations in maternal and fetal plasma and total drug content in fetal tissue as a function of time (Samtani *et al.*, 2004). Furthermore, corticosteroid effects during pregnancy are driven by both the endogenous and exogenous corticosteroids. Finally, it is important to assess free corticosteroid levels in plasma because it is the unbound drug that is accessible to various organs for mediating steroid effects.

The study of corticosteroid concentrations in pregnancy requires the development of a highly sensitive assay for the following reasons: (a) dexamethasone because of its lipophilic nature is extensively distributed into tissue space and has a volume of distribution equal to total body weight (Samtani *et al.*, 2004; Samtani and Jusko, 2005). The high distributive property produces low plasma drug concentrations. (b) Dexamethasone is one of the most potent corticosteroids available and hence the doses are low (0.1–1 mg/kg), which contributes to low plasma concentrations during pharmacokinetic studies. (c) Examination of fetal pharmacokinetics is challenging because the placenta creates a barrier to maternally administered corticosteroids and produces a fetal to maternal concentration gradient of 0.2 in the rat (Samtani *et al.*, 2004). Moreover, the tiny size of the rat fetus poses the limitation of small sample sizes of fetal plasma, which necessitates the development of highly sensitive assay methodology. (d) Measurement of the endogenous glucocorticoid after dexamethasone administration is difficult because the exogenous steroid causes adrenal suppression by negative feedback producing extremely low corticosterone concentrations in plasma.

Commonly used HPLC assays for corticosteroids have a lower limit of quantification of 10 ng/mL (Jusko *et al.*, 1994) and involve laborious liquid–liquid extraction sample preparation (Samtani *et al.*, 2005). Other methods based on gas chromatography/mass spectrometry and fluorescence detection, although offering highly specific and sensitive analysis, require derivatization. Bioanalytical methods based on LC/MS/MS represent the most specific and sensitive method for steroid analysis (Lai *et al.*, 2002). To our knowledge, assay methodology with exquisite sensitivity and selectivity for simultaneously monitoring dexamethasone and endogenous glucocorticoid temporal profiles are not available. We therefore aimed to develop assays for analysis of total and free dexamethasone and endogenous corticosterone in plasma and dexamethasone content in fetal tissue of rats, which is our animal model for prenatal glucocorticoid therapy. Solid-phase extraction and LC/MS/MS combine the attributes of rapidity, easy processing, accuracy, specificity, sensitivity and precision without the need for sample derivatization. We therefore assayed dexamethasone and corticosterone in rat biological samples using a SPE-LC/MS/MS method.

EXPERIMENTAL

Chemicals

Dexamethasone, corticosterone, formic acid and prednisolone were purchased from Sigma-Aldrich (St Louis, MO). Water and acetonitrile were from Burdick & Jackson (Muskegon, MI, USA). Methanol was obtained from EMD Chemicals (Gibbstown, NJ, USA). Solvents used during sample preparation and chromatographic separations were of HPLC grade. Phosphoric acid was from JT Baker (Phillipsburg, NJ, USA) and ammonium formate was supplied by Fluka Biochemika (Buchs, Switzerland).

Preparation of standards and controls

Drug-free rat plasma (EDTA) was purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Plasma based standards and quality control standards were prepared from rat plasma, which had been stripped of endogenous corticosterone using Norit-A pharmaceutical-grade neutral decolorizing carbon (Amend Drug and Chemical Co., Irvington, NJ, USA). Charcoal was added to rat plasma at the concentration of 4 g/100 mL. The suspension was stirred at room temperature for 8 h and centrifuged overnight at 35,300g in a Beckman J2-HS centrifuge at 4°C. Plasma was filtered using non-sterile Acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA) attached to a 20 cm³ syringe in the following sequence: 5, 1.2 and 0.45 µm. Methanolic stock solutions of corticosteroids were added to polypropylene tubes and dried under nitrogen. Appropriate volumes of stripped plasma were added, the tubes vortexed, and standards were aliquoted at >0.5 mL into 1.5 mL polypropylene tubes and stored at -20°C. Eight standards were normally prepared that covered a concentration range of 0.2–100 ng/mL for dexamethasone and 2–1000 ng/mL for corticosterone. Three controls were prepared in a similar manner and their concentrations were fixed at a medium concentration and at concentrations above the lowest standard and below the highest standard.

Fetuses from control pregnant Wistar rats of different gestational ages were frozen in liquid nitrogen and stored at -80°C. For generation of pooled blank fetal tissue homogenate used in setting up standards and controls, entire fetuses were weighed and homogenized (4 mL/g tissue) in ice-cold phosphate-buffered saline (KCl 2.7 mM, KH₂PO₄ 1.5 mM, NaCl 138 mM, Na₂HPO₄ 8.1 mM, pH 7.4) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA). Blank plasma ultrafiltrate was obtained by spinning a 15 mL starting volume of rat plasma in Amicon ultra-15 centrifugal filter units (Millipore, MA, USA) with a 30 kDa molecular weight cut-off filter in a swinging bucket rotor at 1000g for 45 min at 37°C. This procedure produced 7 mL blank ultrafiltrate and the ultrafiltrate from multiple spins were pooled to generate enough blank matrix for generation of standards and controls. Ultrafiltrate and tissue homogenate dexamethasone standards and controls were prepared in an identical fashion as stripped plasma. Tissue homogenates were aliquoted in exact 0.5 mL quantities in the sample preparation plastic tubes, while ultrafiltrates were aliquoted in 0.25 mL quantities in polypropylene tubes and stored at -20°C.

Solid-phase extraction

Sample processing was carried out in lab plasticware to prevent the known adsorption of steroids to glassware (Makin *et al.*, 1995). Non-specific adsorption can affect analyte sensitivity and recovery in the low-level analysis described in this work and the hydrophobicity of the polypropylene plastic surface helps reduce sample adsorption problems (Tsutsumi *et al.*, 2003). Sample preparation involved adding 0.5 mL 4% phosphoric acid to 0.5 mL plasma sample in polypropylene tubes. This makes the samples less viscous and can free up the protein bound drug in plasma (Ding and Neue, 1999). Added was 50 μ L of a methanolic stock (1 μ g/mL) of prednisolone as internal standard. After thorough mixing, samples were centrifuged at 8000g for 20 min and then subjected to solid phase extraction using Oasis HLB, 1 cm³ 30 mg cartridges (Waters Corporation, Milford, MA, USA). The extraction was carried out on a Vac Elut SPS 24 solid-phase extraction manifold (Varian, Palo Alto, CA, USA). The samples were extracted using the generic Oasis HLB procedure recommended by the manufacturer for 30 mg cartridges. Briefly, the SPE cartridge was preconditioned with 1 mL of methanol, followed by 1 mL of water. One milliliter of the processed sample was pulled through the cartridge, the cartridge was washed with 1 mL of methanol–water (5:95, v/v) and elution was performed with 1 mL methanol. The methanolic eluant was dried at 50°C under a gentle nitrogen stream and the dried residue was dissolved in reconstitution solvent consisting of 50% (v/v) water containing 0.1% (v/v) formic acid and 10 mM ammonium formate, and 50% (v/v) acetonitrile. The reconstituted samples were transferred into 0.5 mL polypropylene tubes, centrifuged at 16,000g for 10 min at 4°C and finally transferred to autosampler vials with glass inserts (Agilent Technologies, Wilmington, DE, USA).

Sample preparation for the fetal tissue involved addition of 50 μ L 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 min and the supernatant siphoned off in to 50 mL poly-propylene 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, USA). The extraction procedure was then identical to that described for plasma, except that the funnel shaped Vac RC cartridges accommodated a 20 mL processed sample instead of the 1 mL sample described for plasma.

Sample preparation for plasma ultrafiltrates involved adding 0.75 mL HPLC water to a 0.2 mL sample in polypropylene tubes. Sample volume was restricted to 200 μ L because the maximum volume of ultrafiltrate obtained during rat pharmacokinetic studies ranged between 100 and 200 μ L. Added was 50 μ L of a methanolic stock (1 μ g/mL) of prednisolone as internal standard and then the 1 mL processed ultrafiltrate sample was handled identically to the plasma samples.

Liquid chromatography tandem mass spectrometry

Chromatography was performed on a C₈ Hydrobond AQ column (particle size 3 μ M, 2.1 $\times$ 150 mm, MAC-MOD Analytical Inc, Chadds Ford, PA, USA) equipped with a

ColumnSaver pre-column filter (MAC-MOD Analytical Inc). The mobile phase flow rate was 0.2 mL/min with eluant A consisting of 10 mM ammonium formate in 0.1% v/v formic acid and eluant B consisting of acetonitrile. The eluant A–eluant B mobile phase flow design was as follows: 40:60 v/v for 0–4.5 min, 10:90 v/v for 4.6–6.0 min to allow system cleanup, followed by a 40:60 v/v equilibration step for 4 min. The system was equipped with an Agilent Technologies model 1100 autosampler (Palo Alto, CA, USA), dual pump and an Applied Biosystems PE/Sciex API 3000 mass spectrometer (Foster City, CA, USA) using a turbo-ion spray source. The system control and data analysis were executed using the Analyst software (Applied Biosystems, Version 1.4). The mass spectrometer was operated in the positive ionization mode.

Tandem mass spectrometry parameter optimization

At the start of method development, experiments were performed to optimize the detection parameters during multiple reaction monitoring (MRM). Each steroid was dissolved at a concentration of 1 µg/mL in the reconstitution solvent described above. The solutions were directly infused into the mass spectrometer using the turbo-ion spray source at a flow rate of 10 µL/min via a syringe pump (kdScientific Inc., New Hope, PA, USA). The total ion spectrum for the precursor ion was obtained over the m/z range of 250–1000, while the product ion spectrum was in the m/z range of 50 – 400. After identification of a unique precursor and product ion for each analyte, the detection parameters were optimized for the ion-transitions initially using the Analyst software 'Quantitative Optimization' wizard. This was followed by further optimization in the manual tune mode.

Calibration and validation

Standards were run on a daily basis for assay calibration and integration of peak areas were performed by the Analyst software (Applied Biosystems, Foster City, CA, USA). Quadratic regression curves of analyte to internal standard area ratio vs concentration with a weighting factor of $1/Y^2$ for plasma and tissue homogenate were constructed. A weighting factor of $1/X^2$ was chosen for the plasma ultrafiltrate because it provided a better fit of the calibration data. The goodness of fit for the regression curve was judged by the R^2 coefficient of determination value. Inter- and intra-assay accuracy (percentage error) and precision (CV%) were assessed through replicate analysis of control samples containing known amounts of steroids. Assay recovery was computed by comparing peak areas of control samples that underwent extraction vs post-extraction spiked matrix blank at high and low steroid concentrations. The assay lower limit of quantification was determined as the standard that consistently predicted steroid concentration within 20% of its targeted value with a CV% <20% and had a signal-to-noise ratio of at least five. Stability of cortico-steroids in plasma/stock solutions and during freeze–thaw cycles was not investigated because a wealth of data accumulated over several decades has indicated that these steroids are extremely stable in a variety of matrices (Volin, 1995).

We intended to assess the accuracy of the method in different lots of plasma to evaluate matrix effects on steroid analysis. However, it is costly and labor intensive to prepare separate lots of plasma treated for removal of corticosterone. As an alternative we utilized the newly developed method to re-analyze samples collected at sacrifice from six different

rats that were used as part of a recently published dexamethasone pharmacokinetic study (Samtani and Jusko, 2005). Such samples were previously analyzed by a well-validated, robust and highly specific normal phase HPLC-UV method, with a lower limit of quantification of 5 ng/mL (Jusko *et al.*, 1994; Samtani and Jusko, 2005). Samples from these six animals were stored at -20°C during the interim period.

Improvement of a commercially available radioimmuno-assay for corticosterone

It will be shown later that, although the tandem mass spectrometry assay provides sensitive and specific analysis for corticosterone, it could offer a lower limit of quantification of only 2 ng/mL with 0.5 mL plasma sample. During the evaluation of fetal disposition of endogenous corticosterone, 0.5 mL of plasma is not available. Furthermore during pharmacokinetic studies with dexamethasone the endogenous fetal corticosterone can be suppressed to very low levels requiring extremely sensitive analysis in small sample volumes. Limitations with the LC/MS/MS method therefore necessitated evaluation of other methods for sensitive analysis of corticosterone. Examination of the literature revealed that a variety of commercially available enzyme immunoassays and radioimmunoassays are available for corticosterone. In general radioimmunoassays offered better sensitivity than enzyme immunoassays. Furthermore, evaluation of several commercially available kits indicated that radioimmunoassays offered greater accuracy and precision. The radioimmunoassay that we found to be most useful for our purposes was the ImmuChemTM Double Antibody ^{125}I kit from MP Biomedicals (Costa Mesa, CA, USA) which requires only 5 μL plasma samples for corticosterone analysis. Furthermore, in our experience and in other published reports this kit has been found to be suitable for measurement of free corticosterone in plasma ultrafiltrates (Pacak *et al.*, 1995; Taymans *et al.*, 1997). To achieve measurable levels of free corticosterone 25 μL of ultrafiltrate samples are diluted 1:10 with assay buffer.

We made important modifications to the assay procedure to improve the sensitivity of the kit. The kit includes standards that extend only down to 25 ng/mL because this is the lowest expected concentration in normal rats and mice. We incorporated two additional standards, 12.5 and 6.3 ng/mL, into the curve by diluting the lowest standard (25 ng/mL) provided with the kit. The recommended dilution in the kit insert is 1 in 200 for rodent plasma samples, which is necessary to accommodate normal rat samples within the range of the assay. However, lower dilutions may be feasible, which may allow improvement in sensitivity. One low and one high corticosterone rat plasma sample from one of our recent circadian studies (Yao *et al.*, 2006) were analyzed to test 1:100 and 1:50 dilution protocols vs the recommended 1:200 dilution in triplicate. Apart from the dilution assessment and incorporation of the additional standards, the procedure provided in the kit insert was followed unchanged. Samples were counted in the 1272 CliniGamma counter from LKB Wallac with a counting time of 2 min/tube.

The kit insert indicates that the corticosterone anti-serum has 100% cross-reactivity with corticosterone and $<0.01\%$ cross-reactivity with dexamethasone. However, interaction of the antiserum with other steroids in our samples (corticosterone and dexamethasone metabolites) can compromise the validity of the results. We therefore evaluated the

accuracy, precision and specificity of the kit for corticosterone. Inter and intra-assay precision was assessed by repeated evaluation of circadian study samples (Yao *et al.*, 2006) that spanned >10 fold range of corticosterone concentrations (50–600 ng/mL). The accuracy and specificity of the assay across the entire range spanning the physiologic and adrenosuppression state were analyzed by assaying plasma samples from previous pharmacokinetic and circadian studies. Plasma samples with low corticosterone concentrations were obtained from a study involving female rats exhibiting adrenal suppression due to the treatment with 1 mg/kg dexamethasone (Samtani and Jusko, 2005). Plasma samples with intermediate corticosterone concentrations were obtained from a recent circadian rhythm study (Yao *et al.*, 2006). Finally, plasma samples with high corticosterone concentrations were obtained from a pharmacokinetic study where control female rats were sacrificed at the peak of the circadian cycle, which exhibit the highest level of corticosterone seen in rats. All samples from previous studies have in the past been analyzed by the normal phase HPLC-UV assay described above (Jusko *et al.*, 1994; Samtani and Jusko, 2005). Comparison of results from the two assays would give information regarding the specificity and accuracy of the radioimmunoassay kit.

Animals and dosing procedure for pharmacokinetic study

To demonstrate the utility of the procedures presented in this report, corticosteroid measurements were made in maternal/fetal samples with unknown concentrations from a pharmacokinetic study. Thirty time-pregnant Wistar rats were purchased from Harlan–Sprague–Dawley Inc. (Indianapolis, IN, USA). This study utilized a methodological approach, which has been termed the ‘giant rat’ experiment (Jin *et al.*, 2004), where a group of animals is dosed with the drug and blood/tissue samples are obtained by sacrificing three rats at each time point. 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 h light/dark cycle. A time period of 6 days was allowed for acclimatization. Rats had free access to rat chow and drinking water. 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.

The study began on gestational day 18 at which time these rats weighed 330–440 g. Animals were given six 0.4 mg/kg doses of dexamethasone in the form of dexamethasone sodium phosphate (Phoenix Scientific Inc., St Joseph, MO, USA). These doses were injected intramuscularly between 8 and 9 a.m. and between 8 and 9 p.m. on gestational days 18, 19 and 20. Three animals were sacrificed at each of the following time points: 1, 6 and 9 h after the first dose on gestational day 18; 10 min, and 2.5 and 9 h on gestational day 19 after the third dose; 0.5, 4 and 9 h on gestational day 20 after the fifth dose; and 12 h after the sixth dose on gestational day 21. 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. Blood from all the fetuses belonging to each animal 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 animal

was frozen in liquid nitrogen and stored at -80°C for evaluation of total fetal dexamethasone content. Maternal plasma samples were expected to have dexamethasone concentrations around 100 ng/ml and were diluted (75 μL made up to 500 μL), while most fetal plasma samples because of limited sample volume had to be made up to 500 μL with blank stripped plasma for LC/MS/MS analysis. The dilution of samples and dexamethasone-induced adrenal suppression made measurement of corticosterone extremely difficult by LC/MS/MS. Furthermore, corticosterone is extensively bound to albumin and corticosteroid binding globulin in plasma, which produces extremely low free concentrations that are difficult to capture by LC/MS/MS. Thus, all corticosterone data were obtained using the commercial radioimmunoassay kit.

Free steroid concentrations were evaluated by the method of ultrafiltration using the Amicon Centrifree Device (Millipore, MA, USA) with a 30 kDa molecular weight cut-off filter. A sample of 300–400 μL of fetal plasma was spun at 1000g in a fixed angle rotor for 6 min, while 0.5 mL maternal plasma was spun for 15 min at 37°C . Preliminary experiments indicated that filtration of up to 50% of the sample did not affect binding equilibrium and binding of the steroids to the ultrafiltration device was negligible. The ultrafiltration conditions described above produced a filtrate volume that was 30–40% of the plasma volume added to the upper chamber of the Centrifree device. Samples with limited ultrafiltrate volume had to be made up to 200 μL with blank plasma ultrafiltrate.

RESULTS

Tandem mass spectrometry detection parameters

Comparison of signal intensities for the steroids in positive vs negative mode gave better results with positive ionization because of efficient electrospray ionization of positively charged steroids. The method was developed using a turboionspray source because it offered higher sensitivity than a heated nebulizer source. The higher sensitivity was achievable using electrospray ionization because it allowed reduced source fragmentation of the precursor ions. Figure 1 depicts structures of dexamethasone, corticosterone, and prednisolone. Figures 2(a), 3(a) and 4(a) represent full scan mass spectra of dexamethasone, corticosterone and prednisolone. Similarly, Figures 2(b), 3(b) and 4(b) represent product ion spectra for the three analytes. The protonated molecular ion of dexamethasone at m/z 393.4, by loss of a hydrofluoric acid molecule (molecular weight = 20), gives the major fragment ion at m/z 373.2. The protonated molecular ion of corticosterone at m/z 347.1, by neutral loss of water (molecular weight = 18), generates the product ion at m/z 329.1. The MRM transitions of 393.4 to 373.3 and 347.1 to 329.1 were chosen for the quantitation of dexamethasone and corticosterone, respectively. The optimized instrument detection settings for these analytes are presented in Table 1.

Liquid chromatography

The assay specificity relied on the unique analyte fragmentation pattern and chromatographic retention. The chosen sample clean-up procedure was SPE because it makes matrix interferences less likely when using turboionspray for analyte fragmentation. Furthermore, the clean-up procedure produced an extremely clean extract, which is evident

from the typical chromatogram of blank stripped rat plasma and a spiked plasma standard (Fig. 5). Figure 6 shows a representative chromatogram of a maternal plasma sample from the pharmacokinetic study.

LC/MS/MS assay characteristics

A variety of LC/MS/MS methods have been developed as a screening method for dexamethasone in doping analysis involving urine, feces, edible tissues and milk (Cherlet *et al.*, 2004). However, LC/MS/MS assays for analyzing dexamethasone in plasma are fairly limited. Furthermore, there is only a single LC/MS/MS method described for measuring corticosterone in mouse tissues (Ronquist-Nii and Edlund, 2005). The advantage of the assay described in this report is that it simultaneously analyzes both the endogenous and exogenous steroids in rat plasma and has been extended to fetal tissues and plasma ultrafiltrates for dexamethasone analysis. The assay was adapted from a method described for analysis of betamethasone in sheep plasma (Samtani *et al.*, 2005). During the preparation of this article, two other methods describing LC/MS/MS analysis of dexamethasone in plasma were published (Luo *et al.*, 2005; Taylor *et al.*, 2004). These methods monitor different product ions in tandem mass spectrometry for dexamethasone and make use of longer liquid–liquid extraction-based sample preparation methods. However, both these methods are similar to our assay in terms of assay sensitivity, accuracy, precision and quantifiable concentration range, which will be described below.

The LC/MS/MS assay produced standard curves with $R^2 = 0.99$ within the concentration range of 0.2–100 ng/mL for dexamethasone and 2–1000 ng/mL for corticosterone in plasma. Similar calibration curves were obtained for dexamethasone in fetal tissue homogenate within the ranges of 0.2–100 and 0.5–200 ng/mL in plasma ultrafiltrate. Calibration parameters for the two analytes in various matrices are presented in Table 2. The assay had an inter- and intra-assay accuracy and precision of $\pm 20\%$, and offered almost complete extraction recovery for dexamethasone and corticosterone from rat plasma (Table 3). The limited availability of blank rat fetal tissues restricted the number of replicate controls ($n = 3$) used for studying the accuracy/precision/recovery of the assay in fetal tissue homogenate. However, the limited number of fetal tissue replicate controls indicated that the assay can produce inter- and intra-assay accuracy and precision of $\pm 15\%$, and offer almost complete extraction recovery for dexamethasone (Table 4). The recovery of dexamethasone from complex matrices such as plasma and tissue homogenate was found to be complete and hence recovery from plasma ultrafiltrate was not investigated. It was also found that preparing large batches of blank rat plasma ultrafiltrate was cost-prohibitive. The limited number of ultrafiltrate replicate controls ($n = 3$) indicated that the assay could produce inter- and intra-assay accuracy and precision of $\pm 20\%$ for this matrix (Table 4).

We aimed to assess the accuracy of the method in independent plasma matrices but it was costly to obtain independent matrices of plasma from which corticosterone had been completely removed. We therefore chose to analyze rat samples analyzed for dexamethasone and corticosterone from a previous study. The results from the current method were compared with those previously obtained using a validated HPLC-UV method and the data are shown in Table 5. It can be seen that results for the two steroids using the two different

methods are in very good agreement, indicating that variability in the source of the matrix has a negligible effect on LC/MS/MS assay performance.

Radioimmunoassay characteristics

Studies done to assess inter- and intra-assay precision involving repeated analysis of rat samples spanning a >10-fold concentration range yielded a coefficient of variation under 14%. A test of different dilutions to increase the sensitivity of the assay indicated that the 1:100 dilution gave comparable results to the 1:200 dilution, but the 1:50 dilution produced lower concentrations (Table 6). With a 1:100 dilution of 5 μ L plasma samples it was possible to achieve a lower limit of quantification of 3 ng/mL, while the lower limit of quantification with plasma ultrafiltrates involving 1:10 dilution of 25 μ L samples was 0.3 ng/mL. Regression analysis was used to compare the radioimmunoassay results with results from previous pharmacokinetic and circadian rhythm studies involving the HPLC-UV assay. Regression analysis gave an R^2 of 0.99 ($n = 16$) over the range studied and the results are presented in Fig. 7. A statistical consideration is to look at the 95% confidence interval for the intercept and slope from the regression analysis. The intervals can be used to test the hypothesis that the regression line is not significantly different from the line of unity, which is also plotted in Fig. 7. If the confidence interval for the slope and intercept include the theoretical values of one and zero then the hypothesis is accepted. This expectation was realized (Fig. 7) and there is reasonable confidence that the results from the two assays are in good agreement. Thus, the radioimmunoassay offers accurate and specific analysis of corticosterone.

Pharmacokinetic study results

Total and unbound dexamethasone profiles in maternal plasma during the multiple dosing study are shown in Fig. 8(A). Dexamethasone appeared very rapidly in the maternal circulation after intramuscular administration of dexamethasone sodium phosphate. This is evident from the maternal total dexamethasone concentration of 200–300 ng/mL observed at 1 h, and 10 and 30 min after the first, third and fifth doses of the steroid. Maternal administration of dexamethasone caused adrenal suppression, which lead to lowering of the corticosterone concentrations that could still be captured by the radioimmunoassay [Fig. 8(B)]. Dexamethasone concentrations in the fetal circulation were lower than the maternal concentrations, which is not surprising because the placenta creates a barrier to maternally administered corticosteroids [Fig. 9(A)]. However, dexamethasone transfer across the placenta was fairly rapid because it could be detected in the fetal circulation at the first sacrifice time point of 1 h after the first steroid dose. The fetal tissue dexamethasone content (ng/gm tissue) was slightly higher than the fetal plasma concentrations [Fig. 9(A)], which agrees with the recently reported fetal volume of distribution of 1.2 mL/g (Samtani *et al.*, 2004). Similar to the maternal circulation the concentration of corticosterone declined in the fetal circulation upon exposure to dexamethasone [Fig. 9(B)]. On all the different days of the study dexamethasone concentrations declined with a half-life of approximately 3 h in maternal and fetal circulations, which is excellent agreement with recently published results (Samtani *et al.*, 2004; Samtani and Jusko, 2005). The free fraction of dexamethasone in the maternal circulation was about 20%, indicating that it is moderately bound to its plasma binding protein albumin. In contrast the free fraction in the fetal circulation was

approximately 40%. This could possibly occur because the fetal plasma protein content is far lower than maternal plasma and because fetal albumin starts to rise very late in gestation in fetal rats (Tam and Chan, 1977).

CONCLUSIONS

Combination of a SPE procedure with chromatographic separation on a C₈ column and mass spectrometric detection in the MRM mode provided a rapid and specific method for the measurement of dexamethasone and corticosterone in rat plasma. The method was extended to rat fetal tissue and plasma ultrafiltrate for dexamethasone. The extraction procedure used to isolate analytes resulted in complete recovery of steroids from biomatrices. The validation experiments indicated that the assay was accurate and precise. Validation included testing inter- and intra-assay reliability, accuracy in different matrices, quantification of samples with unknown concentrations, and standard curve reproducibility. Using a 500 μ L plasma sample limits of quantification of 0.2 and 2 ng/mL were achievable for dexamethasone and corticosterone. The sensitivity of the LC/MS/MS assay for corticosterone was not satisfactory for pharmacokinetic studies involving dexamethasone. This led us to investigate the suitability of a commercially available radioimmunoassay kit. Through extensive testing the kit was found to offer specific, accurate and precise analysis of corticosterone. With minor modifications the kit provided a limit of quantification of 3 ng/mL with 5 μ L plasma samples and 0.3 ng/mL with 25 μ L plasma ultrafiltrate. The applicability of the developed procedures was demonstrated by determination of corticosteroids in rat biofluids and fetal tissue from a pharmacokinetic study. Knowledge of these steroid profiles may potentially help in the optimization of corticosteroid use during pregnancy.

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Figure 1.

Structures of the analytes and internal standard obtained from the vendor at www.sigmaaldrich.com, Sigma-Aldrich Co., accessed August 2005.

Figure 2.
(A) Full scan spectrum of dexamethasone in the positive ion mode, (B) Product ion spectrum of precursor ion with m/z 393.

Figure 3.

(A) Positive ion full-scan spectrum of corticosterone, (B) Product ion spectrum of precursor ion with m/z 347.

Figure 4.

(A) Positive ion full-scan spectrum of the internal standard prednisolone, (B) Product ion spectrum of precursor ion with m/z 361.

Figure 5.

(A) Typical MRM chromatogram of blank stripped rat plasma at transitions m/z 361→343 (representative of prednisolone), 393→373 (representative of dexamethasone), and 347→329 (representative of corticosterone), (B) Typical MRM chromatogram of blank stripped rat plasma spiked with 80 ng/mL dexamethasone, 800 ng/mL corticosterone and 100 ng/mL prednisolone.

Figure 6.

Representative MRM chromatogram of a maternal sample from the pharmacokinetic study in which dexamethasone sodium phosphate was dosed intramuscularly to pregnant rats. The severe adenosuppression induced by dexamethasone is evident from the missing corticosterone peak on the chromatogram.

Figure 7.

Regression analysis to assess radioimmunoassay accuracy and specificity across the entire standard curve range. Slope and intercept with the 95% confidence interval for the regression analysis were 1.04 (0.96 to 1.13) and -0.0791 (-0.228 to 0.0701).

Figure 8. Maternal steroid profiles from the pharmacokinetic study involving six 0.4 mg/kg intramuscular doses of dexamethasone in the form of dexamethasone sodium phosphate injected maternally at the time points indicated by arrows. Total and free plasma dexamethasone (A) and corticosterone (B) profiles are indicated by open and closed circles.

Figure 9. Fetal steroid profiles from the pharmacokinetic study. Total and free dexamethasone (A) and corticosterone (B) profiles are indicated by open and closed circles. Filled triangles (A) indicate the fetal dexamethasone content in terms of ng/gm tissue during the pharmacokinetic study.

Table 1
Retention times, ion transitions, and optimized detection parameters for LC/MS/MS

Compound	Retention time (min)	Ion transitions (<i>m/z</i>)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Focusing potential (V)	Excitation potential (V)
Dexamethasone	2.97	393.4/373.3	35	15	23	300	10
Corticosterone	3.34	347.1/329.1	45	23	23	300	10
Prednisolone	2.59	361.0/343.4	25	15	20	300	10

Table 2

Calibration curve parameters for dexamethasone and corticosterone in various matrices analyzed by LC/MS/MS. The equation used to describe the calibration data was $Y = A \cdot X^2 + B \cdot X + C$, where Y is the analyte/internal standard area ratio, X is analyte concentration, and A – C are calibration parameters. Parameters are presented as mean $\pm$ SD ($n = 3$)

	A	B	C	R²
Dexamethasone in plasma	$-1.64 \times 10^{-5} \pm 3.1 \times 10^{-6}$	$8.71 \times 10^{-3} \pm 3.7 \times 10^{-4}$	$1.02 \times 10^{-4} \pm 1.6 \times 10^{-4}$	$9.99 \times 10^{-1} \pm 7.6 \times 10^{-4}$
Dexamethasone in ultrafiltrate	$-9.66 \times 10^{-7} \pm 1.3 \times 10^{-6}$	$2.72 \times 10^{-3} \pm 1.5 \times 10^{-4}$	$4.50 \times 10^{-4} \pm 2.7 \times 10^{-4}$	$9.99 \times 10^{-1} \pm 7.8 \times 10^{-4}$
Dexamethasone in fetal tissue	$-3.18 \times 10^{-5} \pm 4.6 \times 10^{-6}$	$1.40 \times 10^{-2} \pm 4.2 \times 10^{-3}$	$2.52 \times 10^{-3} \pm 6.6 \times 10^{-4}$	$9.99 \times 10^{-1} \pm 5.8 \times 10^{-4}$
Corticosterone in plasma	$-1.85 \times 10^{-7} \pm 7.8 \times 10^{-8}$	$1.11 \times 10^{-3} \pm 3.0 \times 10^{-4}$	$2.32 \times 10^{-4} \pm 2.9 \times 10^{-4}$	$9.96 \times 10^{-1} \pm 2.3 \times 10^{-4}$

Table 3

Assay validation for quantifying dexamethasone and corticosterone in 0.5 mL rat plasma by LC/MS/MS

Dexamethasone			Corticosterone		
Theoretical concentration (ng/mL)	Accuracy, %error	Precision, CV%	Theoretical concentration (ng/mL)	Accuracy, %error	Precision, CV%
<i>Inter-assay statistics (n = 3)</i>					
0.750	1.57	8.77	7.50	7.47	9.66
25.0	2.63	7.43	250	-5.70	14.6
80.0	16.3	9.47	800	-3.33	13.6
<i>Intra-assay statistics (n = 6)</i>					
0.800	3.87	8.23	7.50	18.3	8.66
25.0	-2.30	9.42	250	5.15	16.2
80.0	12.7	12.9	800	9.70	17.5
<i>Recovery (n = 6)^d</i>					
Theoretical concentration (ng/mL)		Recovery, %	Theoretical concentration (ng/mL)		Recovery, %
0.800		90.5	7.50		83.3
80.0		124	800		101

^d Internal standard prednisolone at 100 ng/mL: 119% (n = 12).

Table 4
Assay validation results for dexamethasone in rat fetal homogenate and plasma ultrafiltrate by LC/MS/MS

Fetal tissue homogenate			Plasma ultrafiltrate		
Theoretical concentration (ng/mL)	Accuracy, %error	Precision, CV%	Theoretical concentration (ng/mL)	Accuracy, %error	Precision, CV%
<i>Inter-assay statistics (n = 3)</i>					
0.750	-5.49	11.60	1.00	8.88	9.90
25.0	-5.55	14.79	50.0	13.0	6.65
80.0	0.29	11.04	150	10.2	8.01
<i>Intra-assay statistics (n = 3)</i>					
0.750	5.43	10.35	1.00	18.0	4.30
25.0	5.67	3.94	50.0	19.5	5.80
80.0	1.37	5.89	150	17.8	2.85
<i>Recovery (n = 3)^d</i>					
Theoretical concentration (ng/mL)			Recovery, %		
1.00			106		
80.0			113		

^dPrednisolone at 100 ng/mL in fetal tissue homogenate: 106% (n = 6).

Table 5

Comparison of UV-HPLC and LC/MS/MS methods in rat plasma

Animal no.	Dexamethasone		Corticosterone	
	UV-HPLC (ng/mL)	LC/MS/MS (ng/mL)	UV-HPLC (ng/mL)	LC/MS/MS (ng/mL)
1	31.4	25.9	8.56	9.29
2	27.8	24.8	9.33	11.9
3	90.3	74.4	11.4	10.9
4	39.7	40.0	13.9	12.8
5	42.2	49.5	7.90	8.39
6	33.0	37.0	11.1	8.08

Table 6

Assessment of different dilutions for radioimmuno-assay of corticosterone

Dilution	Corticosterone concentration (ng/mL)	
	High sample	Low sample
1:200	203.7 ± 1.1	58.7 ± 2.2
1:100	197.9 ± 7.2	56.7 ± 0.6
1:50	183.2 ± 12.0	43.2 ± 1.0*

* *p*-Values < 0.05 were considered statistically significant. The mean values were analyzed for significance using an ANOVA test followed by a Dunnett's *post hoc* test. Data are presented as mean ± SD, *n* = 3.