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Article

HPLC-MS/MS method for determination of betamethasone in human plasma with application to a dichorionic twin pregnancy pharmacokinetic and placental transfers studies

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Betamethasone (BET) is a synthetic glucocorticoid recommended for pregnant women at imminent risk of preterm birth before 34 weeks to reduce neonatal complications. There are different techniques to describe BET plasma quantification. However, none quantified the plasmatic concentration of BET in dichorionic (DC) twin pregnancies using LC-MS. Our objectives were to develop and validate a method for quantifying BET by LC-MS for pharmacokinetic (PK) and placental transfer studies in DC twin pregnancies. Blood samples were collected after intramuscular administration of a single BET dose containing 6 mg disodium phosphate + 6 mg acetate. BET was determined in plasma by liquid-liquid extraction. The method showed linearity in the range of 2-250 ng/mL, as well as precision and accuracy with a coefficient of variation and relative standard errors ≤ 15%. Additionally, the method presented selectivity and did not present matrix or carry-over effect. Stability tests also presented coefficient of variation and relative standard errors ≤ 15%. This is the first study which describe maternal and fetal plasma concentrations of BET in a DC twin pregnancy. The BET PK parameters were AUC0-∞, CL/F, Vd/F, Cmax, Tmax of 292.20 h*ng/mL, 39.08 L/h, 278.72 L, 25.55 ng/mL and 0.58 h, respectively. The placental transfer ratios of umbilical vein/maternal vein and intervillous space/maternal vein were 0.14 and 0.19 and 0.40 and 0.27 for both twins, respectively. However, a clinical study with more subjects is imperative to confirm this higher concentration of BET in the intervillous space.

Keywords: Betamethasone. LC-MS. Twin pregnancy. Placental transfer study. Pharmacokinetic study.

INTRODUCTION

Cortisol is the principal endogenous glucocorticoid in humans, and its synthesis is carried out by the adrenal cortex and regulated by the hypothalamic-pituitary-adrenal axis (Bunte *et al.*, 2018. Betamethasone (BET) is a synthetic glucocorticoid derived from cortisol that binds to specific intracellular receptors and DNA to remodel gene expression (PUBCHEM Database, 2019).

Clinical studies have shown that antenatal corticosteroid administration reduces adverse neonatal outcomes secondary to preterm delivery, such as acute respiratory distress syndrome, intraventricular hemorrhage, necrotizing enterocolitis, sepsis, and neonatal death (Liggins, 1969; Petersen, Nation, Ashley, 1980; Roberts *et al.*, 2017; Roberts, Dalziel, 2006). Therefore,

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administering corticosteroids to women at imminent risk of preterm birth is recommended before 34 weeks gestation (ACOG, 2016; National Institute for Health and Care Excellence, 2015; Skoll *et al*., 2018; WHO, 2015).

Plasma quantification of BET can be performed using different techniques such as ultraviolet detection, radioimmunoassay, and mass spectrometry combined with liquid chromatography (LC-MS) (Ballabh *et al*., 2002; Petersen, Nation, Ashley, 1980; Salem, Alkhatib, Najib, 2011). However, only one study determined the concentration of BET in human plasma samples by the LC-MS method. In addition, this is the first study to describe BET concentrations in maternal and fetal plasma in a DC twin pregnancy using this method (Salem, Alkhatib, Najib, 2011).

Thus, this study aimed to develop and validate a method for quantifying BET by LC-MS for application in pharmacokinetic (PK) and placental transfer studies in DC twin pregnancies after a single intramuscular dose of 12 mg of BET (6mg acetate plus 6mg disodium phosphate).

MATERIAL AND METHODS

CHEMICAL AGENTS AND REAGENTS

Stock betamethasone solutions (99%, Sigma-Aldrich, St. Louis, MO, USA) were prepared in methanol at 50 µg/mL. Working solutions were prepared at 5, 2.5, 1, 0.75, 0.5, 0.25, 0.2, 0.1, 0.05 and 0.04 µg/mL in methanol. The deuterated internal standard (IS), BET acetate (99%, Toronto Research Chemicals, North York, ON, Canada), was prepared in methanol at 1 µg/mL. The solvents methanol (J.T. Baker, Fairfield, EUA), diisopropyl ether (Merck, Darmstadt, Germany), and the reagent ammonium formate (>99%, Sigma-Aldrich, St. Louis, EUA) were HPLC grade. Water purified with the Milli-Q® Plus system (Millipore Corp., Belford, MA, USA) was used throughout the experiment.

CHROMATOGRAPHIC ANALYSIS

BET determination was performed by HPLC-MS/MS system composed of a quaternary pump equipped with Waters e2695 separation module auto-sample injector (Waters Corp, Milford, MA USA), column oven CTOASVP (Shimadzu Corp, Kyoto, Japan), and Quattro Micro API triple quadrupole equipped with an electrospray interface (Waters Corp, Milford, MA, USA). Analytes were separated in a reversed-phase C-8 LiChrospher® (5µm, 125 × 4mm, Merck, Darmstadt, Germany) with C-8 guard-column LiChrospher® 100 RP-8, 5µm, 4x4mm (Merck, Darmstadt, Germany) kept at 28ºC. The mobile phase consisted of a mixture of methanol and ammonium formate 0.05 mM (90:10, v/v) with a flow rate of 0.3 mL/min.

The mass spectrometer system was operated in the positive ionization mode, with a capillary voltage of 3 kV and source and desolvation temperatures of 120 and 300 ºC, respectively. Collision gas (argon) was employed at 1.70 10^{-3} mbar. Cone voltage was kept at 30 V, and the collision energy was kept at 9 eV and 10 eV for BET and IS, respectively. The BET protonated $[M+H]^+$ and their ion products were monitored in the 393 > 373 m/z transition for BET and 438 > 418 m/z for IS. MS conditions were optimized by direct infusion with analytes solutions at 35µg/mL.

SAMPLE PREPARATION

Plasma samples of 500 µL were added to 25 µL of IS and 6 mL of diisopropyl ether. Tubes were mixed for 1 min and centrifugated for 10 min (900 × g). The organic supernatant was separated and evaporated to dryness (Christ®, RVC 2-25 CD plus, Funkenstörungsgrad, Germany). Then, it was reconstituted with 125µL of mobile phase, vortexed for 30 seconds, and transferred to glass insert tubes. Only 60µL of the mixture was injected into the chromatographic system.

METHOD VALIDATION

The analytical method was validated according to the resolution for bioanalytical methods from the European Medicines Agency (EMEA, 2011). Three calibration curves for the analysis of BET in human plasma were constructed using 500µL of blank plasma enriched with 25µL of BET standard solution and IS and submitted to

the extraction process. The linearity included the analysis of the blank plasma (plasma without analyte or IS), the zero plasma (plasma without analyte and with IS), and samples at the following concentrations: 2.0, 2.5, 5.0, 10.0, 12.5, 25.0, 37.5, 50.0, 125.0 and 250ng/mL. The peak areas of BET standards divided by IS ratios were plotted against their respective plasma concentrations using 1/x2 weighting. Quality control (QC) samples were prepared in blank plasma in the concentrations presented in Table I.

The method's selectivity was accessed by comparing the area of interferents in the analytes retention time to the area of the lower limit of quantification (LLOQ) using blank plasma samples from six different sources (including two hemolyzed and lipemic samples). The carry-over effect was evaluated by comparing the area of blank samples before and after the injection of two upper limits of quantification samples (5.000ng/mL). The method was considered selective and with no carry-over effect when interferents areas were < 20% of LLOQ area and < 5% of IS area.

Matrix effect was evaluated using plasma samples from eight different sources: four normal plasma, two lipemic, and two hemolyzed. The plasma samples were spiked with a standard solution of BET at low (LQC) and high (HQC) concentration levels and IS. The same standard solutions were analyzed in methanol with IS. For each sample, a IS normalized matrix factor (IS-NMF) was calculated according to the equation: (BET response in the matrix / IS response in the matrix) divided by (BET solution in standard solution / IS response in standard solution). The matrix effect was considered irrelevant when the coefficient of variation (CV%) of the IS-NMF values was >15%. Similarly, a recovery test was performed according to the equation: (area of analyte/ area of IS spiked before extraction) divided by (area of analyte/area of IS in solution).

Accuracy and precision were evaluated in intra-run (n=6) and inter-runs (n=3) of the QC concentrations (Table I). The method was considered accurate and precise when the coefficients of variation (CV) and relative standard errors (RSE) of QC samples were <15% (or <20 % for the LLOQ). Stability tests were performed in three different storage conditions: post-processing stability, stability after three freeze-thaw cycles, and short-term stability. QC samples were prepared at low and high concentrations (Table I). In the post-processing stability test, the QC samples were extracted and maintained in the autosampler for 24h at 12°C, and they were injected into the chromatographic system after this period.

TABLE I - Validation parameters for determination of betamethasone in human plasma

Parameters		
Matrix effect		
IS-NMF mean (CV%)	9.77	
Recovery	**Mean**	**CV**
LQC	1.00	9.26
HQC	0.97	12.38
Linearity (ng/mL)	2-250	
Linear regression equation	0.0164492.x + 0.0215473	
r^2	0.9938	
Precision (CV) and accuracy (RSE)		
Intra-run (n=6)	**Precision (% CV)**	**Accuracy (% RSE)**

TABLE I - Validation parameters for determination of betamethasone in human plasma

Parameters		
LLOQ (2 ng/mL)	10.42	3.69
LQC (5 ng/mL)	7.58	6.90
MQC (125 ng/mL)	5.92	-5.92
HQC (200 ng/mL)	9.56	-5.82
DQC (500 ng/mL)	3.72	-9.11
Inter-run (n=3)	**Precision (% CV)**	**Accuracy (% RSE)**
LLOQ (2 ng/mL)	11.39	3.67
LQC (5 ng/mL)	5.52	8.90
MQC (125 ng/mL)	7.61	-0.26
HQC (200 ng/mL)	9.72	-0.98
DQC (500 ng/mL)	3.73	-11.08
Stability (n=5)		
Short-term (25°C, 4 h)	**Precision (% CV)**	**Accuracy (% RSE)**
LQC (5 ng/mL)	2.66	9.49
HQC (200 ng/mL)	9.26	4.26
Post-processing (15°C, 12 h)	**Precision (% CV)**	**Accuracy (% RSE)**
LQC (5 ng/mL)	7.58	6.90
HQC (200 ng/mL)	9.56	-5.82
3 freeze-thaw cycles (−70°C, 25°C)	**Precision (% CV)**	**Accuracy (% RSE)**
LQC (5 ng/mL)	5.23	10.38
HQC (200 ng/mL)	8.62	-0.68

IS-NMF: internal standard normalized matrix factor; **CV**: coefficient of variation; **RSE**: relative standard error; **LQC**: Low quality control; **LLOQ**: lower limit of quantification; **MQC**: Medium quality control; **HQC**: High quality control; **DQC**: Dilution quality control.

For the three freeze-thaw cycles, the QC samples were frozen at – 70 °C for at least 24h. After this period, the samples were thawed and frozen for 12h. This process was repeated two times. Then, the samples were extracted and analyzed. Finally, in the short-term stability, the QC samples were prepared and remained at room temperature (25 °C) for 4h. Later, the samples were injected into the chromatography system. The stability results were compared to freshly prepared QC samples.

CLINICAL STUDY

The Hospital Research Ethics Committee approved the study protocol (process number 8676/2017). One woman with a DC twin pregnancy at the 31st week was the patient of the study and received a single intramuscular dose of 12 mg BET esters (disodium phosphate + acetate, Celestone Soluspan®, Mantecorp, Anápolis, Brazil). Samples of 5 mL of maternal blood were collected before and 5, 10, 15, 20, 35 min, and 1, 3, 6, 8, 10, 16, and 24h after BET injection. The samples were collected in heparinized tubes protected from light and with 10µL of 2M dibasic sodium arsenate heptahydrate per mL of blood to inhibit the hydrolysis of the phosphate ester. Then, plasma from each sample was separated by centrifugation (4°C, 21.500 g) earlier than 15 min and transferred to clean tubes with 10 µl of 50% (w/v) potassium fluoride solution per mL of plasma also to inhibit hydrolysis of the phosphate ester (Petersen, Nation, Ashley, 1980; Samtani *et al.*, 2005).

After delivery, approximately 2 hours and 30 minutes after the intramuscular BET injection, blood samples were collected from the umbilical vein, maternal vein, and intervillous space. These blood samples were processed similarly to those previously described for maternal blood. Considering that BET esters are converted to BET, only BET was quantified (Petersen, Nation, Ashley, 1980; Salem, Alkhatib, Najib, 2011). All plasma samples were kept at -70ºC until the PK analysis.

Pharmacokinetic analysis

The BET PK was calculated based on the plasma concentration versus time using Phoenix® WinNonlin®, version 6.3 (Certara, Princeton, USA). A non-compartmental model was chosen based on the lowest deviation for the parameters: area under the curve plasma concentration versus time extrapolated to infinity (AUC), maximum plasma concentration (Cmax), apparent total clearance (CL/F), and apparent volume of distribution (Vd/F). The placental transfer of BET was evaluated by the umbilical cord/maternal vein concentration ratio and intervillous space/maternal vein concentration ratio. The maternal vein concentration of BET was calculated again at the delivery time.

RESULTS AND DISCUSSION

The present study describes for the first time the determination of plasmatic BET concentration in a DC twin pregnancy for PK and placental transfer studies using a deuterated IS by LC-MS method. BET determination in pregnant women was executed only by two authors, using ultraviolet detection or radioimmunoassay (Ballabh *et al.*, 2002). The methods that achieved BET determination using mass spectrometry detection were applied in veterinary studies or healthy volunteers with similar results to the present study regarding LLOQ and sample preparation process (Knych *et al.*, 2017; Salem, Alkhatib, Najib, 2011).

The protonated molecule and its respective products are shown in Figure 1, being the transitions 393>373 for BET, 435>397 for BET acetate, and 438>400 for BET acetate-d3 (IS). Similarly to a previous study, detecting BET acetate in any sample was impossible, and only the metabolite BET was quantified (Petersen, Nation, Ashley, 1980). The stabilizing solutions of dibasic sodium arsenate heptahydrate and potassium fluoride were used to inhibit the conversion of the prodrug BET disodium phosphate to BET, which may cause an overestimation of the plasma concentrations of BET in the first hour of the serial collections (Petersen, Nation, Ashley, 1980; Samtani *et al.*, 2005).

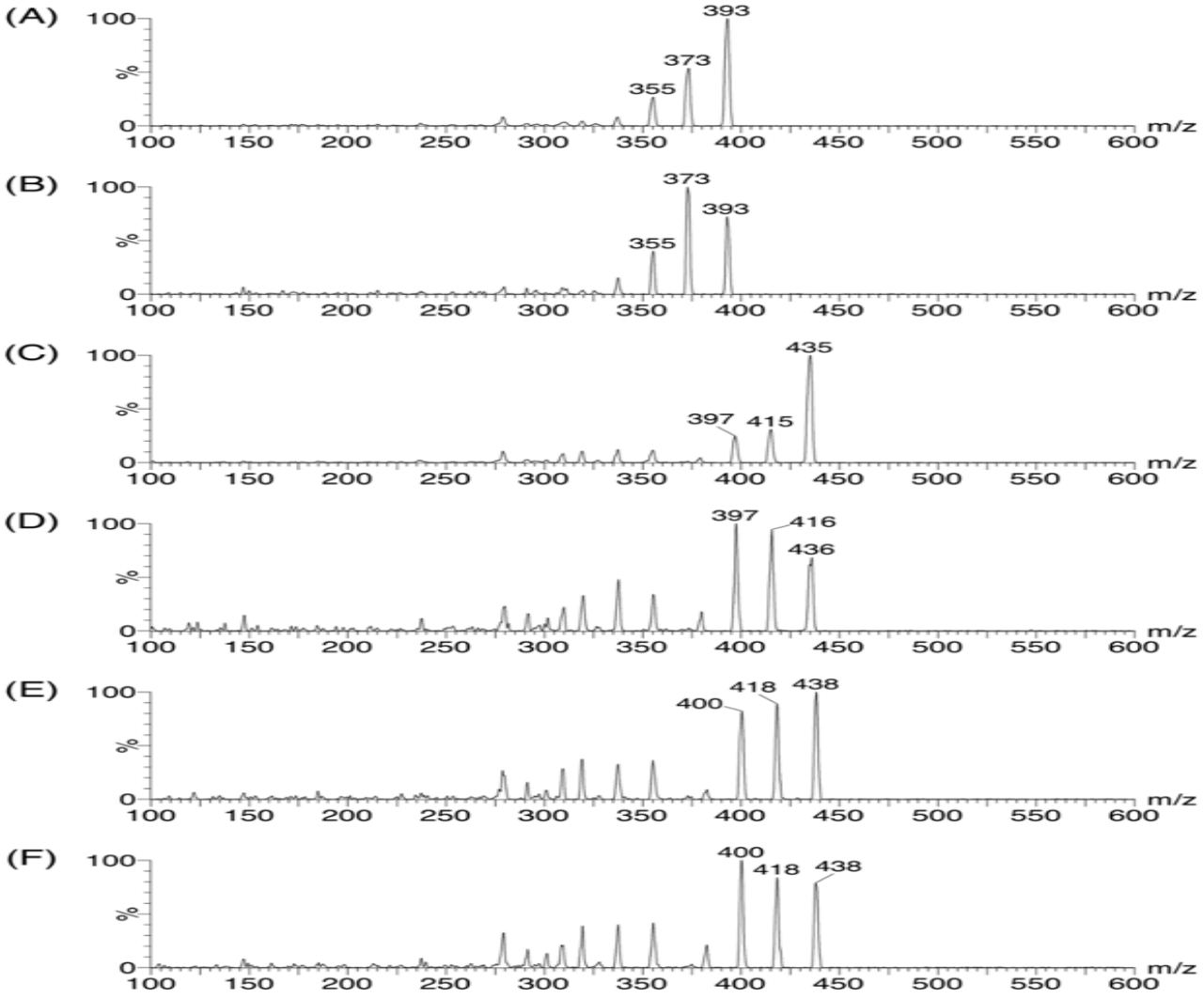

FIGURE 1 – Mass spectra of betamethasone protonated 393>373 (A), betamethasone acetate 435>397 (C) and betamethasone acetate-d3 438>400 (E) and its respective ion products (B, D and F).

The method was selective as the chromatograms of blank plasma spiked at LLOQ, and the pregnant woman sample showed an absence of interferents in the retention time of the analytes (Figure 2). Besides, the method also presented no carry-over effect. Additionally, the quantitative matrix effect evaluation showed that the method had no matrix effect (IS-NMF<15%; Table I). Finally, the method recovery showed values near 100% (Table I), similarly to other studies that also used liquid-liquid extraction for BET determination (4,11). Solid-phase extraction was employed for BET esters determination (11). Acceptable accuracy, precision, and stability were also presented, with CV and RSE<13% for all QC samples.

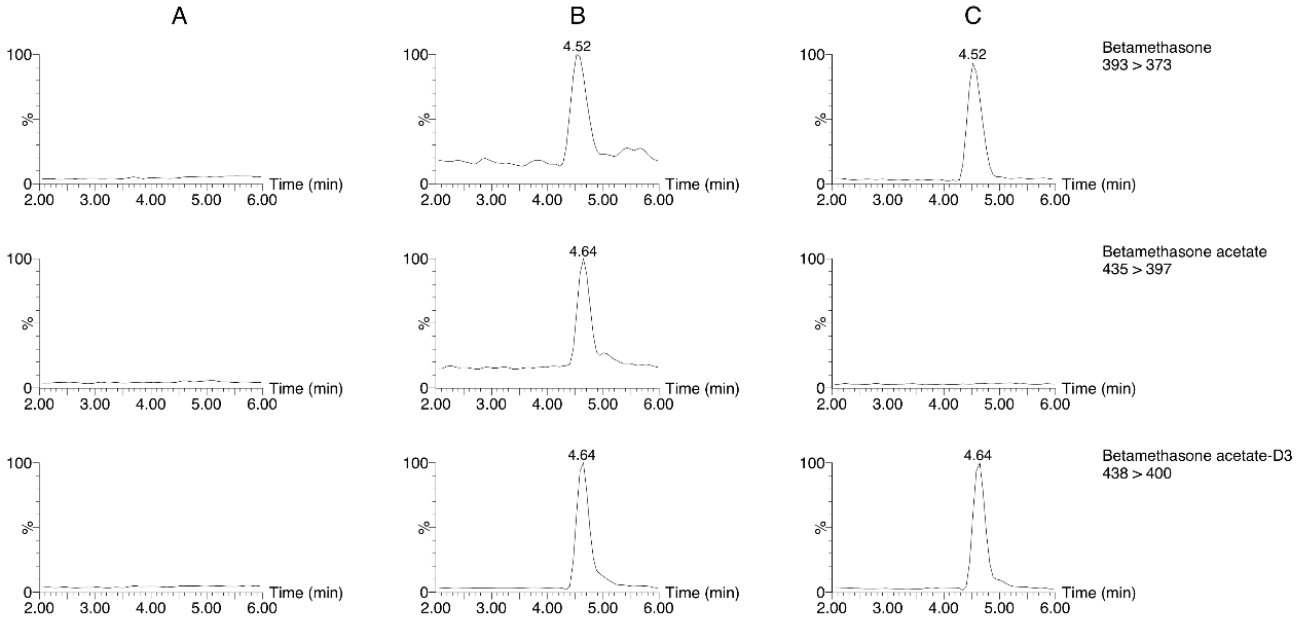

FIGURE 2 – Chromatograms from analysis of betamethasone. (A) Blank plasma; (B) Blank plasma spiked with LLOQ (2 ng/mL) and IS; (C) Pregnant woman sample collected 1h after intramuscular injection of 12 mg of betamethasone.

The method was successfully applied in one DC twin pregnancy for PK and placental transfer studies. The plasma concentration versus time profile of BET is shown in Figure 3. The administration of a single intramuscular dose of 6 mg BET disodium phosphate + 6 mg BET acetate resulted in the following values of BET: AUC0-∞, CL/F, Vd/F, Cmax, Tmax of 292.20 h*ng/mL, 39.08 L/h, 278.72 L, 25.55 ng/mL and 0.58 h, respectively (Table II). The PK parameters are like those in other PK studies considering singleton or twin pregnancies, regardless of BET phosphate stabilization (Ballabh *et al.*, 2002; Petersen *et al.*, 1984). Ballabh *et al.* (2002) reported BET T1/2, CL/F, and Vd/F in singleton and twin pregnancies (12), even though the authors did not mention the BET stabilization. Nonetheless, Petersen *et al.* (1984) described BET Cmax, Tmax, and AUC in singleton pregnancies after 6 mg BET disodium phosphate + 6 mg BET acetate and demonstrated the stabilization of BET phosphate. None of these studies presented all PK parameters for a complete comparison with those in the present study. Placental transfer of BET was like the only study that showed these data (Petersen *et al.*, 1980).

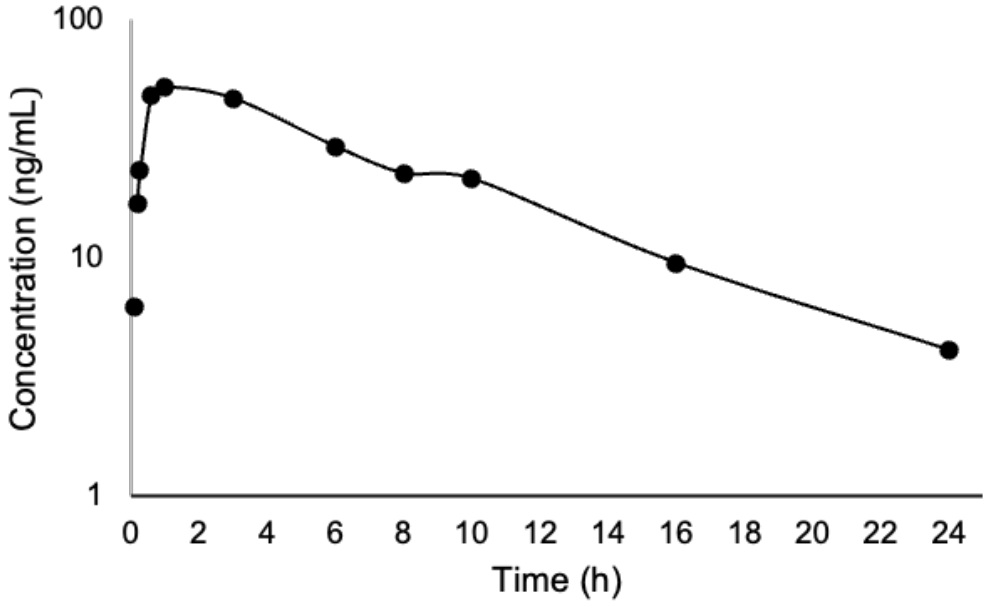

FIGURE 3 - Plasma concentration versus time of betamethasone in a dichorionic twin pregnancy after a single intramuscular dose of 12 mg of betamethasone acetate plus betamethasone phosphate.

Therefore, this study represents the first description of the intervillous space/maternal vein concentration ratio of BET in a DC twin pregnancy (0.27 and 0.40 for twins A and B, respectively). Likewise to other drugs administered to pregnant women, the intervillous space can be a storage depot of BET. It may show higher concentrations than other compartments, which allows a prolonged effect on the fetus (Moises *et al*., 2015). However, a clinical study with more subjects is imperative to confirm the findings in the placental transfer of BET.

TABLE II - Pharmacokinetic parameters of the pregnant woman included in the study after a single intramuscular dose of 12mg of betamethasone (6mg acetate plus 6mg phosphate)

Parameters	Values
$AUC^{0-\infty}$ (h*ng/mL)	292.20
C_{max} (ng/mL)	25.55
T_{max} (h)	0.58
CL/F (L/h)	39.08
Vd/F (L)	278.72
Placental transfer ratios*	
Umbilical vein/maternal vein	0.14; 0.19
Intervillous space/maternal vein	0.40; 0.27
Umbilical vein/intervillous space	0.36; 0.72

$AUC^{0-\infty}$: area under the curve plasma concentration versus time extrapolated to infinity; C_{max}: maximum plasma concentration; CL/F: apparent total clearance; Vd/F: apparent volume of distribution; T_{max}: time to reach C_{max}. *The umbilical vein and the intervillous space collections occurred 2h and 30 min after betamethasone administration. Values of each twin.

CONCLUSION

The validation method showed adequate sensitivity, linearity, accuracy, and precision for PK and placental transfer studies of BET in DC twin pregnancy. The method was recently applied in a larger BET PK investigation in pregnant women.

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